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FACULTY OF MEDICINE, HEALTH & LIFE SCIENCES

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Human keratinocyte migration: Relationship between growth factor and integrin based cell signalling

by

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UNIVERSITY OF SOUTHAMPTON, ABSTRACT
FACULTY OF MEDICINE, HEALTH & LIFE SCIENCES; SCHOOL OF MEDICINE
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HUMAN KERATINOCYTE MIGRATION: RELATIONSHIP BETWEEN GROWTH FACTOR AND INTEGRIN BASED CELL SIGNALING.

By David Eric Sawcer

Following a break in its integrity through injury, the skin must be able to repair the breach to restore its protective barrier function quickly and efficiently. The closure of a full thickness break involves a combination of fibroblast-mediated remodelling and contraction of dermal connective tissue, combined with proliferation and migration of keratinocytes at the wound margin. Control of these processes involves a regulated balance between changing extracellular matrix components (ECM), soluble factors, and cellular elements. Many of the processes involved have been studied in participating cell types, especially fibroblasts. In this work I have investigated human keratinocytes and their migration during wound healing *in vitro*.

Experiments presented were conceived to evaluate in detail the hypothesis that signalling from both integrin-ECM interactions and from soluble growth factors and their receptors work in conjunction to facilitate the migration of keratinocytes in an integrated manner. Two models were used in which keratinocyte movement and behaviour could be modified and quantified: a "scratch wound" in confluent keratinocyte cultures and migration on colloidal gold which allowed individual keratinocytes to be tracked and the distances they moved to be measured. First, keratinocyte responses to different extracellular matrix components and to soluble growth factors present *in vivo* in an acute (days 2-8 post injury) healing wound were assessed in both these model systems. Second, the effects of blocking key signalling pathways (involving FAK, Src and the mitogen-activated protein (MAP) kinases) through which keratinocytes respond to signals from their environment, integrin-ECM and growth factor stimulation, were examined.

In a manner analogous to an acute healing wound *in vivo*, extracellular matrix components influenced keratinocyte migration. Several pro-migratory substrates (ECMs) were identified that enabled keratinocyte migration to varying degrees, type I collagen producing the greatest migration. While on collagen (type I and IV) substrates migration of human keratinocytes occurred with or without the addition of soluble growth factors, the presence of growth factors significantly enhanced migration on both collagens and on other pro-migratory substrates identified. Further, in the presence of integrin-collagen (type I and IV) stimulation alone, keratinocyte migration occurred at a reduced level compared to that observed with both integrin-collagen and growth factor stimulation acting in concert. Growth factor signalling alone proved unable to produce keratinocyte migration.

To explore the involvement of intracellular pathways mediating these effects, expression of selected cellular genes notably FAK and c-Src were inhibited using chemical inhibitors and transfection of dominant negative genes delivered in lentiviral vector constructs. Inhibition of FAK resulted in a significant reduction in keratinocyte migration, while inhibition of Src produced only a limited reduction in migration. Unexpectedly, over-expression of the "wild-type" c-Src gene also appeared to be associated with impairment of migration. Key intracellular mediators of soluble factor signalling (Erk, JNK, and p38) were also evaluated by the same techniques. Erk inhibition reduced migration normally seen both in the presence and absence of growth factors to a level typically seen on non-migratory substrates, while p38 inhibition reduced migration in the presence of growth factors to the same level as that typically seen in their absence. The inhibition of the JNK MAP kinase pathway had no effect on keratinocyte migration. Finally, downstream interaction between the two pathways was investigated and demonstrated. Notably, inhibition of the auto-phosphorylation site on FAK (Tyr-397) both in the presence and absence of growth factors reduced Erk MAP kinase pathway activity to a level typically seen in cells on non-migratory substrates, and keratinocyte migration on type I collagen was similarly reduced. A complex interaction between FAK and Src signalling activity (kinase activity), tyrosine phosphorylation of these kinases and of paxillin, and activation of the Erk MAP kinase pathway appeared to underlie all these findings and was explored.

This work demonstrates that keratinocytes can and do respond to a number of growth factor and integrin signals from their environment by migrating. Further, it has shown that this migration is facilitated within the cell by activity in a number of known pathways that transmit the external signals to the cellular effectors of migration, and in particular that the Erk and p38 MAP kinase pathways and FAK and Src are central to this downstream signalling. By considering experimental conditions analogous to those present in the environment of an acute healing wound (day 2-8 post injury) it is reasonable to suppose the same influences and processes drive keratinocyte migration *in vivo* and *in vitro*.

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Declaration of Authorship

I, David Eric Sawcer,

Declare that the thesis entitled: "Human keratinocyte migration: Relationship between growth factor and integrin based cell signalling" and the work presented in the thesis are both my own, and have been generated by me as the result of my own original research. I confirm that:

- This work was done wholly or mainly while in candidature for a research degree at this University:
- Where any part of the thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
- Where I have consulted the published work of others, this is always clearly attributed;
- Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
- I have acknowledged all main sources of help;
- Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
- None of this work has been published before submission;

Signed:

Date 30th September 2008

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Any omissions or inaccuracies in this work are entirely my own.

Definitions and Abbreviations

Abl	Abelson tyrosine kinase
ADF	Actin depolymerizing factor
AMP	Adenosine monophosphate
ANOVA	Analysis of Variance (Statistical technique)
Arf	Arf subfamily of Ras
Arp	Adaptor protein for Rho and Phospholipase C-δ
ASAP1	Arf-GTPase activating protein
ATP	Adenosine triphosphate
BPE	Bovine Pituitary Extract
BSA	Bovine Serum Albumin
c-CrkII	Large adapter protein (SH2/SH3 containing, non-enzymatic)
c-Myc	Proto-oncogene (encodes a regulation transcription factor)
c-Src	Cellular Src ($p60^{SRC}$) also referred to as Src
Cdc42	Member of Rho subfamily of small GTPases
Cdk2	Cyclin dependant kinase 2
Cdk4	Cyclin dependant kinase 4
DCX	Doublecortin (microtubule-associated protein)
DLK	Dual Leucine Zipper-bearing kinase
DMSO	Dimethyl-sulphoxide
DNA	Deoxyribonucleic acid
DOCK180	Dedicator of cytokinesis 180
ECAM-1	Endothelial cell adhesion molecule
ECL	Enhanced Chemiluminescence
ECM	Extracellular matrix
EDTA	Ethylene Diaminetetraacetic Acid
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
ELMO1	Engulfment and cell motility 1
Erk	Extracellular signal-regulated protein kinase
Erk-1 -2	Erk isoforms, 1(-p44), 2(-p42)
FACS	Fluorescence-activated cell sorter
FAK	Focal adhesion kinase ($p125^{FAK}$)

FAK-F397	FAK dominant negative mutant transgene
FAK-R454	FAK dominant negative mutant transgene
FAK-Wt	Wild type FAK transgene
FAT	Focal adhesion targeting domain
FERM	Protein 4.1, ezrin, radixin and moesin homology domain
FGF1, 2, 7	Fibroblast growth factor 1, 2, 7
FGFR2	Fibroblast growth factor receptor 2
FITC	Flourescein isothiocyanate
Fyn	Member of the Src family of kinases ($p59^{Fyn}$)
GAP	GTPase activating protein
GDI	Inhibitor of guanine nucleotide dissociation
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
GF	Growth factor
GFP	Green fluorescent protein
GRAF	GTPase regulator associated with focal adhesion kinase
Grb2	Growth factor receptor bound protein 2
GTE	Gene transduction efficiency
GTP	Guanosine triphosphate
GTPase	Enzyme that can bind and hydrolyze guanosine triphosphate
HbSAg	Hepatitis B surface antigen
HB-EGF	Heparin binding EGF-like growth factor
HBSS	Hank’s Buffered Salt Solution
HBSS+	HBSS with calcium chloride and magnesium sulphate
HBSS-	HBSS without calcium chloride and magnesium sulphate
HCV	Hepatitis C Virus
HDF	Human Dermal Fibroblast
HEK	Human Epidermal Keratinocyte
HIV-1	Human immunodeficiency virus (pre-dominant form)
HIV-2	Human immunodeficiency virus (second most common form)
HRP	Horse radish peroxidase
HSP27	Heat shock protein 27
Ig	Immunoglobulin (antibody)
Ig-SF	Immunoglobulin super-family

ICAM-1	Inter-cellular adhesion molecule-1
IEG	Immediate early gene
IGF	Insulin-like growth factor
IL-1	Interleukin-1 (cytokine)
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
JNK1, 2, 3	JNK 1, 2, 3 isoforms
JNKK1, 2	JNK MAP kinase kinase 1, 2 isoforms
JNKK2-Dn	JNKK2 Dominant negative mutant transgene
JNKK2-Wt	Wild type JNKK2 transgene
KGF	Keratinocyte growth factor
KGFR	Keratinocyte growth factor receptor
LB	Lysogeny broth (also known as Luria broth)
LD	Leucine domain (protein structural domain)
LIM	Protein structural domain
LPS	Lipopolysaccharides
MAP	Mitogen-activated protein
MAPK	Mitogen-activated protein kinase
MAPKAPK 2/3	MAPK-activated protein kinase 2/3
MAPKK	Mitogen-activated protein kinase kinase
MAPKKK	Mitogen-activated protein kinase kinase kinase
MEK	Erk MAP kinase kinase
MEK1/2	MEK 1, 2 isoforms
MEK1-Dn	MEK1 dominant negative mutant transgene
MEK1-Wt	Wild type MEK1 transgene
MEKK	Mitogen-activated protein kinase kinase
MEKK1, 2	MEKK 1, 2 isoforms
MKK-1,-3, -6	Mitogen-activated protein kinase kinase 1, 3, 6,
MKK- 4	MKK-4 also known as JNKK1
MKK- 7	MKK-7 also known as JNKK2
MI	Migration index
MI_{CG}	Migration index for colloidal gold assay
MI_{IV}	Migration index for in *vitro* assay
MLC	Myosin light chain

MLCK	Myosin light chain kinase
MLK	Mixed lineage kinase
MLK3	MLK 3 isoform
MMP	Matrix metalloproteinase
MMP-1, -13	Matrix metalloproteinase (collagenase 1 and collagenase 3)
MMP-2, -9	Matrix metalloproteinase (gelatinase A and gelatinase B)
MMP-3	Matrix metalloproteinase (stromelysin)
MNK	MAP kinase interacting kinase
mRNA	Messenger RNA (ribonucleic acid)
MTOC	Microtubule organizing centre
Nck	Adaptor protein (SH2/SH3 containing, non-enzymatic)
NCBI	National Centre for Biotechnology Information
NIH	National Institute of Health
NZY^{+}	NZ amine (casein hydrolysate) containing broth
OI1.69	Open Imager v1.69 (software program provided by NIH)
p38	p38 MAP kinase
p38-Dn	Dominant negative mutant transgene
p38-Wt	Wild type p38 transgene
p38-α -β -γ -δ	p38 α, β, γ, δ isoforms
$p56^{lck}$	Member of the Src family
$p130^{CAS}$	Crk associated substrate
PBS	Phosphate-Buffered Saline
pcDNA3	Plasmid vector (5446bp) Manufacturer Invitrogen
pcDNA3.1	Plasmid vector (5428bp) Manufacturer Invitrogen
PDGF	Platelet derived growth factor
PDGFR	Platelet derived growth factor receptor
PI_3 (or PI3)	Phosphoinositide 3-kinase
PIP3	Phosphatidylinositol (3,4,5)-trisphosphate
PKC	Protein kinase C
pRK7	Plasmid vector (4708bp) Vector origin unknown
PRR	Proline rich region (protein structural domain)
pRRLsin	Lentiviral vector (6.6kbp) USC Institute of Genetic Medicine
PSP73	Plasmid vector (2464bp) Manufacturer Promega
PTEN	Phosphatase and tensin homologue: a PIP3 phosphatase

pUC18	Control plasmid for X-10 Gold ultracompetent cells
Rab	Rab subfamily of Ras
Rac	Member of Rho subfamily of small GTPases
Rac-1	Rac isoform
Raf	Erk MAP kinase kinase kinase (Raf-1 isoform)
Ran	Ran subfamily of Ras
Ras	Ras subfamily of small GTPases
Rho	Rho subfamily of Ras (Cdc42, Rac, and Rho are members)
RhoA	Rho isoform
ROCK1	Rho-associated coiled-coil containing protein kinase 1
RSK	Ribosomal protein S6 kinase
SAPK	Stress activated protein kinase
Sar	Sar subfamily of Ras
SB202190	Chemical inhibitor of p38 kinase
SB202474	Control to SB202190 (chemical inhibitor p38 kinase)
SB600125	Chemical inhibitor of 1/2JNK kinase
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SH2, 3	Src homology 2, 3 (protein structural domain)
Shc	Adaptor protein (SH2/SH3 containing, non-enzymatic)
Spir	Member of the WASP homology domain 2 (WH2) family
SMAD	Small mothers against decapentaplegic
SOCS	Suppressor of cytokine signalling
SOS	Son of Sevenless (Guanine nucleotide exchange factor)
Src	Src family of non-receptor tyrosine kinases (c-Src, Fyn, Yes1)
Src-KM	Src dominant negative mutant transgene (Src-K295M)
Src-Wt	Wild type Src transgene
t-PA	Tissue plasminogen activator
TAE	Tris-acetate-EDTA
TAK1	JNK MAP kinase kinase kinase
TBS-BSA	Tris Buffered Saline with Bovine Serum Albumin
TBS-Triton	Tris Buffered Saline with Triton x100
TGFβ (1, 2, 3)	Transforming growth factor β (isoforms 1, 2, 3)
TGFβRI	Transforming growth factor β type I receptor (57kDa)
TGFβRII	Transforming growth factor β type II receptor, (75kDa)

TGFα	Transforming growth factor α
TIMPS	Tissue inhibitors of metalloproteinases
TNFα	Tumour necrosis factor α
Tyr	Tyrosine
u-PA	Urokinase-type plasminogen activator
U0124	Control to chemical inhibitor U0126
U0126	Chemical inhibitor of MEK1/2 kinase
USC	University of Southern California
UV	Ultra violet
v-Src	viral oncogene related to Rous sarcoma virus
VCAM-1	Vascular adhesion molecule-1
VEGF	Vascular endothelial growth factor
WASP	Wiscott-Aldritch syndrome protein family of proteins
WAVE	WASP family verprolin homologous (WAVE) proteins
Yes1	Src kinase family member
ZAP	Zeta-associated protein kinase

Chapter 1. Introduction

1.1 Wound healing epidemiology: An overview

One of the main functions of the skin is to serve as a protective barrier against the environment, while simultaneously facilitating and participating in the maintenance of the integrity of the internal milieu and biological systems. Alongside its protective function and role in homeostasis, such as thermoregulation, the skin also participates in a number of other processes. These additional functions are diverse and include sensory perception, a number of endocrine functions notably in vitamin D metabolism, and immune surveillance. The skin is a highly immunologically active organ system situated for first line response in a number of infectious processes, and is also active in prevention of local malignancy. The skin must serve all these functions and more in parallel.

Injurious stimuli may take several forms, most simply physical trauma. In addition and commonly, injury is caused by, heat (burns), disease (such as venous hypertension and stasis), infection (such as cellulitis), UV radiation and many other agents that may also threaten or cause direct harm to the skin. By whatever means the injury occurs, when injured in its line of duty, the skin must be able to repair itself quickly to prevent further harm and to continue to function in its many roles. More extensive injury to the skin can lead to disability, and in the severest cases even death.

In the United Kingdom (UK) approximately 250,000 people suffer accidental burns each year. Of these, 175,000 attend accident and emergency departments, 13,000 are admitted to hospital and 1,000 patients have burns severe enough to warrant formal fluid resuscitation (Hettiaratchy and Dziewulski, 2005). One third of the cases of all severities and half of the more severe cases are children under the age of 14 years. There are on average 300 deaths from burns each year. These UK figures are representative of most of the developed countries of the world, although in some, such as the United States of America (USA), there is an even higher incidence (1.25 million accidental burns per year) (Brigham and McLoughlin, 1996). Burns are also a major problem in the developing world. It is estimated that there are at least two million burn injuries thought to occur each year in India (population 1 billion). Mortality in the developing world is much higher than it is in the

developed countries (Hettiaratchy and Dziewulski, 2005). Chronic ulceration of the lower limbs is associated with a number of disease states, notably venous hypertension and stasis, but also diabetes mellitus, and less commonly other causes, such as arterial insufficiency of various aetiologies and rheumatoid arthritis. Estimates of prevalence and incidence in the UK vary, but a conservative evaluation recognizes prevalence between 1.5 and 3.0/1000 people that have active leg ulcers due to venous stasis alone. The prevalence increases with age to 20^{+}/1000 in those aged over 80 years (Callam et al., 1985). The annual cost to the National Health Service (NHS) in the UK was estimated at over £300 million in 1992 (Laing, 1992). These estimates do not include the loss of productivity at work attributable to this illness. Prevalence and incidence are similar in other developed countries, such as the USA where it is estimated that 6.5 million have chronic skin ulcers caused by the same diseases, and where it is estimated that the cost of treating delayed wound healing in the elderly is over £4.5 billion per year (Wadman, 2005). In the developing world estimates of such problems are invariably significantly greater. Understanding the processes involved during wound healing and the mechanisms by which this may be delayed or interrupted will hopefully lead to improvements in management, and consequently a reduction in morbidity, mortality, and cost.

1.2 Normal wound healing processes

The normal processes of wound healing have evolved to repair tissue defects quickly and efficiently. A wound in the skin is rapidly plugged with a fibrin clot that immediately restores haemostasis. This is followed by progressive replacement of that dead tissue with a matrix of cells and a collagenous material. Simultaneously, the epidermis proliferates and migrates over the wound surface; sealing the defect more thoroughly and covering up the viable newly formed replacement tissue. Fibroblasts and vascular endothelial cells migrating throughout the wound produce much of the collagenous material in the matrix, and they themselves act as an integral structural component. Inflammatory cells are present throughout wound healing, protecting against infection and contributing actively to the process during all phases. One interesting facet of the reconstruction process is that the matrix formed is contractile. As well as filling in the defect, towards the later stages of wound repair, the tissue contracts, reducing the size of the defect and in doing so contributes to more rapid healing and restoration of function. Remodelling of tissue is integral to wound

healing and can continue for a considerable time after the injury, particularly in the instances where scar formation occurs. The dynamic process of wound healing involves soluble mediators many of which are growth factors (GFs), extracellular matrix (ECM) components, and a variety of parenchymal and blood borne cells.

A well-accepted model of normal acute cutaneous wound healing proceeds through three phases that overlap in time: inflammation, tissue formation, and tissue remodelling (including contraction). In each phase many cellular processes occur such as proliferation, migration, matrix synthesis, and wound contraction. The signals regulating these processes, turning them on and off and adjusting activity levels, come primarily from the repertoire and proportions of soluble mediators present, and the composition of the extracellular matrix in the local environment. While other factors are undoubtedly involved, these two signalling sources play crucial central roles in wound healing and details of their actions and interplay are beginning to emerge.

Acute tissue injury causes the disruption of blood vessels and extravasation of blood constituents which begin the process of wound healing with clot formation that re-establishes haemostasis. In addition to haemostasis, the fibrin clot protects the underlying tissue that would otherwise be exposed and provides a nascent extracellular matrix for initial cellular migration. The clot consists of platelets embedded into a mesh of cross-linked fibrin with small amounts of fibronectin and vitronectin also present. Platelets incorporated into the clot (and increasingly over time parenchymal cells adjacent to and within the site of injury) act as a reservoir for soluble mediators that they secrete. These include platelet derived growth factor (PDGF), epidermal growth factor (EGF), and transforming growth factor β (TGFβ1 & 2). Soluble mediators serve a number of functions in effecting wound healing and in regulation of the processes that are occurring (Coulombe, 2003; Bandyopadhyay et al., 2006).

In the absence of a wound skin cells are exposed to an ultrafiltrate of plasma. Following wounding during haemostasis and the formation of the fibrin clot protein cascade, pathways are activated, notably the coagulation cascades. The resulting fluid contains a different protein composition compared to the original plasma and is termed serum. Serum is also a component of the milieu during the early phase of acute wound healing. It contains many of

the same soluble mediators as those produced by the cells present in the wound and others, such as serum-derived plasmin, that may have a particular functional importance and/or be involved in coordinating the systems of events that are occurring (Bandyopadhyay et al., 2006).

Soluble mediators and fragments of extracellular matrix protein recruit inflammatory cells such as neutrophils and macrophages to the site of the wound by chemotaxis (Zigmond, 1974) and by haptotaxis (Carter, 1967) respectively. These cells characterize the early inflammatory phase. Neutrophils usually begin arriving within minutes of cutaneous injury and unless a significant bacterial infection exists, their infiltration ceases after a few days. Any remaining expended neutrophils are phagocytosed by macrophages. Macrophages arrive slightly later and remain longer in the healing wound; they are present throughout the first few weeks. Macrophages and neutrophils (while present) secrete an impressive array of additional growth factors and cytokines such as interleukin-1 (IL-1), tumour necrosis factor α (TNFα), primarily from neutrophils (Hubner et al., 1996), and transforming growth factor α (TGFα), heparin binding - EGF-like growth factor (HB-EGF), TGFβ3, and vascular endothelial growth factor (VEGF) from macrophages (Rappolee et al., 1988). Macrophages also maintain production of many of the same cytokines initially released by platelets such as PDGF, and TGFβ1 & 2. These soluble factors promote, modulate, and sustain the inflammatory response to the injury. They are important in recruitment of further inflammatory cells. The presence of fibrin clot, platelets, inflammatory cells, and these soluble mediators are characteristic of the inflammatory phase of wound healing (Figure 1).

Later in the inflammatory phase, towards the end of the first few days, the formation of granulation tissue becomes favoured by the combination of cells present and soluble factors, primarily derived from the inflammatory cells that are present. Macrophage-derived factors in particular play a pivotal role in the transition from the inflammatory phase to that of tissue formation (Riches et al., 1996), and the combination of factors present likely acts as a "kick-start" to activity in uninjured parenchymal cells adjacent to the wound (Hunt et al., 2000; Brakebusch, 2005)

(Figure 1)

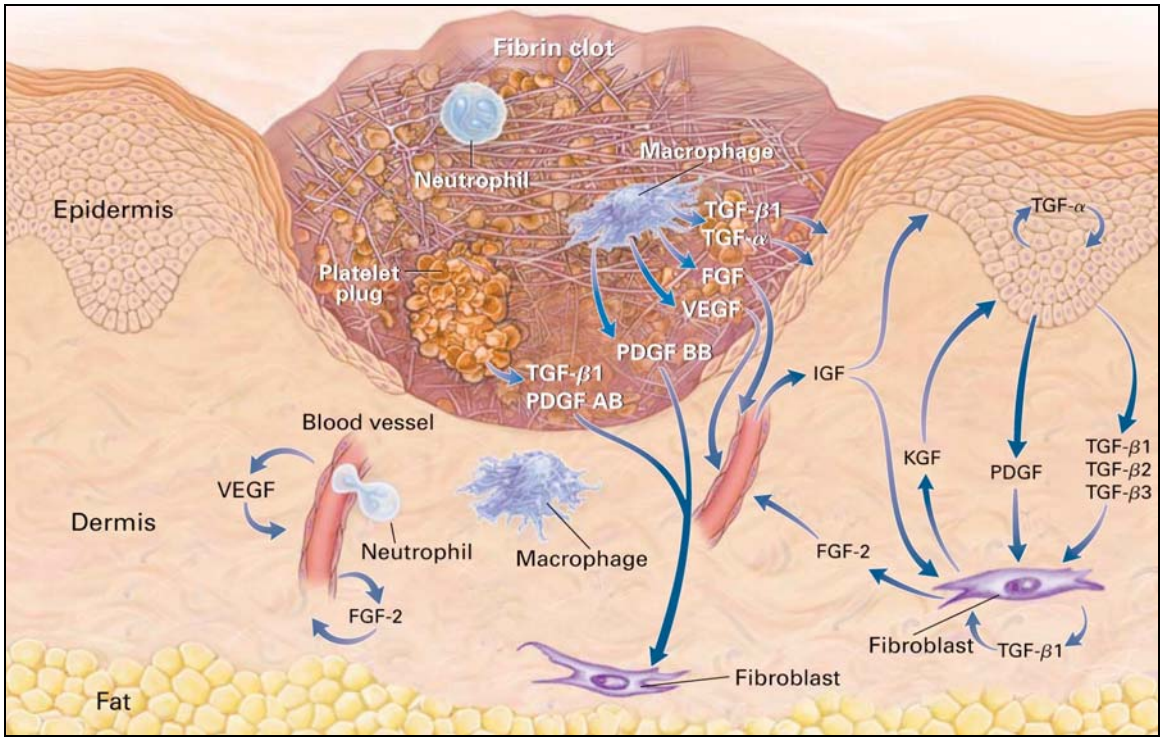


Figure 1: A cutaneous wound showing the important features of the inflammatory phase of acute wound healing. Significant growth factors are shown and include; PDGF (AB and BB), EGF, TGFβ1, 2, and 3, TGFα, Vascular Endothelial Growth Factor (VEGF), Fibroblast Growth Factor (FGF1 and 2), Insulin-like growth factor (IGF), and keratinocyte growth factor (KGF) (Singer and Clarke, 1999).

The next phase of wound healing is characterized by tissue formation and involves simultaneous formation of granulation tissue, neovascularization and re-epithelialization. Granulation tissue begins to replace the fibrin clot in the wound space from the base and margins, and is visible within the wound approximately four days after the acute injury. It comprises macrophages, fibroblasts, and blood vessels suspended in a complex mixture of extracellular matrix components (Figure 2). Fibroblast migration occurs into the fibrin clot of the wound from the margins under stimulation from soluble mediators, chemotaxis, and increasingly by fragments of extracellular-matrix proteins, haptotaxis. Particularly important soluble factors in this regard, include the growth factors PDGF and acidic and basic fibroblast growth factor (FGF1 and FGF2) along with TGFβ. Fibroblasts produce the bulk of the non-cellular portion of granulation tissue with contributions from epithelial and vascular endothelial cells more localized to the sites of activity of those particular cells.

(Figure 2)

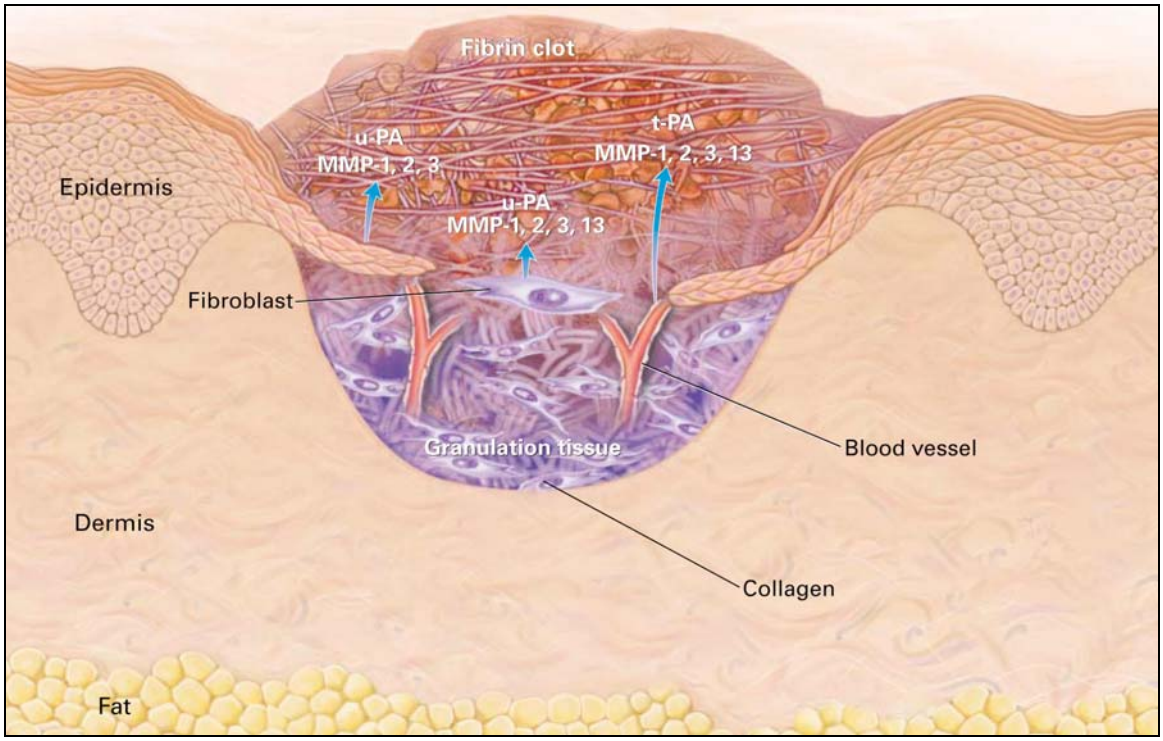


Figure 2: A cutaneous wound showing important features of the tissue formation phase of acute wound healing. Granulation tissue with significant neovascularity lies beneath the fibrin clot. Keratinocytes migrate into the wound dissecting between the two layers. Proteinases thought to be necessary for keratinocyte, fibroblast, and vascular endothelial cell movement are shown. (Urokinase-type plasminogen activator: u-PA; Matrix metalloproteinases 1, 2, 3, and 13: MMP-1, 2, 3, and 13 (collagenase 1, gelatinase A, stromelysin 1, and collagenase 3, respectively); and tissue plasminogen activator: t-PA) (Singer and Clarke, 1999).

The earliest provisional extracellular matrix, produced in large part by fibroblasts, is a mixture of fibrin, type I collagen, fibronectin, and hyaluronic acid (Clark et al., 1982). This provisional matrix becomes progressively more collagenous, containing increasing amounts of type I collagen, over time (days), due to the activity of fibroblasts within the wound. This progressive transition is under the influence of TGFβ1 and is regulated by TGFβ3 (Welch et al., 1990; Bandyopadhyay et al., 2006). Also under the influence of soluble factors such as TGFβ1, some fibroblasts transition to a phenotype referred to as myofibroblasts. Contractile activity in these cells and the changes occurring with collagen processing in the non-cellular matrix material contribute to wound contraction, a phenomena which increases with time (days) during normal wound healing.

The formation of new blood vessels is required to sustain the cells within the newly formed granulation tissue, and neovascularization is a substantial and integral part of the entire process. The mechanisms behind this angiogenesis are complex and rely upon the composition of the extracellular matrix, cellular migration and mitogenic stimulation of vascular endothelial cells (Arnold and West, 1991). These endothelial cells contribute generally to the composition of the extracellular matrix, but most significantly to the extracellular matrix in their local environment. Neovascularization is stimulated by low oxygen tension and high lactic acid in the wound, as well as by soluble factors secreted by epithelial cells and macrophages, such as basic (or acidic) fibroblast growth factor initially (FGF), and subsequently by vascular endothelial growth factor (VEGF) which is secreted by macrophages and keratinocytes (Figure 1).

Re-epithelialization of wounds begins as early as a few hours after injury and by day four is well underway (overlapping with the inflammatory phase described above and integral to the tissue formation phase); it continues until the wound is completely covered. In an acute wound keratinocytes at intact uninjured sites adjacent to the wound edge become involved in wound repair but are initially stably attached basal and suprabasal epithelial cells. These cells would have shown the usual balance of proliferation and differentiation, and as they move up out of the basal layer, committing towards the terminal differentiation that occurs in the outer upper layers of the epidermis. However, in response to epidermal injury, the same keratinocytes at the wound edge previously destined for terminal differentiation, undergo changes in gene expression, biochemistry and morphology referred to as "activation" and become involved in wound repair (Grinnell, 1992). In the healing wound, activated keratinocytes spread outward at a superficial level into the wound space and fibrin clot in a contiguous sheet from surviving skin appendages, such as hair follicles, and from the wound edge (Odland, 1977; Singer and Clarke, 1999). Both proliferation and migration of keratinocytes are necessary for this spreading to occur during wound healing *in vivo* and factors that impede these processes are known to impair the rate of re-epithelialization (Epstein et al., 1893). Two theories of wound re-epithelialization have been proposed: "leap-frogging" (Krawczyk, 1971) and "tractor-tread" (Woodley, 1996). "Leap-frogging" supposes that keratinocyte proliferation occurs away from the wound edge and cells are pushed into a suprabasilar compartment and onto the wound bed. During this process keratinocytes driven into the suprabasilar position down regulate desmosome expression and migrate over basally situated keratinocytes until they themselves make contact with the

wound bed. The “tractor-tread” model proposes that keratinocytes in the basilar position migrate over the wound bed and maintain some desmosomal junctions between cells, resulting in the trailing epidermis being dragged over the wound bed. In this model suprabasally situated keratinocytes appear to play a more ancillary role.

While many of the details of wound re-epithelialization *in vivo* remain to be proven, nonetheless a number of attributes of the process are becoming clear. Activated keratinocyte proliferation during wound healing occurs in the basal and immediate suprabasal layers of intact epithelium at and adjacent to the wound edge and at any remaining appendageal margins. This finding was first suggested as early as 1986 (Bereiter-Hahn, 1986) and more recently confirmed by showing expression of Ki-67, a marker of proliferation, expressed only in keratinocytes at those sites in acute healing wounds *in vivo* (Patel et al., 2006). Analysis of patterns of keratin filament expression has been used to identify a number of distinct populations of keratinocytes within an acute healing wound and, although not one of the techniques used in this thesis, has added significantly to our understanding of the process. In an acute healing wound, activated basal keratinocytes at both the wound margin and on the base of the wound, express keratins K5 and K14 and continue to do so upon completion of re-epithelialization (these keratins are considered markers of basal cell differentiation status). Activated suprabasal keratinocytes show a more complex pattern of keratin expression during acute wound healing that is dependent upon their position in both the wound and within the suprabasal compartment. In general, keratin K10 (a marker of suprabasal differentiation) is expressed in suprabasal keratinocytes at the intact wound edge, while keratinocytes in the suprabasal position covering the wound centre expressed keratins K6, K16 and K17 in a more complex manner (Patel et al., 2006).

Following activation of keratinocytes in an acute healing wound, a number of changes in cellular adhesion have also been reproducibly demonstrated; compared to normally proliferating and differentiating keratinocytes in intact epithelium, activated keratinocytes re-epithelializing the wound bed show alterations in both cell-cell and cell-basement membrane adhesions. At the leading edge of the spreading sheet of keratinocytes in a healing wound a few layers of cells are moving into a region with limited cell contact in the direction of migration. Following behind, there are many more layers of trailing cells that are surrounded in all directions, except at their base, by other similar cells and have some

cell-cell contact with them. Generally, within the spreading sheet of re-epithelializing keratinocytes all cells maintain a degree of cell-cell contact via the interactions of cadherins on the cell surface (Choma et al., 2007; Gilcrease, 2007). However, in both populations (leading and trailing) keratinocytes, there is a regulated reorganization and reduction, in complexity and number, of certain types of the normally well-characterized cell-cell contacts present in unaffected intact epithelium. Examples are, adherens junctions that are intimately linked to the actin cytoskeleton (Ray et al., 2007) and desmosomal junctions that are intimately linked to the keratin cytoskeleton (Green and Jones, 1996). In addition basal keratinocytes on the wound bed show no formation of hemidesmosomes, a structure that, in intact epithelium, firmly attaches the keratin cytoskeleton of the basal cell to the basement membrane (Hintermann and Quaranta, 2004; Gilcrease, 2007). At the same time, the basal keratinocytes of the re-epithelializing sheet form new, albeit transient, adhesions between the actin cytoskeleton and the connective tissue of the healing wound (Zamir and Geiger, 2001; Zaidel-Bar et al., 2004). These transient adhesions, not seen in intact epithelium, are termed focal contacts and as they mature, focal adhesions (Abercrombie et al., 1971). Focal adhesions are assemblies of large numbers of molecules, which at their core are centred on clusters of integrins interacting with surrounding extracellular matrix proteins (Zaidel-Bar et al., 2004). Migrating cells demonstrate a physical and biochemical polarization with formation of actin based cytoskeletal projections called lamellipodia and filopodia that are seen in greatest density at the advancing margin of leading edge cells (Stossel, 1993; Small et al., 2002). During wound healing new focal adhesions form primarily at the most advanced (leading edge) margin within the lamellipodia (Huttenlocher et al., 1995; Burridge et al., 1997). The cell body is translocated over these focal adhesions (that are attached firmly to the underlying extracellular matrix) effectively moving the cell forwards, while the focal adhesion is, relative to the cell body, repositions more “posteriorly” towards the rear of the migrating cell where they are disassembled (Gabbiani et al., 1978).

Formation, translocation and disassembly of focal adhesions are the quintessential processes of cell migration, linking the extracellular matrix of the wound through integrins to the actin cytoskeleton of the cell. Understanding this and other processes central to migration while gaining insight into the influences on them due to soluble growth factors and the role of mediators such as FAK (focal adhesion kinase) and Src (family of non-receptor tyrosine kinases) is the subject of this thesis.

It is worth noting that while keratinocytes in an acute wound *in vivo* migrate as a contiguous sheet, individually migrating keratinocytes display gene expression profiles, biochemical polarization and phenotypical morphology that are characteristic of activated cells at the advancing wound edge *in vivo* (Choma et al., 2004; Frank and Carter, 2004; Harper et al., 2005). Observations from these and similar studies suggest that for individual migrating cells, cellular interactions with soluble factors and with the extracellular matrix are sufficient (without cell-cell interactions) to enable many aspects of the *in vivo* activated phenotype and hence that analysis of individually migrating keratinocytes provides a powerful experimental model system for dissecting integrin and soluble factor signalling pathways. The principal assay of keratinocyte migration used in this thesis is the "colloidal gold migration assay" under the conditions of this assay keratinocytes migrate individually. Results from the colloidal gold migration assay are supplemented by those of the "*in vitro* wound assay" during which keratinocytes migrate as contiguous sheets into an artificially created "wound space". Under conditions of the *in vitro* wound assay, the environment is more closely analogous to that seen in healing of an acute wound *in vivo*. (The colloidal gold migration assay and *in vitro* wound assay are described in detail in Chapter 2).

Activated keratinocytes in an acute wound *in vivo* experience multiple migratory stimuli and the mechanisms regulating migration may differ between keratinocytes at the leading edge of the migrating sheet and the trailing cells following behind. The stimuli for the migration (and proliferation) of epidermal cells during re-epithelialization include absent or altered cell-cell contact at the margin of the wound, exposure to serum rather than the ultrafiltrate of plasma, and the presence of chemotactic and haptotactic stimuli from soluble factors and extracellular matrix components (Miranti and Brugge, 2002; Ridley et al., 2003). The composition of extracellular matrix components in the environment and pattern of expression of integrins on keratinocytes are important factors that influence the processes leading to keratinocyte migration (and wound healing) (Borradori and Sonnenberg, 1999; Choma et al., 2004). Within keratinocytes, FAK and Src are central to these responses (Choma et al., 2007). Also involved and interlinked are the effects of the milieu of soluble mediators that are present and which act through their own receptors and a number of unique and sometimes shared downstream signalling pathways within the migrating cell (Zhu and Assoian, 1995; Sieg et al., 2000; Aplin et al., 2001).

Soluble factors reported to influence migration in keratinocytes include, epidermal growth factor (EGF) from platelets, and the closely linked transforming growth factor α (TGFα), and heparin binding-EGF-like growth factor (HB-EGF) both secreted significantly by macrophages (Li et al., 2006). All three of these soluble factors, act as ligands through the EGF receptor (EGFR). Keratinocyte growth factor (KGF, also known as fibroblast growth factor 7; FGF7) is secreted by fibroblasts and is a powerful keratinocyte mitogen (Werner et al., 1994; Guo et al., 1996) and recently, KGF has been shown to be an important stimulator of keratinocyte migration during wound healing. It acts as a ligand through a constitutively expressed splice variation of the FGF Receptor 2 (FGFR2) (Werner et al., 1992). However, some research suggests that KGF and EGF, TGFα, etc. may have equivalent effects in certain functions, notably achieving the same outcomes in some wound healing processes (Guo et al., 1996). Several other growth factors and cytokines are involved in keratinocyte migration in the regulatory process such as TGFβ1 and 3 and some pro-inflammatory cytokines appear to simulate expression of patterns of integrin expression that facilitate keratinocyte migration (Tsuboi et al., 1993).

While these soluble factors are believed to be the most important amongst those acting to promote migration of keratinocytes, many others are known to contribute, including a number of factors present in serum (Werner et al., 1994; Li et al., 2006). These studies, analyzing the putative pro-migratory factors in serum, have identified a variety of other factors that stimulate migration. They have also demonstrated that whole serum is a more potent stimulator of keratinocyte migration than plasma, and that whole serum is a more potent stimulator of keratinocyte migration than any of the individual constituent pro-migratory soluble factors that have been identified to date. Keratinocytes in unwounded skin experience an ultrafiltrate of plasma and are only exposed to serum at the time of injury and for a period of time during wound healing. It is reasonable to conclude that the mixture of soluble factors present in serum is significant in initiating, promoting and maintaining keratinocyte migration during wound healing *in vivo* (Li et al., 2006).

Changes in integrin expression take place concomitantly with changes in the extracellular matrix composition, suggesting a close interplay of these two groups of molecules during wound healing (Larjava et al., 1993; Borradori and Sonnenberg, 1999; Hynes, 2002; Frank and Carter, 2004; Choma et al., 2007). As they migrate, leading edge keratinocytes are known to deposit components of the extracellular matrix, notably laminin-5, onto the

existing provisional matrix composed of fibrin, type I collagen, fibronectin and hyaluronic acid (Nguyen et al., 2000). The surroundings (as described above), and the extracellular matrix which trailing keratinocytes experience and are in contact with, is different to that of the leading edge cells, containing a far greater proportion of laminin-5, in addition to type I collagen as wound healing progresses (Nguyen et al., 2000; Hintermann and Quaranta, 2004).

The direction of the dissecting path of keratinocyte migration appears to be determined by the array of integrins expressed on their cell membranes (Odland, 1977; Clark et al., 1996). The pattern of integrin expression on migratory keratinocytes favours movement on type I collagen and laminin-5 and to a lesser extent fibronectin and vitronectin (Clark et al., 1982; Clark et al., 1996). Expression of integrin receptors on epidermal (and other) cells allows them to interact with a variety of extracellular matrix proteins present (Gilcrease, 2007). Expression of particular repertoires of integrins on keratinocytes is a manifestation of the migrating phenotype, as is their distribution over the cell surface (e.g. migrating leading edge cells highly up-regulate and express many integrins including α2β1, and show a particular distribution of α3β1 and α6β4 integrins) (Goldfinger et al., 1999; Russell et al., 2003; Choma et al., 2004; Gilcrease, 2007). Expression of the integrin β_1 subunit is significantly increased in keratinocytes throughout wound healing in both leading and trailing cells. The β_1-subunit is primarily associated with α_2 and α_3 subunits, which mediate integrin interactions with type I collagen and laminin-5 respectively, and play key roles in keratinocyte migration (Nguyen et al., 2000; Nguyen et al., 2001). During re-epithelialization the β_1-subunit is associated with other α-subunits, such as the α_5 subunit ($\alpha_5\beta_1$; the fibronectin receptor) that is up regulated primarily in fibroblasts but also in some migrating keratinocytes (Laukaitis et al., 2001; Gilcrease, 2007). Migrating keratinocytes in a wound also express α_v integrins that are not present in the unaffected unwounded epithelium (Clark et al., 1996). In association with β_6-subunit this is know to interact with fibronectin and in association with the β_3-subunit this is known to interact with vitronectin, both fibronectin and vitronectin are components of the earliest extracellular matrix (Kiosses et al., 2001). All keratinocytes also highly expressed the $\alpha_6\beta_4$ integrin during migration (which mediates integrin-laminin and integrin-kalinin interactions and may serve a control or regulatory function) (Niessen et al., 1994; Nguyen et al., 2001). Many of the up-regulated migratory integrins are localized to cellular protrusions such as lamellipodia and filopodia (Goldfinger et al., 1999; Nguyen et al., 2001). In a non-migratory state β_1-

integrins (e.g. $\alpha 3\beta 1$) are located primarily on the cell-cell region of the plasma membrane and $\alpha_6\beta_4$ integrins are located on the basal surface adjacent to the basement membrane in association with hemidesmosomes. Migratory keratinocytes at the leading edge show a reversal of this pattern and those migratory cells in the trailing portion show a mixture of both $\alpha_6\beta_4$ and β_1-integrins on the basal surface (Goldfinger et al., 1999).

An equally important facilitator of migration is the active proteolytic system. Proteolysis facilitates migration by cleaving a path through the wound, and also is the process involved in modifying the properties of the extracellular matrix as it is deposited and remodelled (both type I collagen and laminin are modified extensively by proteolysis) (Xu and Clarke, 2000). A variety of matrix metalloproteinases (MMPs) secreted by migrating cells such as macrophages, epithelial cells, endothelial cells, and fibroblasts, in addition to serum-derived plasmin, and tissue plasminogen activator are involved in these processes (Madlener et al., 1998; Xu and Clarke, 2000). Briefly, the migrating keratinocyte sheets cross the wound bed, progressing between the eschar of the wound and the viable granulation tissue on newly synthesized extracellular matrix. Proteolysis involving matrix metalloproteinases (MMPs 1, and 13 (colaginases 1 and 3 respectively), MMPs 2 and 9 (gelatinases A and B respectively), and MMP 3 (stromelysin), serum-derived plasmin, and t-PA is essential in this process (Madlener et al., 1998) (Figure 2). MMP-9 (gelatinase B) cleaves collagen IV, and VII, and is thought to be involved in the release of basal keratinocytes from the basement membrane zone adjacent to the wound as they start to migrate. MMP-1 (interstitial collagenase 1) is up regulated in cells at the leading edge of migration and likely plays an important role in facilitating migration across the wound and into the fibrin clot. Up-regulation of MMP-1 is under control pathways mediated via interactions between integrins and the surrounding extracellular matrix.

In the final phase of wound healing tissue remodelling supervenes (Bereiter-Hahn, 1986; Singer and Clarke, 1999). Once an abundant collagen matrix has been deposited in the wound, fibroblast production of collagen decreases, and a relatively acellular scar progressively replaces the fibroblast-rich granulation tissue. Cells in the wound (notably fibroblasts) undergo apoptosis that is triggered by unknown signals (Desmouliere et al., 1995). Collagen remodelling during the transition from granulation tissue to scar is dependent on continued synthesis and catabolism of collagen the later occurring at a progressively lower rate. The degradation of collagen in the wound is controlled by many

of the same proteolytic enzymes that allow for migration through the wound (Xu and Clarke, 2000). In fact, the various phases of wound repair appear to rely on distinct combinations of matrix metalloproteinases and tissue inhibitors of metalloproteinases (TIMPS) (Mignatti et al., 1996; Madlener et al., 1998).

Completion of re-epithelialization is accompanied by a number of changes within the newly formed epithelium. There is synthesis of a new basement membrane and restoration of the profile of gene expression, and of the biochemical and morphological changes in the keratinocytes present; from migratory (those previously moving across the wound bed) and proliferative (those adjacent and at the wound margin) to that of stably attached basal and suprabasal epithelial cells that again show the usual balance of proliferation and differentiation as they move up out of the basal layer, committing towards the terminal differentiation that occurs in the outer upper layers of the epidermis. During the migratory phase, motile keratinocytes produce laminin-5 (particularly those at the leading edge), and kalinin (an epithelium-specific basement membrane adhesion molecule that is a component of the anchoring filaments of hemidesmosomes) (Rousselle et al., 1991; Nguyen et al., 2000). However, other typical components of the basement membrane zone, other collagens (type IV and VII), and other laminins (1 and 10), are not expressed until migration ceases (Clark et al., 1982). When the epithelial sheets join and merge, migration subsides and the missing basement membrane components are synthesized throughout the entire wound area proceeding from the edges toward the centre (migrations ceases first at the wound edges and last where the opposing sheets of migrating cells meet).

With the completion of re-epithelialization epidermal cells revert to a non-migratory phenotype and become firmly attached to the newly formed basement membrane. Integrin expression returns to that of the non-migratory phenotype, with reversal of the changes previously described, including redistribution of β_1-integrins (e.g. $\alpha 3\beta 1$) to locations primarily on the cell-cell region of the plasma membrane and $\alpha_6\beta_4$ integrins to locations on the basal surface adjacent to the basement membrane (Goldfinger et al., 1999). At the same time there is reappearance of hemidesmosomes, linking the basal keratinocytes firmly to the newly formed basement membrane, and formation of increased numbers of fully developed cell-cell contacts such as adherens and desmosomal junctions (Gilcrease, 2007). Similarly keratin filament expression returns to the patterns seen in intact skin demonstrating the

typical proliferation and differentiation from basal to suprabasal cell populations (Fuchs, 1990; Coulombe, 1997).

1.3 Molecular basis of cell migration: An outline

Cell migration is a highly organized and integrated multi-step process. Migration is predominant during embryogenesis and in the adult during wound repair as described above. It is also a feature of the pathogenesis of several known diseases, notably cancer. Complex assembly/disassembly and regulatory pathways are involved that spatially and temporally integrate the component processes of migration. In unwounded (non-migratory) epithelium the keratin cytoskeleton of keratinocytes is attached firmly to the extracellular basement membrane via integrins (e.g. $\alpha_6\beta_4$ in association with hemidesmosomes). Under the same conditions cell-cell adhesion between keratinocytes occurs by a number of junctional structures notably those centred on proteins in the cadherin family including; adherens junctions (E-cadherin) which link to the actin cytoskeleton and desmosomal junctions (desmoglein and desmocollin) which link to the keratin cytoskeleton. However during healing of an acute wound, the actin cytoskeleton of activated migratory keratinocytes becomes linked to the extracellular matrix via integrins (e.g. $\alpha_2\beta_1$ and $\alpha_3\beta_1$) in association with focal adhesion formation and the translocation of the cell over this link to the extracellular matrix to which the focal adhesions are attached leads to keratinocyte migration. In the migratory state, cell-cell adhesion is still maintained by cadherins, notably E-Cadherin (Hashizume et al., 1996), and is supplemented by the interactions of integrins such as $\alpha_6\beta_4$ and $\alpha_3\beta_1$(Goldfinger et al., 1999; Friedl and Wolf, 2003). Together these interactions maintain keratinocyte adhesion during collective epithelial migration.

For a cell to migrate, it must be polarized physically and in terms of the molecular processes occurring (that is the molecular processes occurring at the front and back of a migrating cell are different) (Ridley et al., 2003). Establishing and maintaining cell polarity in response to extracellular stimuli is mediated by interlinked processes involving Rho family GTPases (such as, Rac-1, Cdc42, and Rho-A), phosphoinositide 3-kinases (PI3-kinases), integrins, microtubules, and vesicular transport systems (detailed below). A useful molecular model of cell migration is that of a cyclical process of repeating elements that result in net movement of the cell forwards (Lauffenburger and Horwitz, 1996). Initial polarization occurs in response to chemotactic and haptotactic stimuli to migrate. In the presence of a

chemotactic agent, PIP3 (phosphatidylinositol (3,4,5)-trisphosphate) is produced at the leading edge through the localization of PI3-kinase to the leading edge and its activity at that site, and by the localization of PTEN (a PIP3 phosphatase) to the cell margins and rear (Funamoto et al., 2002). (PTEN and myosin II are implicated in restricting protrusions to the cell front). The cell then becomes physically polarized extending membrane protrusions in the direction of migration, broad flat lamellipodia or finger-like filopodia. Within lamellipodia, actin filaments form a branching "dendritic" network, whereas in filopodia they are organized into long parallel bundles (Schaub et al., 2007).

Rho family small GTPases are pivotal regulators of actin stress fibre formation and control the formation of lamellipodia and filopodia as well as of focal adhesion formation and organization (Etienne-Manneville and Hall, 2002). Rac (Rac-1) and Cdc42 are active at the front of the cell (most likely due to the action of several GTPase regulators which are activated locally by PI3-kinase production of PIP3) (Ridley et al., 2003). Targets of Rac and Cdc42 that mediate actin polymerization and the formation of protrusions are the WASP/WAVE family of Arp2/3 complex activators (Bompard and Caron, 2004). Rac stimulates lamellipodia extension by activating WAVE proteins and Cdc42 binds to WASP proteins, which *in vitro* stimulates the Arp2/3 complex to induce dendritic actin polymerization (Welch and Mullins, 2002; Bompard and Caron, 2004). Protrusion formation is driven mechanically by actin polymerization that in lamellipodia is mediated by the Arp2/3 complex. The Arp complex binds to the sides or tip of a pre-existing actin filament and induces the formation of a new daughter filament that branches off (Welch and Mullins, 2002). Regulation of Arp2/3 complex-mediated motility is complex and involves heterodimeric capping protein and the ADF/cofilin family of proteins, which have been proposed to promote lamellipodial protrusion by driving both actin assembly and disassembly (Carlier et al., 1999; Loisel et al., 1999; Ghosh et al., 2004).

Cdc42 is also implicated in generating polarity by localizing the microtubule-organizing centre (MTOC) and Golgi apparatus in front of the nucleus, oriented toward the leading edge. Cdc42-induced MTOC orientation may contribute to polarized migration by facilitating microtubule growth into the lamella and microtubule-mediated delivery of Golgi-derived vesicle trafficking toward the leading edge, providing membrane and associated proteins needed for forward protrusion (Etienne-Manneville and Hall, 2002; Rodriguez et al., 2003).

In the presence of enhanced Rac activity a number of processes have been identified that help to maintain polarized morphology by cellular protrusion formation. Firstly, Rac activity can stimulate recruitment and/or activation of PI3-Kinase at the plasma membrane, which in turn as described above can activate several GTPase regulators of Rac via PIP3 (Ridley et al., 2003). Secondly, Rac and microtubular activity may form a positive feed back loop in which microtubule polymerization activates Rac, and Rac in turn stabilizes microtubules (Rodriguez et al., 2003). Thirdly, Rac is activated (and recruited to the membrane) by integrin engagement with extracellular matrix protein. This leads to focal adhesion formation (at the leading edge) that in turn enhances recruitment and clustering of integrins to the same site at the leading edge of lamellipodia (Schwartz and Shatti, 2000; Kiosses et al., 2001)

Focal adhesions serve to stabilize cellular protrusions and act as sites of traction for migration remaining in place as the cell moves forward. Ultimately, focal adhesions are repositioned rearward under the action of the mechanical changes in the cytoskeleton and then disassembled. In some instances disassembly (turnover) occurs in the front of the cell (near to the origin of the protrusion) with the release allowing formation of new protrusions. In other cases disassembly is at the rear of the cell after the cell has translocated entirely over the focal adhesion (producing forwards migration). Focal adhesion disassembly and turnover is under the control of proteins within the intracellular focal adhesion complex notably FAK and Src and others such as paxillin and extracellular signal-regulated protein kinase, (Erk).

Having reviewed wound healing and outlined its molecular basis before proceeding to detail the elements of that process to be investigated, an overview of cell receptor signalling, second messengers and of pertinent soluble growth factor receptors and their mechanisms of action is presented. In addition cellular adhesion molecules are reviewed focusing on integrins and their links to focal adhesion dynamics and their interactions with key components FAK and Src. FAK and Src along with soluble growth factors are central to this investigation.

1.4 Cell surface signalling: General principles and cutaneous receptors

Molecular communication between cells is fundamental to the existence and wellbeing of multicellular organisms, is similar between individual or groups of cells and their environment. Aberrant communication contributes to the pathogenesis and pathophysiology of many skin diseases, including neoplastic conditions. Proliferation, differentiation, migration, and cell death, amongst many other activities demonstrated by individual cells are all processes responsive to stimuli from neighbouring cells and the local environment. Despite the diversity of these processes and the larger diversity of pathways involved in them, the fundamental model of a single element within the process is simple, consisting of a signal, a cellular receptor, a transduction system into the cell, and a downstream effector system within the cell in each case (Schwertz, 1994). Schematically, a cell surface receptor has three domains, the extracellular ligand binding domain, a transmembrane region (typically hydrophobic) and an intracellular effector domain (a scheme analogous to the larger pathway as a whole). In many cell surface receptors the intracellular domain has auto-phosphorylation activity, contains a binding site(s) for downstream effectors and has a tyrosine kinase domain. The degree of auto-phosphorylation, the affinity of the internal binding sites and the kinase activity expressed are determined by external influences, notably the state of ligand binding at the extracellular domain.

A group of enzymes referred to as protein tyrosine kinases play an important role in the regulation of many cellular processes. They are sub-classified into two groups, receptor tyrosine kinase and non-receptor tyrosine kinases. Receptor tyrosine kinases demonstrate the typical arrangement of cell surface receptor consisting of a single polypeptide chain with extracellular, transmembrane and intracellular segments. (All of the known peptide growth factor receptors, including EGFR, KGFR, and PDGFR are directly or indirectly coupled receptor tyrosine kinases and will be discussed in more detail below).

1.5 Second Messengers: General principles and cutaneous systems

Within cells, ligand activated receptors influence downstream signalling mechanisms that lead to a cascade of change, ultimately mediating the intended effect under normal circumstances. Amplification, inhibition, and feedback are characteristic features of these complex cascades. Many cellular elements are the end stage targets of these cascades including effects on regulation of nuclear transcription and modification of functional and

structural membrane and cytosolic proteins. Activated integrins (integrin receptors) can link to elements of the cellular cytoskeleton and participate directly in effector activity. Uniquely, integrins are involved in both in the control and effector functions in some of the pathways that they influence.

Elements of the downstream cascades include proteins with specific conserved binding sites such as SH2 (Src homology 2) and SH3 (Src homology 3) domains (or affinity for such regions). These downstream effectors may also have kinase activity and are referred to as non-receptor tyrosine kinases or non-receptor serine/threonine, kinases depending on the type of kinase activity exhibited. Those that do not are referred to by other names according to their function, such as adaptor proteins. Other elements of the downstream cascades include, the G-protein systems (heterotrimeric G-protein receptor systems and small GTPase systems, discussed below), the adenylate cyclase and cyclic-AMP (adenosine monophosphate) system (which is frequently linked to G-protein activation), the phospholipases (such as phospholipase A2, that are commonly activated by G-proteins and also by receptor tyrosine kinases) and the lipid kinases such as PI3 Kinase (phosphatidylinositol 3 kinase), which are involved in some manner in almost all receptor tyrosine kinase systems.

The PI-kinases are categorized into three families with phosphatidylinositol 3-kinase (PI3-kinase) being the most important in migration. PI3-kinase is an enzyme-complex containing an 85kDa regulatory subunit and an 110kDa catalytic subunit that phosphorylates hydroxyl groups on the inositol ring of phosphatidylinositol. Phosphatidylinositol contains five free hydroxyl groups that are available for phosphorylation by phosphoinositide kinases, and for de-phosphorylation by phospholipases. Amongst the most important lipid product is phosphatidylinositol (3,4,5)-trisphosphate (PIP3) that results from the activity of PI3-Kinases on phosphatidylinositol (4,5)-bisphosphate. Stimulation of the cells with growth factors causes rapid increase in PI3-kinase activity and increased association of PI3-kinase with tyrosine-phosphorylated growth factor receptors. The lipid products of PI3-kinase (e.g. PIP3) act as second messengers by binding to and activating a diverse array of cellular target proteins. These events constitute the start of a complex signalling cascade, which ultimately results in the mediation of cellular activities such as proliferation, differentiation, lamellipodia formation, migration, and adhesion (Vanhaesebroeck et al., 1996).

The Rho family of small GTPases (20-25kDa) are guanine nucleotide (GTP)-binding proteins. The family includes the Rho subclass (the most widely studied is RhoA), the Rac subclass (the most widely studied is Rac-1) and the Cdc42 subclass (the most widely studied is Cdc42). Rho small GTPases were originally identified and characterized during the search for proteins homologous to the Ras proto-oncoprotein (Satoh et al., 1992) and are classified within the Ras superfamily which is composed of more than 50 proteins in six main families: Ras, Rho, Arf, Sar, Ran and Rab. Each of the proteins exists in two inter-convertible forms: GDP-bound (guanosine diphosphate- bound), which is inactive and GTP-bound (guanosine triphosphate-bound) which is the active form. Their activity is regulated by guanine nucleotide exchange factors (GEFs) that turn them on, GTPase activating proteins (GAPs) that turn them off, and guanine nucleotide dissociation inhibitors (GDIs) that can function as sequestering agents typically down-regulating their activity (Ellenbroek and Collard, 2007). In turn the activities of many of these proteins are regulated by a wide variety of other signalling systems including the PI-kinases (PI3 kinase) and FAK and Src-family kinases. In resting cells Rho GTPases exist mostly in GDP-bound form and in complex with Rho GDIs in the cytosol. When activated (the GTP-bound form) of Rho GTPases are typically localized at cell membranes. Small GTPases of the Rho family control cell growth, migration, and organization of cell cytoskeleton amongst other activities, and they are also involved in transformation and metastasis (Jaffe and Hall, 2002).

The mitogen-activated protein (MAP) kinase cascades are families of protein kinases (non-receptor serine/threonine kinases) that utilize sequential kinase activation to regulate various cellular processes, including cell proliferation, migration, differentiation, and cellular response to physical stress (they are discussed in more detail in Chapter 5). Initiation of the MAP Kinase cascade can result from direct receptor stimulation by ligand, such as EGF and the EGFR interaction, or indirectly via other mechanisms such as integrin interaction with extracellular matrix (discussed in more detail in Chapter 6). The cumulative effect of activation of the MAP Kinase cascade is the phosphorylation and regulation of many cellular signalling molecules, including cell surface proteins, cytoskeletal components, and membrane-bound and cytoplasmic kinases. In addition and importantly there is activation of intranuclear transcription by downstream effects of activated MAP kinases that can migrate into the nucleus. These cellular changes in turn lead to the intended cellular response to the initial stimuli (Lin et al., 1993; Johnson and Lapadat, 2002).

1.6 Extracellular soluble factors related to keratinocyte migration and their pathways in wound healing

The model of wound healing described above is a dynamic process involving interplay between soluble mediators (many of which are growth factors), extracellular matrix components, and a variety of parenchymal and blood borne cells. It proceeds through three phases that overlap in time: inflammation, tissue formation, and tissue remodelling as previously described. At each stage different combinations of these elements are present and are functionally important. Important soluble factors influencing keratinocyte migration include EGF largely from platelets, TGFα and HB-EGF both secreted primarily by macrophages which all act through the EGF receptor (Derynck, 1986; Cha et al., 1996; Li et al., 2006). TGFβ1 and 3 and a number of pro-inflammatory cytokines appear to simulate expression of patterns of integrin expression which facilitate keratinocyte migration (Tsuboi et al., 1993) and many other soluble factors are known to contribute including a number of factors identified in serum (Werner et al., 1994; Li et al., 2006).

1.6.1 EGF TGFα and HB-EGF and the EGF Receptor

As early as 1987 both EGF and TGFα were proposed as pro-migratory factors (Barandon and Green, 1987), but experimental design did not allow for the potential confounding effect of the proliferation induced by these agents, which left the conclusions in doubt. *In vitro* migration assays experiments (Chen et al., 1993) and later those using the colloidal gold migration assays (Cha et al., 1996) subsequently allowed unequivocal demonstration that these factors are indeed pro-migratory. The *in vitro* wound assays and colloidal gold migration assays will be used extensively in later experiments.

In responsive cells, all known effects of the soluble factors EGF, TGFα, and HB-EGF are mediated through binding to a specific membrane receptor; the EGF receptor. This receptor is a single- chain glycoprotein (1186 amino acids) containing the three characteristic functional domains mentioned above, an extracellular, glycosylated portion that binds EGF, a small transmembrane portion (lipophilic), and a cytoplasmic portion that has the intrinsic tyrosine kinase activity and multiple sites that can be phosphorylated (creating binding sites such as those motifs recognized by SH2 domains). The receptor when bound to ligand homo- (or on occasion hetero-) dimerizes and undergoes a conformational change that leads to auto-phosphorylation and an enhanced kinase activity intracellularly. (The binding

affinities of EGF or TGFα and to some degree the kinase activity of the receptor are both regulated by the phosphorylation of the receptor by other protein kinases). Downstream signalling of the activated receptor includes interactions with Ras and activation of the MAP kinase pathway as well as PI3 kinase pathways and localization to focal adhesions by interaction with FAK, linking the receptor to integrin-ECM stimulation. (Ridley et al., 1995).

1.6.2 TGFβ and Receptors

TGFβ is expressed in five isoforms, three of which are important in wound healing (TGFβ1, TGFβ2, and TGFβ3). The presence of several isoforms is not thought to represent biological "reserve" in this case (Bandyopadhyay et al., 2006), but rather the balance among the isoforms present may be critical in regulating a given effect. For example in the case of scarring, reduction in TGF-1 and -2 during wound healing reduces scarring an effect that can be reproduced by exogenous application of TGF-3, which down-regulates the other two TGFβ isoforms (Martin, 1997). All of the signals from the TGFβ family are transduced by receptor serine/threonine kinases. The TGFβ receptors important in wound healing are a heterodimeric complex of type I receptor TGFβRI (57kDa) and type II receptor TGFβRII (75kDa) (Derynck, 1986). When activated by ligand binding the most well characterized downstream response of the receptor complex is to phosphorylate SMAD (Small Mothers Against Decapentaplegic) proteins that dimerize (e.g. $SMAD_{2/3}$ with $SMAD_4$) and migrate into the nucleus, where they activate target gene transcription in association with DNA-binding partners. Distinct repertoires of receptors, SMAD proteins, and DNA-binding partners seemingly underlie the biological responses of TGFβ isoforms (Roberts and Russo, 2000). Activated TGFβ receptor complexes are also capable of producing biological effects by mechanisms independent of SMAD, involving other signalling systems such lipid kinases (PI3 kinase) and the small GTPases (Zhang et al., 2005). The effects of TGFβ are diverse and include many changes known to favour migration such as secretion of pro-migratory substrate (e.g. fibronectin), up-regulation of pro-migratory integrins e.g. $\alpha_5\beta_1$, $\alpha_v\beta_6$ and $\alpha_v\beta_3$ (Kiosses et al., 2001; Laukaitis et al., 2001) and alterations in secretion of proteolytic enzymes such as MMP-9 (Gelatinase B or type IV Collagenase) (Massague, 1990).

1.7 Cell adhesion molecules: An overview

Cell adhesion is crucial in the formation, maintenance, and function of coherent multi-cellular structures. There are two major types of cell adhesion seen in multi-cellular organisms: cell-cell adhesion in which physical bonds are formed between adjacent cells, and cell-matrix adhesion in which cells bind to adhesive proteins extracellularly such as to an extracellular matrix. These two types of adhesion are mediated by a variety of adhesion molecules most of which are transmembrane proteins with domains extending into both the extracellular space and the intracellular space. These adhesion molecules fall into four major categories: Cadherins, Immunoglobulin (Ig)-like adhesion molecules, Selectins, and Integrins.

Cadherins are the main mediators of Ca^{2+}-dependent cell-cell adhesion. Cadherin-mediated adhesion is accomplished by a homophilic interaction between cadherin molecules on surface of adjacent cells. Keratinocytes grown in culture on an extracellular matrix, and those cells at the leading edge of an advancing sheet of cells extending across a wound during the healing process, develop lamellipodia and filopodia. When the filopodia of two opposing migrating cells make contact, the plasma membrane of each becomes interdigitated in the other. At the tip of the filopodia protrusions, there is a rapid accumulation of E-cadherin, and α-catenin in complex. Accumulation of progressively more of these complexes and the addition of other structural proteins such as β-catenin, and vinculin, link the actin cytoskeleton of the two cells and can lead to the formation of an adherens junction between them (Vasioukhin et al., 2000).

The immunoglobulin super-family (Ig-SF) of cell adhesion molecules is expressed in a wide variety of cell types, including epithelial and endothelial cells and leukocytes. They are characterized by the presence of one or more Ig-like domains in their extracellular region and can function by both homophilic and heterophilic binding. Their heterogeneous pattern of expression is reflected in the many and diverse biological processes in which they are involved, such as central nervous system development, and the immune responses. Important members of this family in relation to wound healing include inter-cellular adhesion molecule-1 (ICAM-1) and vascular adhesion molecule-1 (VCAM-1) which facilitate the interaction between vascular endothelial cells and leucocytes migrating into the wound, particularly during the inflammatory phase of wound healing.

Selectins are expressed primarily on leukocytes and endothelial cells. They play an important role in the immune response and wound healing. In contrast to other cell adhesion molecules, selectins bind to carbohydrate ligands. Hence, the resulting binding forces are relatively weak. L-selectins are expressed on most leukocytes and E-selectins are inducible on vascular endothelium following stimulation with cytokines. This distribution and weak interaction binding allows selectin-mediated interactions between leukocytes and endothelial cells that promote rolling of the leukocytes along the endothelium.

Integrins belong to a super-family of non-covalently bound heterodimeric membrane receptor glycoproteins. They are composed of a variable α-subunit of 150-170kDa and a conserved 95kDa β-subunit. 19 α-subunits and 8 β-subunits have been identified in mammalian cells to date, which in various combinations give rise to the 24 different integrins that have been characterized. Although both subunits are required for adhesion, the binding specificity to extracellular matrix components primarily depends on the extracellular portion of the α-subunit. Intracellular interactions (with various proteins) occur primarily with the cytosolic portion of the β-subunits (Hynes, 2002). While generally classified as adhesion molecules, integrins also play an important role in signal transduction. Signal transduction through integrins occurs in two directions: from the extracellular microenvironment into the cell (outside-in signalling), and from the cytoplasm to the extracellular domain of the receptor (inside-out signalling). Integrin ligation to extracellular matrix is the first step in the formation of the focal adhesions (discussed below). Integrin-ECM binding transmits by conformational change a signal to the carboxyl-terminal intracellular region of the integrin receptor, increasing affinity at that site for binding proteins such as talin (outside-in signalling). Binding by talin and other proteins, as focal adhesions are elaborated, causes a further conformational change in the integrins involved that is transmitted to the amino-terminal extracellular region. In this case the effect of binding inside the cell is to create a conformation of high affinity for ligand binding outside the cell, as long as the intracellular biding persists (inside-out signalling).

Following extracellular matrix ligand binding to integrins, integrins form into clusters around which intracellular focal adhesions form. Clustering of integrins is most simply due to cross-linking between integrins that occurs with binding to extracellular matrix proteins, but it is also facilitated by the interactions between the multiple proteins assembled within the developing focal adhesion (the activity of PI-3 kinases, and protein kinase C (PKC) are

also involved). One effect of this clustering is to form a region of enhanced binding affinity to extracellular matrix that reinforces the initial integrin-ECM binding interaction and increases integrin-ECM occupancy at that site (inside-out signalling). Importantly, the processes that govern assembly and disassembly of focal adhesions regulate integrin clustering, affinity and occupancy and this in turn regulates the strength of attachment of the cell to the extracellular matrix.

1.8 Focal adhesions, Integrins, FAK and Src

Most cultured cells adhere to the underlying substrate through distinct regions of their plasma membrane known as focal contacts, or focal adhesions. Fully established, these adhesions are small, typically 2-6 μm long and 0.25-0.5 μm wide and were first seen as structures with electron microscopy (Abercrombie et al., 1971). At these contact sites clusters of integrins are bound to extracellular matrix proteins. On the cytoplasmic side of integrin clusters, integrins interact with various proteins such as talin, FAK, and paxillin; associated with them is an elaborate network of other proteins that develops, forming what can be visualized as the focal adhesion. Importantly, these focal adhesions are linked to the cellular cytoskeleton via actin stress fibres (bundles of actin and myosin II) (Figure 3). Some authors draw a clearer distinction between types of adhesion, smaller focal contacts being generally considered as rapidly forming, following integrin-ECM stimulation, they are first seen distally in cellular protrusions, they are translated proximally (with migration) and mature into focal adhesions. Some adhesions appear to stabilize (associated with increasing levels of α-actin), mature and become more complex (elaborating more fully the array of proteins present) and these adhesions are disassembled or torn loose at the cellular tail (Zamir and Geiger, 2001). However most focal adhesions are disassembled proximally in the cellular protrusions (i.e. they are highly turned-over). Compositional changes occur over time in these adhesions as they mature, possibly linked to physical tension. Generally focal adhesions contain greater proportions of actin stress fibres, and more mature adhesions have greater proportions of α-actin. During migration tension in the cytoskeleton is transmitted through the actin stress fibres, accumulated proteins and integrin clusters of the adhesion to the extracellular matrix. The strength of these adhesions is determined by their constituents and is crucial to successful migration. For convenience the term focal adhesion will be used (unless it is necessary to make particular reference to the other nomenclature).

As well as functionally acting as a physical link between the cell and environment, focal adhesions are important generators of integrin mediated signalling.

(Figure 3)

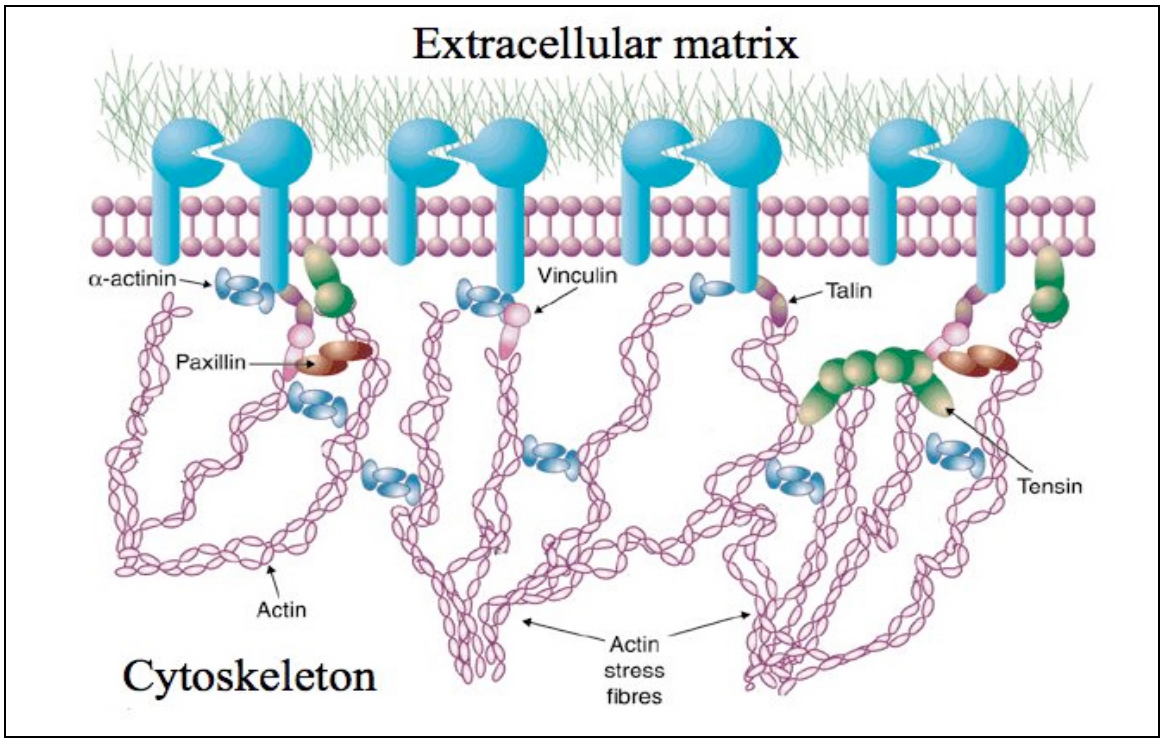


Figure 3: A schematic representation of the integrin-mediated link between extracellular matrix and the intracellular actin cytoskeleton in a focal adhesion: Integrin binds to extracellular matrix ligand extracellularly. Schematically shown is cross-linking of integrins and ECM extracellularly within an integrin cluster of a developing focal adhesion. Intracellularly, integrin β-subunits interact with talin and subsequently other important cytoskeletal proteins including paxillin, vinculin, and tensin. These interactions link extracellular matrix via integrin to the actin stress fibres of the cytoskeleton. α-actin is also shown and is present only in mature focal adhesions rather than those undergoing dynamic turnover. α-actin acts to stabilize the focal adhesion (modified from: Miranti C 2002).

Like all cell signalling interactions the information gathered must be transmitted into the cell before any effects can be elaborated. Integrins do not have enzymatic activity. Signals are transmitted from the extracellular environment via integrins and are elaborated in the cytoplasm by the profile of proteins that accumulate in focal adhesions beneath the ligand bound integrin clusters, and in particular by the relative levels of tyrosine phosphorylation of the proteins present (as well as by other activities distributed amongst the proteins present). Over 50 proteins have been linked to fully developed focal adhesions. Some

proteins relevant to focal adhesion formation and migration are schematically illustrated in (Figure 4). The signals from integrin-ECM interactions have been shown *in vivo* and *in vitro* to be linked to a number of cellular processes survival, proliferation and migration. They are undoubtedly involved in the regulation of the formation, dissolution and composition of the focal adhesions themselves. In addition to their composition (linked to the strength of attachment and intracellular signalling), focal adhesion turnover (assembly and disassembly) is crucial to migration.

(Figure 4)

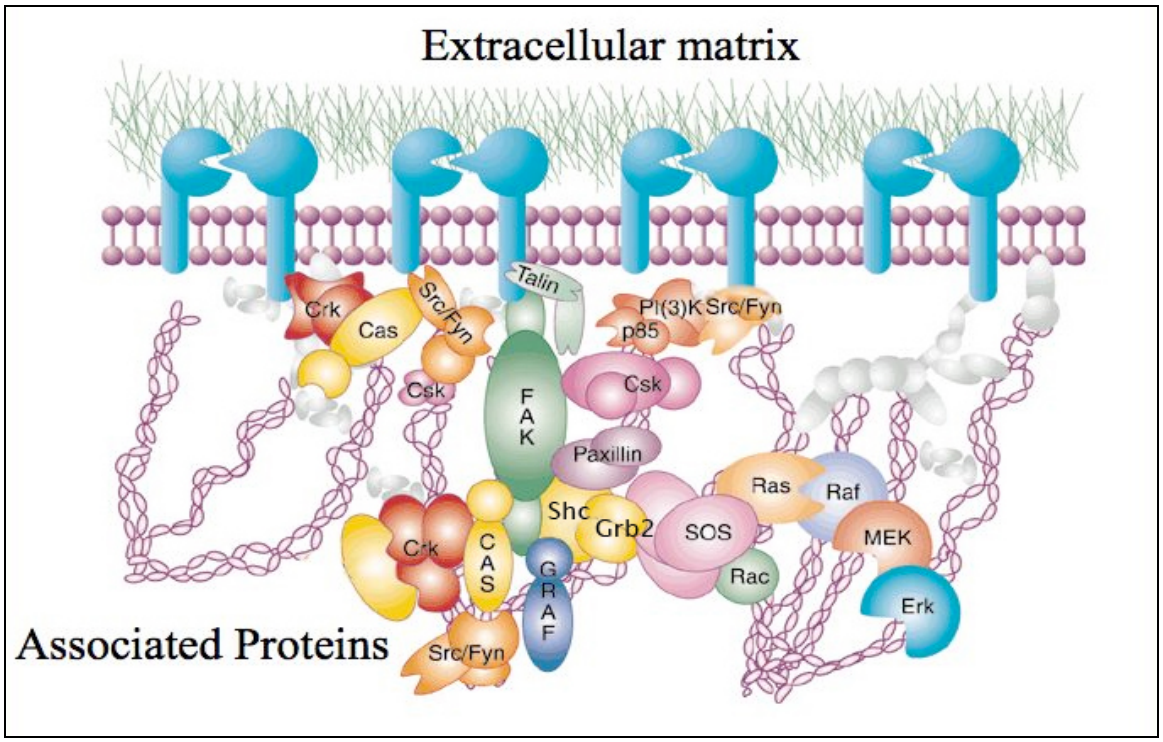


Figure 4: A schematic representation showing important proteins commonly associated with focal adhesion formation (linked to migration) and associated with signal transduction from the extracellular matrix into the cell. Integrin binds to ECM ligand extracellularly. Intracellularly talin and paxillin link integrin to FAK that in turn interacts with cellular Src (c-Src) and/or another Src family-member, Fyn. Many additional proteins have been shown to localize to focal adhesions and some them are shown; adaptor protein Shc, growth factor receptor bound protein 2 (Grb2), Son of Sevenless (SOS) (and elements downstream of the Ras, Rac, signal pathways such as Raf, MEK, and Erk), Cas, Crk, Csk, and Graf, and some cell surface receptor proteins such as the p85 subunit of PI3-kinase (PI_3 kinase). See text for more details (modified from: Miranti C 2002). (Not shown is the attachment of FAK to the EGF receptor discussed later).

There is considerable evidence to support of the importance of FAK (focal adhesion kinase) and Src family members (c-Src ($p60^{SRC}$), Fyn ($p59^{FYN}$), and Yes1) in promoting cell migration, although to date there is no consensus on what downstream signalling events from these proteins is required for the initiation of cell migration and all details of the interactions of these proteins remains to be elaborated. A number of different models have been proposed by which FAK-Src function can promote cell migration events. One hypothesis is that FAK and Src are recruited to focal contacts following integrin-ECM stimulation where they act individually and jointly (in a FAK-Src complex).

The activity of FAK and Src promotes a number of tyrosine phosphorylation events many of which lead to changes required for cellular migration but also that lead over time to degradation of FAK from the adhesion (by unknown means) and inactivation of Src. Loss of function of these key proteins from the adhesion is associated with its remodelling and dissolution (proximally). Subsequently with continued integrin stimulation new adhesions are formed (distally) and the process repeats providing for turnover of focal adhesion sites, a key feature of migration (Fincham and Frame, 1998). Other work has focused on the role of tyrosine phosphorylation of FAK in conjunction with paxillin, (and $p130^{CAS}$) that links to, and feeds back negatively on the activity of the FAK-Src complex (which likely is responsible for most of the tyrosine phosphorylation). This interaction may be regulated by cycles of tyrosine phosphorylation and de-phosphorylation within a number of proteins under the influence of FAK and Src that in turn lead to assembly and disassembly (turnover) of focal contacts. This process is also linked to cyclical changes in the activity of Erk.

1.8.1 Focal adhesion kinase: FAK

FAK was identified in 1992 simultaneously by several investigators (Guan and Shalloway, 1992; Hanks et al., 1992; Schaller et al., 1992). It was characterized as a 125kDa tyrosine phosphorylated protein that localized to sites of integrin clustering and subsequent formation of focal adhesions (Hildebrand et al., 1993). It has been the focus of considerable effort since that time, illuminating its role as a mediator of many downstream processes related to integrins and other signalling pathways. FAK has been linked to migration events and studied extensively in fibroblasts though there has been more limited study of the role of FAK in keratinocyte and migration. In burn wounds FAK expression has been shown to be elevated in actively migrating human keratinocytes (Hauck et al., 2002b). Pathologically elevated FAK expression has been seen in several human tumour cell lines (Kornberg,

1998). In most of these tumours high levels of FAK expression correlate with enhance tumour invasion and in some cases (e.g. prostate carcinoma cell lines) inhibition of FAK (Slack et al., 2001) or Src minimized this phenomenon (Hauck et al., 2002a). Tumour cell invasion likely depends upon several processes including cell shape, focal adhesion turnover, and proteolysis (Schlaepfer et al., 2004). As such it is reasonable to assume FAK related pathways may be involved in any or all of these functions through effects on actin polymerization, the disassembly (or assembly) of focal contacts, and the regulation of protease activation or secretion (Hauck et al., 2002a).

There are many important integrin-ECM stimulated signalling events involving the FAK and the Src that involve a number of proteins shown to be associated with focal adhesions, important interaction sites within FAK and Src are shown in (Figure 5).

(Figure 5)

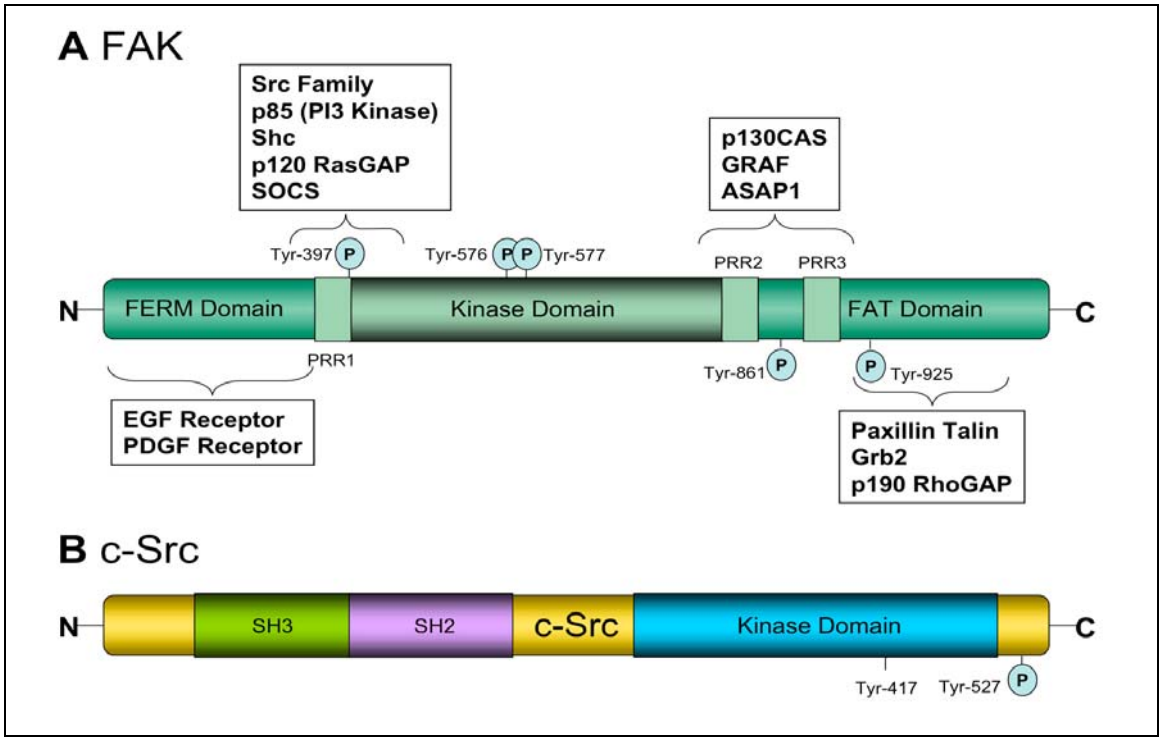


Figure 5: Schematic representation of FAK and c-Src showing the major binding sites and domains. A: Key FAK domains and binding sites (discussed within the text) include FERM (protein 4.1, ezrin, radixin and moesin homology) domain, FAT (focal adhesion targeting) domain, and the tyrosine phosphorylation kinase domain. Also shown are several (though not all) sites of tyrosine phosphorylation of FAK (Tyr-397 the auto-phosphorylation site, and Tyr-576 and Tyr-577 phosphorylation sites within the kinase domain) and the three-proline rich regions (PRR1, 2, and 3). Binding sites of significant proteins and receptors linked to cellular migration that interact with

various regions of FAK are also shown. B: Key c-Src key domains include; the SH3 and SH2 domains and the tyrosine phospho-kinase domain. Also shown are two key regulatory phosphorylation sites (Tyr-417 which, when phosphorylated, result in activation of Src and Tyr-527 which results in its deactivation). Note: Numbering of the residues here refers to chicken c-Src.

FAK ($p125^{FAK}$) is one of the first proteins to be recruited to focal adhesions as they form (Kirchner et al., 2003). Recruitment and activation (by auto-phosphorylation and further tyrosine phosphorylation) of FAK can occur in response to a number of stimuli most notably integrin binding. FAK recruitment to sites of integrin clustering occurs via a region of the molecule termed FAT (focal adhesion targeting region) shown in (Figure 5: A). This region interacts with the β subunits of integrins in clusters (indirectly) by binding to structural proteins such as talin and paxillin and thereafter FAK stabilizes its position and also provides a link to other components of the cellular cytoskeleton (Klingbeil et al., 2001). Paxillin a structural adaptor protein containing a number of different domains by which it can interact such as a proline-rich site for SH3 domain binding, two N-terminal leucine-rich domains known as LD motifs, and four zinc-finger LIM domains that are important for paxillin targeting to focal contacts. FAK binding to paxillin is mediated by an interaction between the two-leucine rich regions (LD motifs) in paxillin and hydrophobic regions in the FAT domain of FAK (Hayashi et al., 2001). Talin is a structural protein that can associate with β-integrin cytoplasmic tails.

Src family members (c-Src, Fyn and Yes1) are non-receptor tyrosine kinases. c-Src is derived from the proto-oncogene that bears the same name and is the cellular progenitor of the first viral oncogene discovered, v-Src that is related to the Rous sarcoma virus. Src is translocated to focal adhesions (peripherally) by an actin dependant process from sites within the cell, a process that does not depend on either its kinase activity or myristrylation site (Fincham and Frame, 1998). Following this Src associates with the cell membrane and can be activated by several types of cell surface or cytoplasmic receptors including growth factor receptors (e.g. PDGF receptor) G-protein-coupled receptors (e.g. β-adrenergic receptor) and an integrin bound to extracellular matrix. In each case, Src is activated by binding of a ligand to the SH2 and or SH3 domains (Figure 5: B). In the case of integrin-ECM signalling, the primary SH2 ligand of Src is a phosphorylated tyrosine residue (Tyr-

397), the auto-phosphorylated site of FAK, forming a FAK-Src complex (Schaller et al., 1994).

Src bound to FAK at Tyr-397 mediates phosphorylation of FAK at many sites, Tyr-576 and Tyr-577 (required for maximal kinase activity of FAK), Tyr-861 and Tyr-925. Src-mediated phosphorylation of FAK at Tyr-925 creates a binding site on FAK for the SH2 domain of the Grb2 adaptor protein (Schlaepfer and Hunter, 1996). In addition integrin activation of both FAK and Src can also promote tyrosine phosphorylation of Shc (an adaptor protein) at Tyr-317, creating a SH2 binding site on Shc to which Grb2 can bind. Grb2 binding to FAK or Shc can potentiate the translocation of the GDP-GTP exchange protein SOS to the plasma membrane in turn leading to enhanced GTP exchange on Ras. The activation of the Erk MAP kinase cascade is one target for the actions of GTP-bound Ras (Figure 6). [Integrin-stimulated Ras may also activate PI-3 kinase which may also facilitate the coupling of Ras to the Raf-1 kinase leading to the enhanced activation of Erk MAP kinase pathway (King et al., 1997)].

(Figure 6)

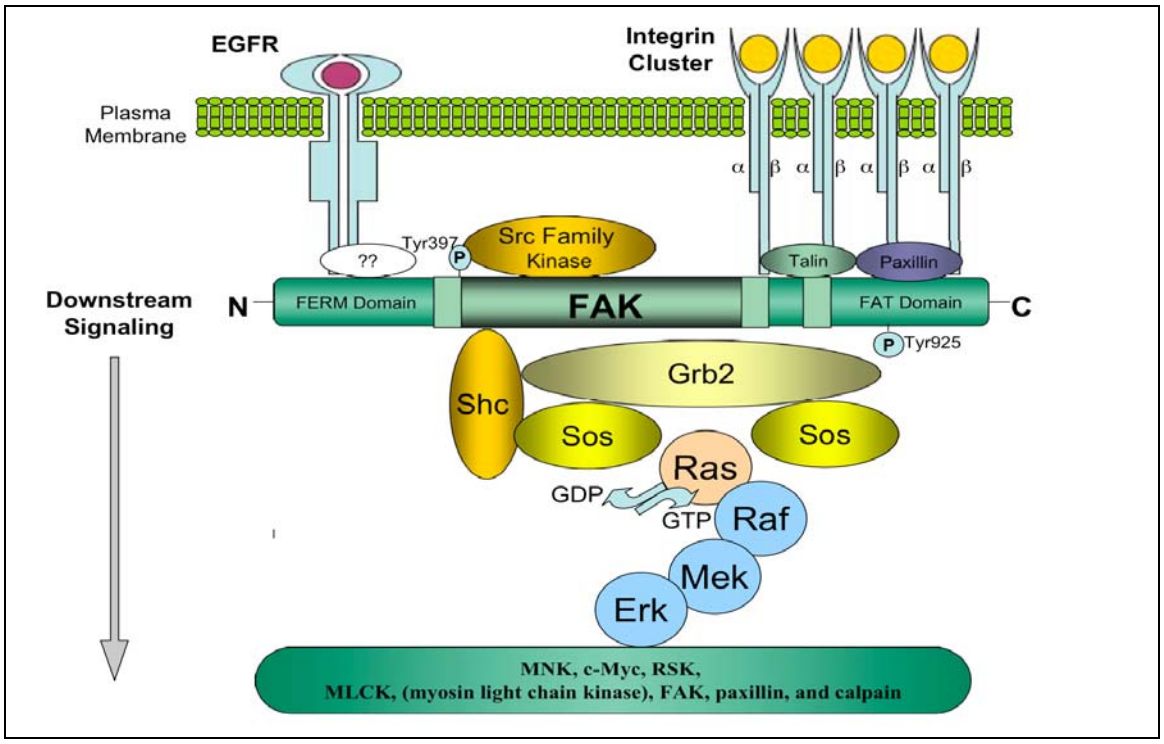


Figure 6: Schematic representation of activation of the Erk MAP kinase pathway by integrin-ECM stimulated signalling and Grb2-SOS binding. Following integrin-ECM stimulation the FAT domain of FAK interacts with talin, paxillin and integrin, linking FAK to clustered integrin. FAK auto-phosphorylates at Tyr-397 and binds Src family kinases at this site. Src is translocated to these sites

following integrin stimulation and binding to FAK leads to increased levels of tyrosine phosphorylation in FAK (including phosphorylation of Try-925) and of Shc (including phosphorylation of Tyr-317 not shown). Grb2 binds (via its SH2 domain) to Shc at phospho-Tyr-317 and to FAK at phospho-Tyr-925 that in turn recruits SOS to the focal adhesion. Grb2-SOS enhanced GTP exchange on Ras recruits Raf-1 and the Erk MAP Kinase pathway is activated (that is one known target for the actions of GTP-bound Ras). Relevant effectors of the Erk MAP kinase pathway are shown at the lowest level in the diagram (MAP kinase interacting kinase (MNK), Proto-oncogene (c-Myc), ribosomal protein S6 kinase (RSK), myosin light chain kinase (MLCK), FAK, paxillin and calpain). Also shown is the EGFR. Stimulation of this receptor by growth factor can activate the same MAP kinase pathway and EGFR is recruited to focal adhesions by interaction with sites in the FERM domain of FAK (possibly via an intermediary represented as "??"). Further Grb2-SOS activity on Ras (enhanced GTP exchange) may require association with the cytoplasmic tail of the EGFR (by either mechanism of activation).

Stimulation, by integrin-ECM interaction can lead to parallel changes in both FAK and paxillin (and $p130^{CAS}$) tyrosine phosphorylation, an effect that is a consequence of both FAK and Src activity jointly. These changes have been shown to be dependent on the integrity of the FAK auto-phosphorylation site Tyr-397 (Schaller and Parsons, 1995). All three proteins FAK-Src-Paxillin can co-localize or independently localize to focal adhesions (Hildebrand et al., 1993; Yamada and Miyamoto, 1995). Several phosphorylation interactions play key roles in modulating FAK interactions with integrin and paxillin, such as tyrosine phosphorylation of Tyr-118 on paxillin by FAK and or Src in complex.

Tyr-118 phosphorylation of paxillin leads to binding of a number of other proteins to this site of particular importance is Erk. Erk has been shown to bind to Tyr-118 and then to phosphorylate FAK on serine residues, such as Ser-910 within the FAT domain both *in vivo* and *in vitro* (Hunger-Glaser et al., 2003). The effect of this is to favour dissociation of FAK from paxillin and adhesion disassembly (Hunger-Glaser et al., 2004). However, in addition activated Erk kinase (at different times) phosphorylates paxillin in a manner that can favour FAK-paxillin association and adhesion assembly.

These intriguing and in places complimentary observations suggest that there may be a complex regulation of the integrin-FAK-paxillin complex by the FAK, the FAK-Src

complex and Erk activation during focal adhesion assembly and disassembly in cellular migration that is discussed further in Chapter 6.

Proteins such as $p130^{CAS}$ (Crk associated substrate) are also considered targets for FAK phosphorylation and binds to FAK at PRR2 (Figure 5) via its SH3 domain. Similarly to paxillin, a number of studies have demonstrated increased tyrosine phosphorylation $p130^{CAS}$ that parallel changes in FAK following integrin stimulation. Src-family association with FAK at sites of integrin clustering can potentiate the tyrosine phosphorylation of $p130^{CAS}$ (and paxillin) and this phosphorylation leads to SH2 mediated binding of the large adapter protein c-CrkII (Crk) (Parsons, 2003). While the downstream mediators of these proteins have not been well characterized Crk and Nck adaptor proteins binding to tyrosine phosphorylated $p130^{CAS}$ may lead to enhanced cell migration through the activation of pathways potentially involving the Rac-1 GTPase or the c-Jun N-terminal kinase (JNK) MAP kinase cascade. Such activation is linked to protrusion formation and focal adhesion assembly. FAK-Src-$p130^{CAS}$ -Crk interactions in response to integrin activation are involved in a number of functions such as; cell proliferation, survival, and migration. Deregulated, $p130^{CAS}$-Crk signalling is implicated in the progression of certain cancers and in developmental defects in humans.

Summarizing the remaining interactions with FAK shown in (Figure 5): the N-terminal domain of FAK is referred to as the FERM (protein 4.1, ezrin, radixin and moesin homology) domain. Binding within this region has been documented to several soluble growth factor receptors such as EGFR and PDGFR (Sieg et al., 2000; Mitra et al., 2005). At Tyr-397 (the FAK auto-phosphorylation site) as well as the interactions with Src family members and the adaptor protein Shc, detailed above, this site has also been shown to interact with p85 the regulatory subunit of PI-3 kinase, which as mentioned, plays an important role in growth factor receptor signal relay and in the mediation of lamellipodia formation during spreading and in migration. Another growth factor singling regulator, suppressor of cytokine signalling, (SOCS) also binds at this site, as does the p120RasGAP that is closely linked to p190RhoGAP that binds within the FAT domain of FAK. These Ras GAPs (GTPase-activating proteins) are involved in maintenance of cell polarization by regulation of actin stress fibre activity amongst other effects. Finally, within the C-terminal domain of FAK are two proline rich domains (PRR2 and PRR3) that are sites mediating interactions and binding of SH3 domains (such as those present in $p130^{CAS}$), the GTPase

regulator associated with FAK, GRAF, and the Arf-GTPase activating protein, ASAP1 bind to this area.

1.9 Human keratinocyte migration and the relationship between soluble growth factor and integrin based cell signalling: Hypotheses

The hypothesis tested in this thesis is that signalling from both integrin-ECM interactions and from soluble growth factors and their receptors work in conjunction to facilitate the migration of keratinocytes. Acting together it is proposed that they will produce optimal (augmented) migration, while acting alone integrin-ECM initiated signalling might still result in migration, albeit at a reduced level. Growth factor signalling acting alone will prove unable to produce migration. During migration of cells other than keratinocytes, integrins have been shown to mediate their effects through signalling pathways that critically involve the activities of FAK and Src kinases in the assembly, translocation and most significantly disassembly of focal adhesions, while the contribution of growth factors to migration is via activation of one or more of the MAP kinase pathways. The involvement of FAK, Src and the MAP kinase pathways is hypothesized to be central to keratinocyte migration (in both the presence and absence of growth factors) and activity in these pathways during migration as well as interactions between these two signalling systems is proposed to be present and will be explored.

The *in vitro* wound assay and colloidal gold migration assay are used to evaluate keratinocyte migration under a number of conditions. In Chapter 3 these assays are described in detail and in particular a protocol for the *in vitro* wound assay is proposed that identifies and minimizes some of the known limitations of this assay. In addition the results of the experiments performed to optimize the *in vitro* assay are used to demonstrate the statistical methods applied in the analysis of the result from later chapters.

In Chapter 4 migration of human keratinocytes is studied under conditions chosen to reflect those present at times during the healing of an acute *in vivo* wound. In particular the effects of different extracellular matrix substrates, those that occur as components of *in vivo* wound matrix, and of similarly chosen growth factors are evaluated in their contribution to the initiation and continued migration of human keratinocytes. In this chapter the hypothesis is that in the presence of type I collagen (and possibly other similar substrates known to be

present in the *in vivo* healing wound) migration of keratinocytes will occur with or without the addition of soluble growth factors, and in addition that the presence of soluble factors would significantly augment (enhance) keratinocyte migration.

Further exploration of the role of soluble growth factor signalling during keratinocyte migration on type I collagen is presented in Chapter 5. In particular involvement of the MAP kinase pathways Erk, p38, and JNK as downstream mediators are considered. In order to begin to understand the relationship between activities in these pathways during keratinocyte migration, the simplest initial questions considered here is which, if any, of the three well characterized MAP kinase pathways are active during keratinocyte migration and, if active, which pathways appear to be necessary. The presence or absence of activity in each pathway is demonstrated in keratinocytes migrating on pro-migratory type I collagen and non-migratory poly-L lysine, in the presence and absence of soluble growth factors. Under these conditions, the role of various the MAP kinase pathways in keratinocyte migration is investigated by blocking their activity either with selected dominant negative genes or use of chemical inhibitors (both chosen for inhibition of elements of the MAP kinase pathways). In particular in this chapter the hypothesis that an intact Erk MAP kinase pathway will prove to be essential to keratinocyte migration in the presence and importantly in the absence of soluble growth factors is considered (and if true demonstrating one critical link between integrin-ECM and growth factor stimulated migration). Further, it is hypothesized that the augmented migration proposed to follow growth factor stimulation depends on the presence of an intact Erk MAP kinase pathway and possibly the p38, but not JNK, SAPK MAP kinase pathways.

Experiments presented in Chapter 6 evaluate the roles of FAK and Src as central mediators of integrin-ECM stimulated keratinocyte migration. Firstly, the hypothesis that inhibition of a key binding and activation site of FAK (Tyr-397 the auto-phosphorylation site) and/or inhibition of its kinase activity will significantly decrease keratinocyte migration on type I collagen is explored. Two dominant negative genes that inhibit FAK in the desired manner are used in this evaluation. It was anticipated that an intact FAK auto-phosphorylation site would prove to be essential to keratinocyte migration. Similarly it is hypothesized that inhibition of the kinase activity of Src by use of dominant negative and chemical inhibitors will also have an effect on keratinocyte migration under the same conditions. In particular it is anticipated that such inhibition (of FAK and Src by these means) will affect migration on

type I collagen both in the presence and absence of growth factors. Western blot is used to evaluate the effects of over-expression of FAK and Src transgenes ("wild-type" that are unaltered and with normal function and dominant negative inhibitors; FAK-F397, FAK-R454 and Src-KM) on tyrosine phosphorylation of FAK and of significant known downstream mediators of migration such as paxillin. In the presence of these same transgenes and by the same means (Western blot) activity in the MAP kinase pathways (Erk, p38 and JNK) is demonstrated. Under these conditions it is hypothesized that the changes in FAK phosphotyrosine levels and paxillin phosphorylation (at Tyr-118) are predictably altered and directly linked to changes in keratinocyte migration. Similarly, it is anticipated that activity in the Erk MAP kinase pathway, and possibly in the stress activated protein kinase (SAPK) pathways; JNK and p38 will prove to be linked to changes in FAK and Src activity and again to changes in migration. Once again in all cases this is hypothesized as being likely to occur in the presence and absence of growth factors and if true will demonstrate a critical link between integrin-ECM and growth factor stimulated migration. Finally in light of the findings of the preceding experiments (particularly in relation to the results observed following Src-Wt over-expression), an experiment is performed to show what if any effect on migration there might be from chemical inhibition of the p38 MAP kinase pathway in keratinocytes transfected with the FAK and Src "wild-type" transgenes and kinase deficient dominant negative transgenes (FAK-R454 and Src-KM) on type I collagen. The hypothesis considered in this experiment is that the p38 MAP kinase pathway might prove more critical to migration under conditions that are associated with greater actin-myosin activity and greater turnover of focal adhesions (a circumstance that can reasonably be considered to be present in the case of Src "wild-type" over-expression).

Chapter 2. Materials and Methods

2.1 Human keratinocyte passage

The majority of experiments performed used Human Epidermal Keratinocytes (HEKs) derived from primary cultures of a single donor neonatal foreskin and provided from the supplier as "cryopreserved primaries" (Clonetics cat # CC-2503). For each experiment performed in this thesis frozen cells were requested from our laboratory core facility at USC (University of Southern California) where they were routinely stored. Upon thawing, these keratinocytes were ≥70% viable (Trypan blue exclusion assay) and had an expected lifespan of 16-25 population doublings when handled according to the supplier's guidelines. After thawing cells were re-suspended in a suitable medium (see below) and immediately placed in an incubator, 5% CO_2, 95% humidified air, at 37.0^{O}C. Some control experiments used Human Dermal Fibroblasts (HDFs) isolated from a single donor neonatal foreskin, cryopreserved at the end of the primary culture (Cascade Biologics cat#: C-004-5C). The principles and practice of handling fibroblasts were the same as those used for keratinocytes, unless otherwise mentioned.

The medium used for initial incubation of keratinocytes upon thawing, and for routine subculture thereafter, was EpiLife Medium (Cascade Biologics cat # M-EPI-500). 0.06M calcium chloride (supplied with medium) was added drop-wise while gently mixing per supplier's guidelines, to result in a final concentration 0.06mM of calcium chloride. This reconstitution was prepared under sterile conditions in a Class II safety cabinet with filtered laminar airflow and the reagents were warmed to 37.0^{O}C before use.

During keratinocyte subculture the medium was conditioned by addition of the Human Keratinocyte Growth Supplement kit (Cascade Biologics HKGS kit S-001-K). The final concentrations of components of the medium when reconstituted per supplier's guidelines with the full HKGS kit was: Bovine Pituitary Extract (BPE) 0.2% v/v (1:500 dilution); Insulin (Bovine) 5μg/ml; Hydrocortisone 0.18μg/ml; Transferrin (Bovine) 5μg/ml; 1xPSA (penicillin, streptomycin, amphotericin B), and human Epidermal Growth Factor (EGF) 0.2ng/ml. The medium reconstituted in this way was used for serum-free subculture, unconditioned media being Growth Factors negative (GF-) and conditioned media Growth Factors positive (GF+). The medium GF+ or GF- formulated in this manner is generally referred to as "1x". Upon thawing of HDFs, the medium used for initial incubation and for

their routine subculture, was Medium 106 (Cascade Biologics cat # M-106-500). This was reconstituted per the supplier's guidelines to produce GF- medium. Addition of the Low Serum Growth Supplement Kit (Cascade Biologics cat # S-003-K) produced GF+ medium. HDF GF+ medium reconstituted per the supplier's guidelines contained; foetal bovine serum 2% v/v, hydrocortisone (1 μg/ml), EGF 10ng/ml, human basic fibroblast growth factor (3 ng/ml) and heparin (10 μg/ml) stabilized with BSA (Bovine Serum Albumin) and 1xPSA (penicillin, streptomycin, amphotericin B). Other purchased media that were used included: Human Serum (pooled), separated from clotted human whole blood, sterile-filtered, and tested for mycoplasma and viruses (Sigma-Aldrich cat # H1388); Human Plasma (pooled), separated from whole blood collected with anticoagulants (3.8% trisodium citrate), centrifuged, filtered (0.45 μm), tested for pathogens and lyophilized (Sigma-Aldrich cat # P9523). Specific details of subsequent conditioning of any medium beyond that described above will accompany the details of each related experiment.

The keratinocytes (and HDFs) were typically subcultured at ≈70-80% confluence (grown in P100 and P60 plastic cell culture dishes). Several subcultures (passages) following thawing were typically required to provide the necessary number of cells for each experiment. All keratinocytes (and HDFs) used in experiments were in the range 3-4 passages. Subculture was performed under aseptic conditions in a Class II cabinet. The existing medium was aspirated and the attached cells were washed with 10ml, warmed ($37.0^{O}C$), Hank's Buffered Salt Solution without calcium chloride and magnesium sulphate (HBSS-) (Sigma-Aldrich or from our laboratory core facility at USC, made to the same specifications). The HBSS- was then aspirated and the attached cells "Trypsinized" (detached) by addition of warmed ($37.0^{O}C$) 0.05% trypsin/ethylene diaminetetraacetic acid (Trypsin EDTA) (Invitrogen cat #25300062, or from our laboratory core facility at USC, made to the same specifications). Trypsin EDTA was added at ≈1ml per $25cm^2$ (of cell culture plate area) and the culture plate and gently rocked to evenly coat the plate. The culture plate was then returned to the incubator at $37.0^{O}C$ for 5-7 minutes; after 5 minutes plates were examined under light microscopy (100x) for evidence of detachment. Cells showing evidence of detachment became increasingly rounded and shiny as they lost their attachments to the culture plate. Trypsinization was allowed to continue until ≥90% of cells showed the effects of the trypsin EDTA (signs of detachment). At this point the plate was rapped on a soft surface to release the cells. Re-inspection under microscopy (100x) typically revealed most cells were then detached from the culture plate. If not, a further 30 seconds trypsinization in the incubator

was allowed and the process repeated until most cells were detached. Once ≥90% of cells detached, the trypsin was neutralized by adding an equal volume of warmed Trypsin Neutralizing Solution (GIBCO Unconjugated Soybean Trypsin Inhibitor, Invitrogen cat # 17075029, or from our laboratory core facility at USC, made to the same specifications) to the plate with gentle rocking. The mixture was then transferred by pipette to a 15ml plastic centrifuge tube and centrifuged at 1200rmp (220 x g) for 5 minutes. The supernatant (liquid) was carefully aspirated, avoiding any disturbance or contact with the cell pellet at the base of the tube. 3ml of serum free EpiLife medium (GF-) was added to the keratinocyte pellet. The cells were re-suspended in the medium by flicking the tube once or twice and gently swirling the contents. When the pellet was thoroughly disrupted, the cells were uniformly mixed by continued gentle swirling and prior to each aspiration for analysis by repeatedly pipetting up and down a narrow bore pipette 5 times.

A cell count was performed using a two-chamber haemocytometer (Cascade Biologics). Ten μl of re-suspended cells were added to one chamber avoiding air bubbles. Cells were counted in the four quadrants of the haemocytometer. Each quadrant has an area $1mm^2$ and is divided into an array of 16 squares (4x4). Cells in contact with boundary lines were counted. An average value per quadrant was calculated = N. (All cells present in all of the four quadrants were counted and the number divided by 4 = N). The total cell concentration (number of viable and non-viable cells per ml) was determined by the formula: Total Cell Concentration = $N \times 10^4$ cells/ml. Typically N was in the range of 30 to 85, and the total cell concentration was in the range 300,000 to 850,000 cells/ml. (Note: total cells per P100 at 70-80% confluence was of the order of 2,550,000 cells and total cells per P60 plate at 70-80% confluence was of the order of 900,000 cells).

2.1.1 Assessment of cell viability

Assessment of cell viability was performed in each case using a Trypan blue exclusion assay. The Trypan blue significantly stains only cells without intact membranes i.e. non-viable cells. 50μl of 0.4% Trypan blue (Sigma-Aldrich) was added to a 1.5ml Eppendorf tube. An equal amount, 50μl, of the agitated (thoroughly mixed) cell suspension was added to the same tube which was then mixed and allowed to stand for 3 minutes. Ten μl of cell suspension was counted in a haemocytometer; the total cells present (stained and non-stained) in all of the four quadrants were counted = N_{s+ns}. The total stained cells present in all of the four quadrants were counted = N_s. The percentage cell viability was given by the

formula: Percentage Cell Viability = (N_{s+ns} -N_s)/ N_{s+ns}) x 100. Percentage cell viability for routine keratinocyte passage was typically 80-90%. All cells from plates showing less than 80% viability were discarded. The final viable cell concentration was calculated according to the formula: Viable Cell Conc. = (Total Cell Conc. x Percentage Cell Viability) / 100. Typically, viable cell concentrations were in the range: 650,000-700,000 cells/ml for a P100 cell culture plate and 230,000-250,000 cells/ml for a P60 plate. Typical harvest yielded in the order of; total Viable Cells 2-2,100,000 cells for a P100 cell culture plate and 700-750,000 cells for a P60 cell culture plate.

For routine subculture (and most experiments), inoculation of the cell suspension onto new cell culture plates was at 3000 cells/cm^2 of growing area (e.g. P100 (78.5 cm^2) culture plates 235,000 cells, and P60 (28.0 cm^2) culture plates 84,000 cells). The volume containing this number of re-suspended cells was calculated using the Viable Cell Count. After thorough and uniform mixing (by continued gentle swirling and prior to each plating pipetting up and down a narrow bore pipette 5 times) the appropriate volume (typically ≤1ml) was pipetted into a sterile 15ml test tube and the total volume in the tube was made up with conditioned GF+ medium to 10ml for P100 and 4ml for P60 culture plates. Uniformly across each cell culture plate this mixture of cells and medium were added. The new plates were returned to the incubator. Light microscopic inspection was performed (100x) at 1 to 2 hours to ensure the keratinocytes were adherent and appeared sufficient in number. Until that time unused re-suspended (GF+) keratinocytes were stored in the incubator. A further cell culture plate could be produced if insufficient adherent cells were detected. Typically however, at that time any unused keratinocytes were discarded. During cell growth, cells remained in the incubator at 5% CO_2, 95% humidified air, and 37.0^{O}C, but were removed daily for light microscopic (100x) monitoring. The medium was changed every two to three days under sterile conditions in the Class II safety cabinet. Old medium was aspirated off and freshly prepared pre-warmed 37.0^{O}C medium of the same type (typically GF+ for routine subculture) was added and the cells were returned to the incubator.

2.2 Colloidal gold migration assay

The colloidal gold migration assay used throughout these experiments represents a modified form of an assay of cellular migration first described in 1977 (Albrecht-Buehler, 1977). The assay was used initially to investigate the pattern and direction of migrating fibroblasts but

was shortly thereafter adapted for the quantitative analysis of cell motility, and used for that purpose in a number of other cell types, including endothelial cells (Zetter, 1980; Bowersox and Sorgente, 1982). Since that time the assay has been continually updated in technique (Zetter, 1980; Woodley et al., 1988; Scott et al., 2000) and as technology has evolved (Li et al., 2001; Li et al., 2004a).

This assay is based on the phenomenon that migrating cells are able to push to one side, collect (on their dorsal surface) and/or phagocytose small particles in their path on the substratum on which they move (Abercrombie et al., 1970; Albrecht-Buehler and Goldman, 1976). This activity generates particle-free tracks ("phagokinetic tracks") on a densely particle-coated migratory substrate that can act as a permanent record of cellular movement. In the original method, as described by Albrecht-Buehler (Albrecht-Buehler, 1977), glass cover slips were coated with bovine serum albumin and then with particles of colloidal gold. Cells seeded onto the gold monolayers migrated, and as they did so cleared gold particle-free phagokinetic tracks. Quantification of this cellular migration is now achieved using computer-assisted image analysis. By averaging the phagokinetic track area left by a number of cells, it is possible to obtain an accurate assessment of the overall migratory activity in a given experimental environment (Zetter, 1980). Statistical analysis can then be used to indicate the presence of subpopulations of cells that may differ in their movement, under otherwise identical conditions, and/or may be used to define a particular cell subset or a specific migration-influencing factor that is present and identifiable under the defined conditions of the assay.

While the basic protocol of the colloidal gold assay used in these experiments is the same as that described by (Albrecht-Buehler, 1977) and modified by (Zetter, 1980; Woodley et al., 1988; Scott et al., 2000); (Li et al., 2001; Li et al., 2004a) further modifications in protocol and analysis were made for use in these experiments and are detailed below.

Initial preparation for each assay was fundamentally the same for each migration experiment, that is, while the conditions of any given experiment varied, as will be described, the quantities of the reagents listed below were altered in proportion only to ensure sufficient reagents were available for experiments to be performed. The Colloidal Gold Migration assay required preparation of fresh solutions of Bovine Serum Albumin (BSA) 1% and 0.1% w/v formaldehyde. 1% BSA was prepared by dissolving 0.5g of BSA

powder (BSA, Standard Grade; VWR SeraCare Life Sciences) in 50ml of purified distilled H_2O by slowly mixing for 30 minutes. The solution was then allowed to stand for a further 1-hour at room temperature before use. 0.1% formaldehyde was prepared by the addition of 135μL of stock formaldehyde solution (37% w/v formaldehyde solution in H_2O, Sigma-Aldrich) to 50ml purified distilled H_2O. Gold chloride solution was prepared using Hydrogen Tetrachloroaurate(III) (Also known as Tetrachloroauric(III) acid, molecular formula $HAuCl_4 \cdot 3H_2O$, Sigma-Aldrich) 0.342g dissolved in 50ml purified distilled H_2O. This was vigorously vortexed until fully dissolved. Sodium carbonate solution was prepared using sodium carbonate anhydrous powder (Molecular Formula Na_2CO_3, Sigma-Aldrich) 0.194g dissolved in 50ml purified distilled H_2O.

Clean round glass cover-slips 2.2cm diameter (VWR International) were grasped with forceps at one edge and dipped into the solution of 1% BSA. With great care the glass slip was repeatedly dipped into the solution and withdrawn, over a period of several minutes. The BSA slowly adhered to the glass and this process provided for a better and more uniform coating. Excess BSA was allowed to drain off the cover slip at an angle and the slip was then dipped once (in and out) of a fresh aliquot of 100% ethanol. The slips thus prepared were then dried gently but rapidly with a hand held hair dryer at medium settings (temperature approximately $85^{O}C$ at 10-15cm from the dryer). Once coated and dried each glass slip was placed into one well of a 12 well cell culture plate (well diameter 2.3cm) (VWR International). The BSA coated glass-slips in the 12 well cell culture plates were then coated with gold salt. Small particles of a mixture of gold salts were deposited onto the BSA on each glass slip as a solution of colloidal gold salt freshly prepared was placed on the slips and allowed to cool. The colloidal gold salt solution was prepared by mixing reagents in the following proportions and manner. 1ml of the solution was sufficient to coat each glass slip in each well.

In an 125ml Erlenmeyer flask the following reagents were mixed at room temperature; 20.8ml of purified distilled H_2O, 12.0ml of the sodium carbonate (Na_2CO_3) solution as prepared above, and 3.6ml of Gold Chloride ($HAuCl_4 \cdot 3H_2O$) solution, also as prepared above. Over a naked Bunsen burner flame the mixture was gradually heated while continually, but very gently swirling the liquid (rapid heating or vigorous shaking would result in a failure of the gold particles to precipitate out). Immediately, as the solution reached the boiling point it was removed from the heat and 3.6ml of the 0.1% w/v

formaldehyde was added (an equal volume to that of the Gold Chloride solution) while continuing very gentle swirling. The successful preparation of a colloidal gold salt solution was indicated by the solution turning brownish in colour. When held up to a bright room light the colour was blue-purple. This mixture was then immediately pipetted into the wells of the 12 well cell culture plates containing the BSA coated glass slips, 1ml per well. The plates were allowed to incubate undisturbed (and covered) during which time gold particles were deposited onto the BSA coated glass slips. Incubation was continued either for 45-60 minutes at room temperature (in a designated area for storage), for migration experiments that were run immediately, or overnight at $4^{O}C$ (in a cell culture storage refrigerator), for experiments that were to be performed the next day.

Culture plates containing deposited gold salt required handing with extreme care and in a sterile manner. Contamination and or minor mechanical agitation would easily disrupt the gold salt layer and render the experiment void. During all subsequent handling, the cell culture plates were at all times in an incubator, designated clean storage area, or cell culture storage refrigerator and were manipulated under sterile conditions in a Class II safety cabinet with filtered laminar airflow, unless otherwise specified. After incubation the residual liquid (remaining colloidal gold salt solution) was carefully aspirated from each well. Each well was then rinsed with Hank's Buffered Salt Solution with calcium chloride and magnesium sulphate (HBSS+) (Sigma-Aldrich or from our laboratory core facility at USC made to the same specifications). 1ml of HBSS+ was added to each well and the plate was gently rocked to-and-fro for 10-15s. This was then aspirated and a further 1ml of HBSS+ was added to protect the gold salt and prevent the slips from drying out. Microscopic visual inspection of the wells was then performed using a 10x eyepiece and 5-10x objective lenses (Olympus CK2 inverted phase contrast microscope, or Zeiss Axioplan-2 optical microscope). A uniform orange brown speckled appearance on a black background covering the surface of the slides followed a successful preparation. Glass slips showing a heterogeneous coating (uneven density of speckling, areas of no specking or confluent areas of brown colour without speckling, other than at the very edges of the glass slips) were noted and were not used.

Further preparation for a colloidal gold migration assay varied according to the details of the experiment being performed. In each case per the parameters of the experiment there were:

1- Deposition of one, or more, extracellular matrix (ECM) components and suitable control(s) substrates onto the gold salt layer in the required number of wells specified in the experimental design.
2- Conditioning of media, which were prepared to represent the chosen environment(s) in which migration was to occur along with suitable control environment(s) as specified in the experimental design.
3- Plating primary human keratinocytes on to 12 well cell culture plates, approximately 3000cells/well at passage three or four, onto the substrates listed in 1, and suspended in the conditioned media listed in 2.
(Note: The keratinocytes assayed may be prepared with or without prior manipulation and or pre-treatment. Furthermore, there may also be additional post plating manipulation and or exposure. In both cases these interventions will be described along with general details for each experiment).

Deposition of substrates onto the gold salt layer required preparation of fresh solutions of each substrate component designated for each well, according to the experimental design. The concentration used for each substrate varied. Each substrate was prepared from stock materials by dilution in HBSS+ per manufacturer guidelines (Table 1).

(Table 1)

Substrate	*Concentration for deposition over a 2 hour period*	*Concentration for deposition over a 24 hour period*
Collagen I	40µg/ml	20µg/ml
Collagen IV	40µg/ml	20µg/ml
Fibronectin	10µg/ml	5µg/ml
Vitronectin	10µg/ml	5µg/ml
Laminin-1	80µg/ml	40µg/ml
Laminin-5	10µg/ml	5µg/ml
Poly L-Lysine	10µg/ml	5µg/ml

Table 1: Concentrations of substrates in solution used for deposition. (Substrates were deposited over 2 or 24 hours).

Details of which substrate was deposited in any given well of a cell culture plate and of any variations in concentrations used will be described with the details of each experiment.
Type I Collagen was derived from rat-tail tendon and Type IV Collagen from mouse-tail

tendon (BD Biosciences cat #354236 and #354233 respectively). Fibronectin and Vitronectin were recombinant proteins of human origin (BD Biosciences cat #354008 and #354238 respectively). Laminin-5 was rat in origin and contains homologous chains to human Laminin-5, $\alpha 3\ \gamma 2\ \beta 3$ at ≈160kDa and $\gamma 2'$ at ≈98kDa and $\gamma 2x$ at ≈64kDa (Chemicon cat # CC145). Laminin-1 was obtained from the from our laboratory at USC, it was mouse in origin and prepared according to previously described techniques (Woodley et al., 1988). Poly-L lysine is a synthetic substrate used as a negative control to migration in many migration experiments (Sigma Aldrich cat # 25988-63-0). On Poly-L lysine keratinocytes show adherent morphology and adhesion formation, but they do not migrate, either in the presence or absence of growth factors.

Immediately after preparation of gold salt-coated glass-slips, 1ml of HBSS+ was added to each well to protect the gold salt layer. Prior to substrate deposition this was carefully and slowly aspirated from the lower margin of the glass slip with the 12 well culture plate tilted to around 30^{O} degrees (the exact angle was varied so that there was minimal exposure of the gold salt to the air at any time). 1ml of each substrate solution of an appropriate type and concentration was added to each designated well in the culture plate, per the details of the experimental design. The cell culture plates were then stored to allow substrate deposition: for experiments being performed on the same day, 2-hours undisturbed were required in a designated area at room temperature and for experiments due to be performed on the following day plates were stored for 24-hours in a cell culture fridge at $4^{O}C$. Repeat microscopic visual inspection of the wells was performed (10x eyepiece and 5-10x objective lenses) and any glass-slips showing a disrupted coating were noted and were not used.

During this time, the cells to be used in the experiment were prepared. Details of the subculture procedure and any other prior manipulation of the cells during preparation for the experiment are discussed elsewhere or in association with the details accompanying each experiment. Keratinocytes were subcultured to passage 3 or 4 to provide the correct number of cells for the planned experiment. One day prior to performance of an experiment the existing cell culture medium was changed to GF-, and the keratinocytes were "starved" overnight (16-hours). Medium was aspirated carefully and slowly from the lower margin of the P100 or P60 cell culture plates tilted to around 30^{O} degrees (tilted to achieve minimal exposure to the air at any time). 10ml (P100) or 4ml (P60) of pre-warmed $37.0^{O}C$ GF- medium was carefully added to each plate and the cell culture plates were returned to the

incubator. (Substrate deposition might also have been performed at that time, 24-hour deposition).

On the day of the experiment, media were prepared and conditioned to meet the chosen environment(s) in which migration was to occur along with suitable control environment(s). These media were warmed to 37.0^{O}C. More specific details of media conditioning will accompany details of each experiment. The cells "starved overnight" were trypsinized and re-suspended in a total of 3ml GF- medium, in the same manner described for subculture passage. A Trypan blue exclusion assay was performed and a viable cell concentration determined. (Substrate deposition might also have been performed at that time, 2-hour deposition). Using the viable cell concentration for each cell type in the experiment, a volume containing 3000 cells (3000 cells per well) was calculated V_{3000}. This volume was multiplied by the number of wells, N_W, designated in the experimental design to receive this cell type with any one of the conditioned media that were used. The re-suspended cells of the type chosen were thoroughly and uniformly mixed and this volume = V_{3000} x N_W was drawn-up. It was then added to a volume of the one chosen conditioned media equal to the number of wells that needed to be filled (volume of one conditioned media needed =1 x N_W in ml). This volume was placed in a 15ml test tube T_1. This was repeated for all cell types and all conditioned media variations covered in the details of the experiment. ($T_1, T_2, \ldots T_n$) each contained $N_{W1}, N_{W2}, \ldots N_{Wn}$ ml of a matched cell type and a matched conditioned media. After the appropriate time for deposition (2 or 24 hours) the remaining substrate solution in each well was aspirated as above. The matched cell types and conditioned media combinations (in $T_1, T_2, \ldots T_n$) were re-mixed and 1ml of each was added to each well detailed to receive this combination as outlined in the experimental design. This process ensured sufficient cells and medium, of a matched cell type and medium conditioning, to cover the designated substrates, to create the required environment(s) for the experiment. The 12 well cell culture plates were returned to the incubator. After 2-hours incubation, a repeat microscopic visual inspection of the wells was performed and cover slips showing a disrupted gold salt coating, low numbers of adherent cells, or other abnormalities were noted and were not used. Post-plating manipulation and or treatment after this point were infrequent. If such intervention was made, information is provided along with details of each experiment.

Once all plates were processed and inspected they were incubated for over six hours and up to twenty-four hours. During this time the plated cells migrated. Prior studies have shown that this time is sufficient to allow keratinocyte migration to reach a maximum, which under optimal conditions occurs as early as six hours (Li et al., 2001). This time is also known to be well below replication time for primary human keratinocytes.

2.3 *In vitro* wound migration assay ("Scratch assay")

The *in vitro* wound assay was first described by (Matthay et al., 1993). It has been modified subsequently (Zhang and Kramer, 1996; O'Toole, 1997), and is currently used widely according to a variety of different experimental protocols. These protocols differ primarily in the concentrations of substrate deposited, the manner and timing of the substrate deposition in relation to the seeding of the cells to be studied, along with different approaches to the manufacture of the wound.

2.3.1 *In vitro* wound migration assay: Protocol 1

The significant feature of this protocol is substrate deposition prior to plating of cells and prior to creation of the wound. Sterile 12 well cell culture plates were washed with HBSS+ and the residual HBSS+ removed. Designated substrates were prepared and deposited in the same manner as for the colloidal gold migration assay; using the same concentrations and for the same deposition times (2 or 24 hours) (Table 1). Note: The substrates were deposited onto the plastic base of 12 well cell culture plates.

Keratinocytes at passage 3 or 4 were prepared in the same manner as for the colloidal gold salt migration assay (including any necessary prior manipulation according to the details of the experiment). One day prior to execution of an experiment, the existing cell culture medium was aspirated and replaced with GF- medium. The keratinocytes were "starved" overnight (16-hours) in this medium. The starved keratinocytes were then trypsinized, tested for viability (Trypan blue exclusion assay), counted, and re-suspended in GF- medium as described above (Section 2.2). They were then seeded onto the substrate coated wells of the 12 well cell culture plates at 40,000 viable cells/well. (1ml of cells in GF- medium per well was used: 1 well is 3.8cm^2). This provided near confluent 80-90% coverage, of the base of each well. The cell culture plates were returned to the incubator for two to three hours and examined under microscopy (100x) to ensure there was near

confluence of attached cells. Wells not showing this level of coverage were excluded from further experimentation. By microscopy (100x) a uniform wound ("scratch") was made physically through the layer of cells at the base of each well. A single uniform defect in the cell layer was best made using the pointed end of a 1000μl pipette tip. This light flexible tip used with minimal pressure caused limited if any damage to underlying substrate while making a reliable and reproducible wound.

The width of the wound in the keratinocyte monolayer was 2-3mm. Wells showing inadequate or irregular wounds were excluded from further experimentation. After wounding the wells were washed twice with Phosphate-buffered saline (PBS) (Invitrogen cat # 70013032, or from our laboratory core facility at USC, made to the same specifications) to remove cellular debris. The residual PBS was aspirated. Media conditioned to meet the chosen environment(s) in which migration was to occur along with suitable control environment(s) was prepared and 1ml added to each designated well (matching cells in media to substrate per the details of the experiment). Mitomycin-C 5μg/ml, a potent inhibitor of cellular proliferation, was added to each medium prepared to prevent possible keratinocyte proliferation during the 16-24 hour experiment. (Mitomycin-C was added at a concentration of 5μg/ml when present in medium overnight 16-24 hours and at 20μg/ml when present in medium for 2-4 hours). Such concentrations of Mitomycin-Care known to produce maximal inhibition of keratinocyte proliferation and did not appear to affect their migration or cause cytotoxicity. (This was demonstrated in otherwise similar experiments (results not shown) performed with and without addition of Mitomycin C and has been shown previously (Daniel and Groves, 2002) and by the Trypan blue exclusion assay). Post-plating manipulation and or treatment after this point were infrequent. If such intervention was made, details will be provided along with details of each experiment. Once all plates were prepared they were incubated for between sixteen hours and twenty-four hours. During this time the plated cells migrated into the wound.

2.3.2 Conditioned media preparation; containing Secreted-ECM

Migrating keratinocytes have been reported to produce limited amounts of extracellular matrix proteins such as laminin-5 (Zhang and Kramer, 1996), fibronectin (O'Keefe et al., 1987), and to a lesser degree collagen IV (Amano et al., 2001; Jussila et al., 2002). During the *in vitro* wound assay this secreted-ECM is mainly deposited locally adjacent to and beneath those cells producing it (Nguyen et al., 2000; Frank and Carter, 2004). In addition

it is believed that the secreted-ECM also spreads to more distant sites in the surrounding medium and is deposited onto the culture plate in exposed areas including the site of any wound that has been produced. When this occurs, the composition of the extracellular matrix on which the keratinocytes are migrating is altered and is to some extent unknown (that is, the extracellular matrix on which migration is occurring may not be precisely that supposed in the experimental conditions and its composition is unknown).

To demonstrate this was occurring and characterize generally any effect on migration such activity may have had, the conditioned media thought to contain secreted-ECM was gathered by collecting the medium surrounding keratinocytes specifically plated for that purpose. Experiments were then performed that compared migration of keratinocytes on wells coated, using the collected conditioned media (thought to contain the secreted-ECM) and the migration produced on wells coated with standard solutions of substrates; Collagen I, and Poly-L lysine (using the colloidal gold migration assay).

The conditioned media containing secreted-ECM was prepared from a subculture of unmodified keratinocytes. These cells were maintained in GF+ medium on uncoated P100 plates to passage 3 and grown to 70-80% confluence before each subculture. The cells were then "starved" overnight (16-hours) in GF- medium (to simulate conditions prior to a typical migration experiment). The cells were then trypsinized, re-suspended in GF+ medium, and seeded in large numbers (1,250,000 viable cells per plate) onto two more fresh uncoated P100 plates pre-washed with HBSS+. This produced approximately 50-60% confluence. This level of confluence was less than that typically used during an *in vitro* wound migration assay (80-90%). The slightly reduced level of confluence allowed more cells to migrate in the presence of the GF+ medium. Under these conditions it was hoped a larger amount of secreted-ECM would be released into the medium for collection and further experimentation. The cells were incubated for 24-hours and the medium was then aspirated and collected and the conditioned media was labelled "secreted-ECM solution" for further experimentation. This conditioned media was used as a substrate depositing solution, 1ml was added to each designated well in the colloidal gold migration assay to allow any substrate present to deposit on the well base.

2.4 Migration data collection and statistical analysis

After completion of migration in either assay (typically 16-hours and no more than 24-hours), the existing medium was aspirated and the wells rinsed with HBSS-. The keratinocytes were then washed with 1 ml of HBSS and fixed with 1% w/v formaldehyde. Plates fixed with 1% w/v formaldehyde could be stored for several weeks at 4^{O}C. Migration (if any) that had occurred in each well was then analyzed.

2.4.1 Colloidal gold migration assay analysis

The migration tracks (phagokinetic tracks) in the gold salt made by keratinocytes moving across the substrate were analyzed using an image capture system and a National Institute of Health (NIH) software program called NIH-Image v1.61 (http://rsb.info.nih.gov/nih-image/download.html). The image capture and analysis system was centred on a microscope assembly, Zeiss Axioplan-2 optical microscope (Figure 7).

(Figure 7)

Figure 7: Image capture and analysis system.

The objective lens was 10x and the objective stage area could be moved in two horizontal dimensions for positioning within the field of view (and vertically for focusing). The 12 well cell culture plates were placed on the objective stage and were transversely illuminated externally for dark-field analysis. The image capture element (CCD device) was Model KP-

MIU (Hitachi-Denshi Ltd). This element received the same image as eyepiece (10x) stage in the microscope assembly (the CCD image could be simultaneously viewed on a computer monitor adjacent to the microscope as well as through the eyepiece). Output from the CCD device was linked by coaxial cable to a PCI video capture card, an ATI Rage 128 Pro. This capture card was installed in a Microsoft Windows XP Dell Dimension (Pentium P4 2.6GHz 1Gb RAM) computer system. The NIH-Image software ran on this system and included drivers for direct connection to the image capture card.

Up to 20 non-overlapping fields (images) from each well, of each cell culture plate, used in an experiment were captured and saved to disc. The first image was taken from the centre of the well and subsequent images were taken proceeding counter clockwise at progressively greater distances from the centre. (Fields containing no discrete phagokinetic tracks were not saved to disc). It was seldom possible to obtain 20 unique non-overlapping fields (images) from a single well that contained discrete phagokinetic tracks. Field images captured and saved for analysis were similar to those shown in (Figure 8).

(Figure 8)

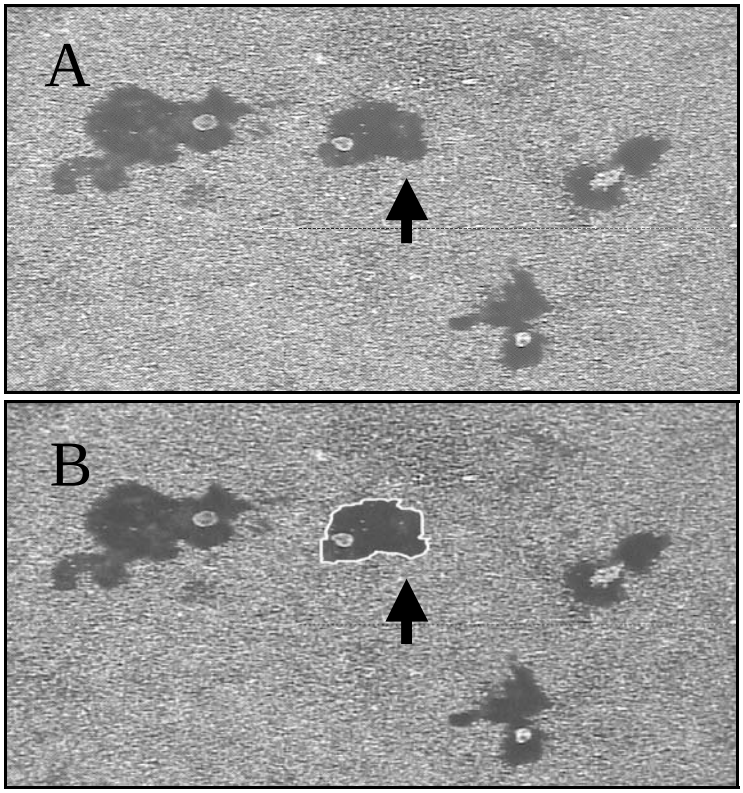


Figure 8: A typical image captured from a gold salt migration experiment showing several phagokinetic tracks (darker areas) made by keratinocytes migrating across particulate gold salt

(speckled grey background) deposited on the surface of a glass cover slip. (Keratinocyte cell bodies are visible as lighter approximately circular areas within the dark phagokinetic track). A: Shows dark-field image as originally captured. B: Shows the same image following addition of a hand-drawn outline (shown in white) around the periphery of one phagokinetic track (indicated by the arrow in each image) of a single keratinocyte prior to further analysis (calculation of the area contained within the outline)

These images were analyzed further within the NIH-Image software. Within the NIH-Image software at start-up and prior to analysis identical input parameters were specified prior to analysis. These parameters were required to ensure recorded values were comparable between images and also comparable to existing standard values (see below). The measured values obtained in the software were reproducible between independent observers (to an accuracy far greater than the limits imposed by other elements of the whole procedure). With an image displayed on the computer screen a line was drawn by hand (mouse and Intuos2 6x8 tablet) around each migration track to be analyzed, one track at a time (Figure 8: B). The NIH-Image software was used to calculate the "area" within the outline according to the scale set by the start-up parameters. This value for area was saved into a Microsoft Excel template. Up to 20 discrete tracks taken from the saved images of a given well were analyzed in this manner. Statistical analysis was performed within Microsoft Excel. A mean value for the area of a track was calculated from the 20 measured values, along with its standard deviation. This average was used to calculate a figure referred to as the Migration Index for Colloidal Gold assay (MI_{CG}) and its standard deviation (Woodley et al., 1988). The MI_{CG} for a given set of experimental conditions was the mean area taken up by the migration tracks, expressed as a percentage of the standardized area of the field of view (fixed by the parameters entered at start-up): MI_{CG} = (Mean Migration track Area of 20 tracks / Area of Standard Field of View) x 100. Typical values for the MI_{CG} were in the range MI_{CG} = 2-40.

At least three independent experiments were performed (three sets of data and three mean MI_{CG}'s) for any given set of experimental conditions. Statistical analysis (ANOVA: Single factor) test was performed on the data sets to determine if the values and MI_{CG} from each independent experiment likely represent data from a single population. Note: The null hypothesis, for the ANOVA test was chosen as: There are no significant differences among

the three mean MI_{CG}. (The alternate hypothesis states that there are significant differences among some or all of the individual means). The alpha level for the ANOVA in each case was chosen as 0.05. The ANOVA test null hypothesis was rejected if $F\text{-}F_{crit} \geq 0$ (and similarly the alternate hypothesis accepted if $F\text{-}F_{crit} < 0$).

Note: F ratios, are the statistic derived from the ANOVA test, they are determined from variance estimates. The significance level, or alpha level, of the test was chosen as 0.05, which is analogous to the choice of $p<0.05$ as being significant. A null hypothesis for a comparison is rejected (or accepted) depending on the F ratio calculated (F) for one data set and that calculated (F_{crit}) for the whole at the chosen level of significance. The null hypothesis is rejected if $F\text{-}F_{crit} \geq 0$. When comparing results from migration assays accepting the null hypothesis was equivalent to the statement that there is no significant difference between the three groups compared, (the data likely represent that from a single population).

In cases where the ANOVA showed that a data set (from any of the repeated experiments) appeared to be inconsistent, suggesting the grouped data sets did not represent results from a single population, the outlying data set was rejected. The associated experiment was then reproduced to obtain a new data set and the ANOVA test was repeated. In cases where the ANOVA null hypothesis was not rejected; then the data and mean MI_{CG} in the three experiments likely represents values from the same population. In that case all data was pooled and an overall average MI_{CG} and its standard deviation was calculated. In no case were less than 20 (and typically greater than 50) data points used to calculate the overall average MI_{CG} and standard deviation. In any instance where less than three reproducible experiments were used, this was noted. Pooled data and overall average MI_{CG} along with its standard deviation was used to display experimental results in the later figures. Separate data from the three independent experiments performed and the results of the ANOVA test are not presented, unless relevant. (Results of some experiments in Chapter 3 are fully shown to demonstrate this statistical analysis).

Further statistical analysis was performed according to the purpose of the experiment. Welch's (unpaired) t-test was used in most cases to compare migration under differing experimental conditions and under control conditions. (The data gathered in each experiment represents a small number of normally distributed continuous variable samples with differing variance that is not paired. Such data is ideally analyzed by the Welch's

(unpaired) t-test). The null hypothesis chosen for the Welch's t-test was chosen as: The mean MI_{CG}'s obtained during experiments under control conditions and those obtained under other experimental conditions do not differ. (The alternate hypothesis states that there are significant differences between these mean MI_{CG}'s). The alpha level for the Welch's t-test was also chosen as 0.05.

2.4.2 *In vitro* wound assay migration analysis

Images of the wound before and after migration were captured with the NIH-Image software under microscopy at a fixed magnification; a representative example image is shown in (Figure 9).

(Figure 9)

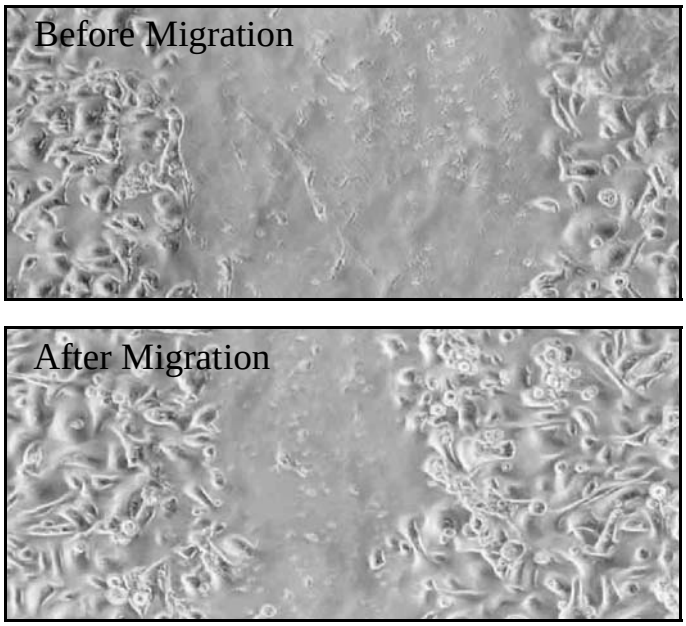


Figure 9: Representative images captured before and after migration during an *in vitro* wound assay are shown. The keratinocytes are migrating towards the centre in sheets, which can be seen at either side of each image. (The images have been aligned so that the left hand sheet of keratinocytes appears in the same vertical position before and after migration).

Up to 5 separate non-overlapping images were captured at points along the length of the wound in each well, of each cell culture plate used in an experiment. The first image was taken from the centre of the well, and subsequent images were taken at first above and then below the previous image, alternating and proceeding across the well/wound. Fields containing damaged or incomplete wound were not saved to disc. In many instances it was not possible to obtain 5 unique non-overlapping fields (images) from a single well that

contained intact wound suitable for further analysis. (The glass cover slip size was 2.2cm in diameter, this allowed for at most 6-7 non-overlapping potential fields to be considered that could be analysed. Analysis of such fields required an image showing approximately 3-4mm of the wound).

Images from the *in vitro* assay give a clear visual depiction of migration into the standardized wound by keratinocytes over the time course of the experiment for qualitative comparisons. Quantification of the migration that occurred during the *in vitro* migration assay was performed within the NIH-Image software (the same software that was used for the colloidal gold migration assays analysis). The same software start-up parameters used for analysis of the colloidal gold migration data were also used for the *in vitro* wound assay data. Again use of these standard parameters ensured comparability of measurements between images. Each captured image was analyzed by superimposing two parallel lines, 2.5mm in length (generated in the software) onto each of the two approaching edges of the wound. The two lines were fixed at the same vertical height but their separation was adjustable to allow alignment with the two wound edges (Figure 10).

(Figure 10)

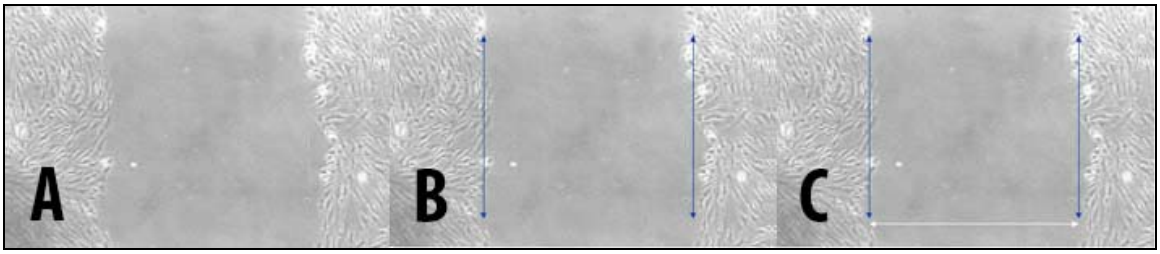


Figure 10: Quantitative analysis of the *in vitro* wound assay. A: Image of a wound prior to migration. B: Two parallel lines (shown in blue) are positioned approximately at the wound margins. C: Measurement of the distance between the two wound edges (shown in white). The process is repeated after migration has occurred.

The distance between the two lines was recorded and this value was saved into a Microsoft Excel template where statistical analysis was performed. An average value for the width of the wound was calculated from the measured values, along with its standard deviation. This average was used to calculate a figure referred to as the Migration Index for *in vitro* assay (MI_{IV}) and its standard deviation. MI_{IV} for a given set of experimental conditions was equal

to one minus the average width of the wound after completion of migration divided by the average width of the wound before migration. [MI_{IV} = 1- (Mean Width of wound after migration is completed / Mean Width of wound before migration begins)]. Typical values for the MI_{IV} ranged from 0.0 to 1.0. (0.0 was the value obtained when no migration occurs i.e. the width of the wound remains unchanged and 1.0 is the value obtained when there is complete closure of the wound by the end of the migration period).

In most cases three independent experiments were performed to obtain three MI_{IV}'s for a given set of experimental conditions and controls (no less than two experiments were performed in any case). As previously described (Section 2.4.1), statistical analysis was performed first using an ANOVA (Single factor) test to determine if the data and mean MI_{IV}'s from each independent experiment was likely to represent values from a single population. (The same null and alternate hypotheses were used). In cases where the ANOVA showed that a data set (from any of the repeated experiments) appeared to be inconsistent, suggesting the grouped data sets did not represent results from a single population, the outlying data set was rejected. The associated experiment was reproduced to obtain a new data set and the ANOVA test was then repeated. In cases where the ANOVA null hypothesis was not rejected; the data and mean MI_{IV}'s in the three experiments likely represents values from the same population. In that case all data was pooled and an overall average MI_{IV} and standard deviation was calculated. In no case were less than 6 (and typically 10 or more) data points used to calculate the average MI_{IV} value and its standard deviation. Pooled data and overall average MI_{IV} along with its standard deviation was used to display experimental results in the later figures. (Separate data from the three independent experiments performed and the results of the ANOVA test are not presented, unless relevant).

Further statistical analysis was performed according to the purpose of the experiment. Welch's (unpaired) t-test was used in most cases to compare migration under differing experimental conditions and under control conditions as described previously (Section 2.4.1). (As was the case for the colloidal gold migration assay the data gathered in each experiment represents a small number of normally distributed continuous variable samples with differing variance that is not paired. The same null and alternate hypotheses were used).

2.5 Protein analysis of cell lysates: Western Blotting

Measurement of the level of expression of a gene product in its phosphorylated and unphosphorylated forms under designated experimental conditions was made using standard Western Blot techniques. (Unless specified, all reagents were manufactured in the USC laboratory core facility). At the appropriate time point in the experiment, existing medium was aspirated and the cell culture plates were washed twice with ice-cold PBS. Lysis buffer was added, 100μl on P60 cell culture plates and 250μl on P100 plates. Using a "rubber policeman," the cells were immediately scraped from the cold cell culture plates and transferred to pre-chilled, labelled, Eppendorf tubes. In a cold room setting (4°C) the samples were vortexed for 15 seconds each and then centrifuged at 14,000 rpm for 5 minutes (Sigma Aldritch Spectrafuge Mini Centrifuge cat#S7191) to form a pellet. The supernatant from each of the tubes was transferred to further pre-chilled similarly labelled, Eppendorf tubes. The protein contents of the lysates were measured in Bradford spectrophotometry protein assays with measurements at wavelength of 595nm per manufacturers guidelines (Bio-Rad cat#500-0201). A volume of lysate containing 50μg of each sample (a volume determined by the results of the Bradford assay) was added to sample buffer sufficient to produce a total volume of 25μl. This was heated to 100°C for 5 minutes. Each sample was then carefully loaded into separate lanes of a stacking gel that was prepared above a 10% sodium dodecyl-sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) resolving gel. 5μl of a standard molecular weight marker (Bio-Rad cat#161-0318) was also loaded into lane two of the stacking gel and 25μl of sample buffer alone was loaded into any unused lanes. The proteins were separated by size with SDS-PAGE. The Enhanced ChemiLuminescence (ECL) Western Blotting system (Amersham Biosciences cat#RPN2106) was then used and the manufacturer's protocol followed. The resolved proteins were transferred, "blotted," onto a nitrocellulose membrane (Amersham Biosciences Hybond ECL cat # RPN2020D), under an applied electric field. The nitrocellulose membrane was then washed on an orbital shaker with Tris Buffered Saline with Bovine Serum Albumin (TBS-BSA) for 5 minutes. This was repeated three times with fresh TBS-BSA on each occasion. Blocking of non-specific binding sites was achieved with 5% non-fat milk in TBS, at room temperature on an orbital shaker for 2-hours (or overnight at 4°C). The membrane was then briefly washed twice with fresh ECL Wash Buffer on each occasion (5 minutes each time on an orbital shaker).

Primary antibodies were chosen to non-phosphorylated and phosphorylated forms of specific proteins. Primary antibodies were monoclonal mouse IgG1 or polyclonal rabbit IgG with appropriate specificity and were diluted in TBS-BSA (or according to manufacturers' guidelines). Note: The ideal concentration of the primary antibody was determined by dot blots of various concentrations of primary antibody 1:100 1:500 1:1000 1:2000 and 1:4000, against a single concentration of diluted secondary antibody 1:1000 in TBS-BSA, of corresponding specificity (mouse or rabbit), according to the ECL Western Blotting protocol. Primary antibodies used were anti-p38α, anti-JNKK2 (MKK7) and anti-FAK (Santa Cruz Biotechnology cat# sc-535, sc-7104 and sc-557), anti-JNK, anti-MEK1/2, anti-phospho p38 and anti-phospho-JNK (Cell Signalling Technology cat# 9252, 9122, 9211 and 9251), anti-Erk1/2 and anti-phospho-Erk1/2 (Invitrogen cat # 44-654G and 36-8800) and anti-Src, anti-Paxillin, anti-phospho-Paxillin (p-Tyr-118) and anti-phospho-Tyrosine (p-Tyr) (Abcam cat# ab22701, ab39537, ab38466 and ab17755).

Incubation of the membrane and primary antibody was performed at room temperature on an orbital shaker for 1-hour (or overnight at 4°C). The nitrocellulose membrane was washed on an orbital shaker with ECL Wash Buffer for 15 minutes. This was repeated three times with fresh wash buffer on each occasion. Secondary antibody (mouse or rabbit) conjugated with horseradish peroxidase (HRP) was diluted in Tris Buffered Saline with Triton (TBS-Triton X-100). (Note: The ideal concentration of the secondary antibody was determined by dot blots of various concentrations of secondary antibody 1:100 1:500 1:1000 1:2000 and 1:4000, against a single dilution of primary antibody, 1:1000, according to the ECL Western Blotting protocol). Incubation of the membrane and secondary antibody was performed at room temperature on an orbital shaker for 1-hour (or overnight at 4°C). The nitrocellulose membrane was washed on an orbital shaker with wash buffer for 5 minutes. This was repeated three times with fresh ECL Wash Buffer on each occasion.

ECL and autoradiography film (Amersham Biosciences Hyperfilm ECL cat#28-9068-36) allowed visualization of a particular protein as a band on the developed film. Images from unsaturated exposures were used to determine relative "fold" difference between bands by densitometry measurements. A mean densitometry reading of a given band was calculated from three different experimental images (corresponding to the same protein in three different experiments performed under the same conditions). To obtain the relative "fold"

increase or decrease, this was compared with similar values of the densitometry readings from control bands or from the same protein band under different experimental conditions.

2.6 Fluorescent staining of cultured cells

The procedure for microscopic visualization of fluorescent staining of cultured cells was that described previously (Chen et al., 2000; Li et al., 2004a). Keratinocytes at passage 3 or 4 were starved overnight (16-hours) in serum-free medium GF-. They were then trypsinized, assayed for viability (Trypan blue exclusion assay) and re-suspended in GF- medium. 35,000 viable cells per well were plated in each well of a 6 well tissue culture plate onto square (2.2cmx2.2cm) glass cover slips that had been pre-coated with selected substrate at the concentrations shown in (Table 1). The keratinocytes were then incubated at 37°C and 5%CO_2 for 4-hours to allow attachment and the existing media aspirated. The keratinocytes were then stimulated per the conditions of the experiment. Typically, this was by addition of GF+ or GF- medium for 15, 30, or 60 minutes. (Any variations from this will be mentioned with the corresponding result). Existing medium was aspirated and the keratinocytes were fixed by the addition of 1.5ml per well, freshly made 3.7% formaldehyde in PBS at room temperature. After 20 minutes this was aspirated and the cover slips were washed with PBS three times. Cells were permeabilized by addition of 1.5ml Triton X-100 (0.2% in PBS) at room temperature. After 10 minutes this was aspirated and the cover slips were washed thoroughly with PBS. (All washing stages were repeated with fresh solution on each occasion three times for ten minutes each time at room temperature).

F-actin was visualized under fluorescence microscopy with Rhodamine-conjugated phalloidin (Invitrogen cat# R415). Washed permeabilized cells (on cover slips in wells) were incubated for 40 minutes at room temperature with Rhodamine-conjugated phalloidin at a concentration of 1μl/ml dilution [in 1% BSA in PBS]. Each well was then washed thoroughly (three times) with PBS, excess liquid was removed and the cover slips air-dried. Cover slips were mounted using anti-fade reagent Prolong Gold Reagent (Invitrogen cat#P36930) and clear nail polish. A drop of anti-fade reagent, was placed onto a clean glass slide and the air-dried cover slips were carefully lowered (with the cell surface downwards) onto the reagent, avoiding trapping any air bubbles. The slide and cover slips were allowed to cure for one day on a flat surface in the dark. Clear nail polish was placed along all edges of the cover slip to form a seal. Fluorescence was visualized using a Zeiss

Axioplan-2 (Carl Zeiss) optical microscope equipped with epifluorescence and photographed with an attached digital camera (AxioCam MRm; Carl Zeiss). In each case the same magnification was used.

Between 75 and 100 cells were analyzed per experimental condition. Subjectively cells were assessed for features of spreading and polarization. The morphology of the cell was noted and the approximate number and distribution of lamellipodia were recorded (the presence and distribution of filopodia were also noted). Cell body asymmetry was also recorded, a polarized morphology was said to exist when the cell body showed a significant difference in length in one axis that was greater (>2x) than any other.

2.7 Lentiviral gene transduction system and chemical inhibitors

Chemical inhibitors and a lentiviral dominant negative gene delivery system (Chen et al., 2003) with various specificities were used to manipulate gene expression and vary gene function under various experimental conditions. There are four main techniques currently in use to study gene function and or alter gene expression in cultured cells. These include: i) the use of chemical inhibitors, addition of chemicals that primarily block the function of a gene product to be studied, ii) Uptake or expression of anti-sense oligonucleotides, that primarily reduce the steady state level of messenger RNAs (mRNAs) for the target gene, and hence expression of its gene product, iii) Development of cell lines with somatic mutations, deletions, or other modifications in a gene and hence the gene product, that alter function, expression, or both, iv) Direct gene delivery systems, either viral or non-viral based that can achieve similar results altering function, expression, or both. Each technique has advantages and disadvantages.

2.7.1 Chemical inhibitors

Chemical inhibitors are simple to use reagents added to culture medium in various predetermined concentrations, at desired interval(s) during an experiment(s). These agents were used to complement the experiments performed with the lentiviral gene delivery system. The chemical inhibitors leave the endogenous stoichiometry of the target molecules relatively unchanged. However, they seldom show absolute specificity (despite manufacturer's assurances) at all concentrations of use and hence, on occasions they may inhibit more than only the intended gene product. The chemical inhibitors were dissolved in

dimethyl-sulphoxide (DMSO), per the manufacturer's guidelines and added to pre-warmed culture media. All inhibitors used were; cell permeable, selective, reversible inhibitors of their respective targets (deviations in selectivity are noted). Details are provided below and in (Table 2).

(Table 2)

Cellular Protein (PubMed reference of corresponding cellular gene)	Inhibitor (Reference: Original submission or analysis)	Inhibitor Function	Comments on Specificity	Inhibitor Control
p38α (Han et al., 1994; Nahas et al., 1996)	SB202190 (Singh et al., 1999)	Competitive inhibition of p38α and p39β kinase activity (with respect to ATP).	No inhibition of Erk or JNK MAP Kinase pathways	SB202474
MEK1 (Zheng and Guan, 1993)	U0126 (Cross et al., 2002)	Non-Competitive inhibition of MEK1 and MEK2 kinase activity (with respect to substrates ATP and Erk1/2)	No inhibition of p38α/β or JNK MAP Kinase pathways	U0124
JNK2 (Lu et al., 1997)	SP600125 (Han et al., 2001)	Competitive inhibition of JNK1 and JNK2 kinase activity (with respect to ATP).	Inhibition of p38α/β and Erk1/2 MAP Kinase pathways at higher concentration	Un-named control provided
FAK (Hanks et al., 1992)	No inhibitor available	---	---	---
c-Src (Weijland et al., 1983)	PP2 (Hanke et al., 1996)	Competitive inhibition of c-Src and other Src family members; Fyn and $p56^{Lck}$ kinase activity	Direct inhibition of FAK is not seen.	PP3

Table 2: Detail and reference information for the cellular proteins and respective chemical inhibitors (and their controls) used in experimentation.

Potential cytotoxicity of the inhibitors to keratinocytes was assessed by Trypan blue exclusion assay and cell proliferation studies, following exposure and removal of the inhibitor. Separately, but from the same subculture, keratinocytes were prepared identically to those used in the migration experiments (plated onto the same substrate, in the presence of the same conditioned media and undergoing any other manipulations identically). These keratinocytes were exposed to each inhibitor at the same concentrations used during the migration experiments and over a time course identical to those cells undergoing experimentation. At the end of the experimental time frame, the same time as completion of the migration experiments, the inhibitor-containing medium was removed and these control keratinocytes were subjected to a Trypan blue exclusion assay before being re-plated for further subculture. After exposure to inhibitors, keratinocytes were said to exhibit signs of cytotoxicity if the percentage cell viability during subculture, assessed by the Trypan blue exclusion assay, was less than that typically achieved for routine keratinocyte passage i.e. <80-90%. A second criterion for cytotoxicity was that the exposed keratinocytes in the subsequent subculture were unable to proliferate up to and above 90% confluence, compared to unexposed controls. If cytotoxicity by either assessment occurred with any of the inhibitors used, at any concentration present in the final migration experiments it is noted in the results section. This was uncommon.

The p38 inhibitor was SB202190 (Calbiochem cat# 559388), and the corresponding negative control was SB202474 (Calbiochem cat#559387) (Singh et al., 1999). This inhibitor competitively (with respect to ATP) inhibits the kinase activity of both p38α/β but has no effect, at the concentrations used, on the Erk (in most cells, including keratinocytes) or JNK mitogen-activated protein kinase (MAPK) signalling pathways. The SB202190 inhibitor was used at concentrations previously described 0, 1, 3, and 10μM (Han et al., 2001) (Table 2).

The MEK 1,2 isoforms, (MEK1/2) inhibitor was U0126 (Calbiochem cat#662005) and the corresponding negative control was U0124 (Calbiochem cat#662006) (Cross et al., 2002). This inhibitor produced non-competitive inhibition of kinase activity with respect to usual substrates adenosine triphosphate (ATP) and Erk. Other potential targets of MEK1/2 such as Abelson tyrosine kinase (Abl), Cyclin dependant kinase 2 (Cdk2), Cyclin dependant kinase 4 (Cdk4), Erk, JNK, mitogen-activated protein kinase kinase (MEKK), mitogen-activated protein kinase kinase 3 (MKK-3), mitogen-activated protein kinase kinase 4 (MKK-4),

mitogen-activated protein kinase kinase 6 (MKK-6), Protein kinase C (PKC), and Erk MAP kinase kinase kinase (Raf) are not inhibited (i.e. have no effect on JNK or isoforms p38α/β in the MAPK signalling pathways at the experimental concentrations). The U0126 inhibitor was used at concentrations previously described 0, 3, 10, and 30μM (Han et al., 2001) (Table 2).

The JNK1/2 inhibitor was SP600125 (Calbiochem cat#420119) and the corresponding negative control (unnamed) (Calbiochem cat#420123) (Han et al., 2001). This inhibitor competitively (with respect to ATP) inhibits the kinase activity of JNK1/2 with a selectivity that is 300 times that of the inhibition of Erk1/2 or p38α/β MAPK signalling pathways at the concentrations used. The inhibitor SP600125 was used at concentrations previously described 0, 10, 30, and 100μM (Han et al., 2001) (Table 2).

The Src inhibitor used was a pyrazolopyrimidine PP2 (Calbiochem cat#529573) and the corresponding negative control was PP3 (Calbiochem cat#529574) (Hanke et al., 1996). This inhibitor competitively inhibits several members of the Src family; c-Src, Fyn, and $p56^{lck}$. Inhibition of other kinases occurs, but only at much higher concentrations EGFR, Janus kinase (JAK) and zeta-associated protein kinase (ZAP). (FAK kinase activity is not inhibited directly by PP2, but inhibition of Src family members by PP2 is associated with reduced phosphorylation at Tyr-576 and Tyr-577 on FAK). The inhibitor PP2 was used at concentrations previously described 0, 3, 10 and 30μM (Hanke et al., 1996) (Table 2).

2.7.2 Lentiviral gene transduction system

There are several means of transfection (introduction of foreign DNA of the desired gene) into eukaryotic cells that are viral (usually referred to as transduction) and non-viral. In primary cell lines non-viral gene delivery systems often result in transient expression of the intended gene product and they typically have lower gene transfection efficiency compared with viral transduction systems (Jiang et al., 1991; Chen et al., 2003). There have been many comparisons of non-viral techniques such as calcium phosphate co-precipitation, liposomal transfer, electroporation, cationic polymer-mediated transfection and proprietary agents such as Lipofectamine. Calcium phosphate co-precipitation and cationic polymer mediated transfection are frequently shown to be the most efficient (Jiang et al., 1991) (and at that time the most cost effective). Other studies have demonstrated that the transfection efficiency of non-viral systems is of the order of 20-30%, of all cells undergoing the

procedure (Staedel et al., 1994; Li et al., 2001) and transgene expression is usually transient. Viral transduction systems have been widely used and typically produce a much higher transfection efficiency; both adenovirus and lentiviral vectors are commonly used *in vitro* (Schagen et al., 2000). The transgene carried by an adenovirus vector is usually not integrated into the host genome and while the efficiency of transfection is high the gene product expression like the non-viral systems often remains transient (Harui et al., 1999; Schagen et al., 2000).

A lentiviral gene transduction system was used to deliver genes into keratinocytes for these experiments. The system was based on the human immunodeficiency virus (HIV) derived lentivirus vector pRRLsin (provided to our lab by Prof. Nori Kasahara, USC Institute of Genetic Medicine). Using this system (performed by Professor Mei Chen in the USC core transfection facility), high gene transduction efficiency was consistently achieved in both primary HEKs and HDFs. Furthermore, sustained expression of the transgene beyond three weeks has been demonstrated consistently for all the genes used (Chen et al., 2003). (This interval is significantly longer than the time course of any migration or other experimentation performed). Keratinocytes intended for experiment were subcultured in sufficient numbers for the intended protocol(s). At 70-80% confluence (p2 or p3; 2^{nd} or 3^{rd} passage) keratinocytes were provided to the USC core transfection facility for transgene delivery. Lentiviral constructs at predetermined concentrations, containing the transgene were also provided to the USC core facility.

Note: All other materials used in the transduction process were supplied by the USC core transfection facility and the procedure described in the following paragraph and illustrated in (Figure 11) was performed at the USC core facility by Professor Mei Chen and not by the candidate.

In the USC core facility the keratinocytes provided were subcultured and re-plated at 40-60% confluence (approximately $2x10^5$ viable cells per 60mm cell culture plates) in GF+ medium, 24-hours before infection. At the same time a recombinant lentiviral stock was produced according to previously described protocols, using human embryonic 293T cells (Figure 11). The final concentration of this was determined by limited experimentation performed in the core facility and was typically of the order of 10μM in serum free medium (Sakoda et al., 1998). At the time of infection in the core facility existing GF+ culture

medium surrounding the keratinocytes was replaced with 2ml of the recombinant lentiviral stock solution and 16μg of polybrene (8μg/ml). The culture plates were then incubated for 6-hours at 37^{O}C. Following this, the medium was discarded, the plates washed and GF+ medium was added.

(Figure 11)

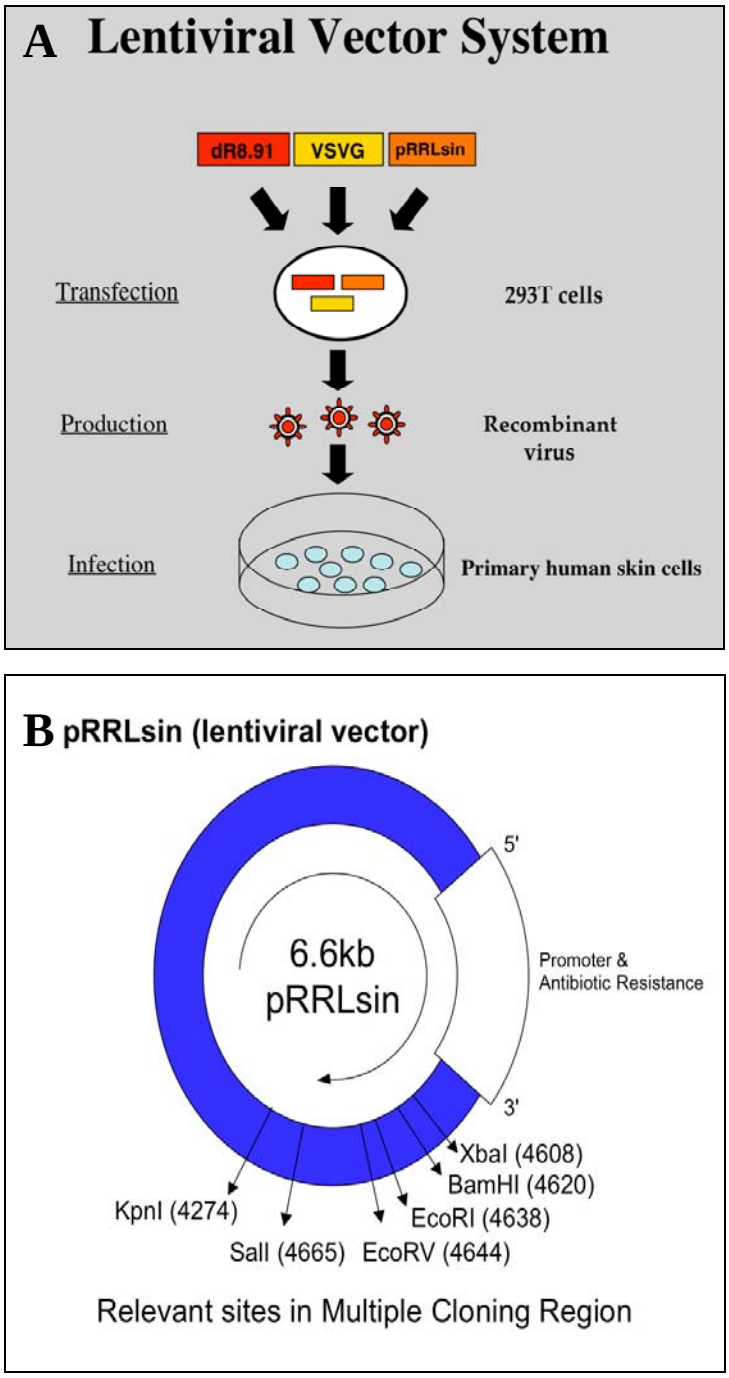


Figure 11: Schematic outline of the lentiviral system used for transduction of selected genes into primary human keratinocytes in preparation for the dominant negative migration assays along with detail of the various regions within the lentiviral vector pRRLsin. A: Transfection of human

embryonic 293T cells is performed with three vectors: dR8.91 (a packaging construct vector), VSVG (an envelope construct vector), and pRRLsin, the lentiviral vector construct that contains the transgene of interest. A recombinant virus is produced and is used to infect the primary human skin cells (keratinocytes) supplied for experimentation. During infection the cells (HEKs or HDFs) incorporate the lentiviral vector construct (and gene of interest) into their genome and are returned for experimentation. Figure A is modified from (Chen et al., 2003). B: Detail of the regions present with the lentiviral vector including the transcription promoter and ampicillin resistance regions and the relevant (see later text) restriction endonuclease sites present in the multiple cloning region.

As a control to the transduction of the transgenes to be evaluated experimentally, pRRLsin-GFP (a lentiviral construct containing green fluorescent protein (GFP) inserted into the vector pRRLsin) was transfected in the USC core facility, into a subculture of the keratinocytes intended for use in subsequent experiments. Production of GFP within these keratinocytes could be visualized by fluorescence using an epiluminescence microscope. Example images [from (Chen et al., 2003)] are shown in (Figure 12) along with an indication of the gene transduction efficiency recorded by fluorescence-activated cell sorting (FACS) analysis, also performed at a USC core facility.

Note: pRRLsin-GFP transduction and calculation of gene transduction efficiency by fluorescence-activated cell sorting was performed by Professor Mei Chen as a service of the USC core transfection facility.

(Figure 12)

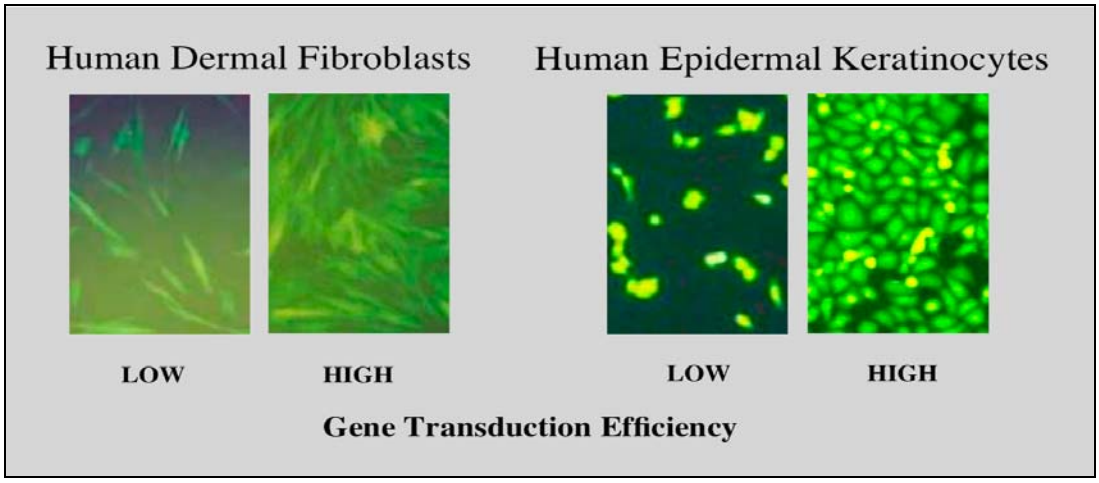


Figure 12: Visualization of green fluorescent protein (GFP) expression in human dermal fibroblasts and human epidermal keratinocytes following GFP transfection with Lipofectamine (left hand side images showing "LOW" gene transduction efficiency for each cell type) and the pRRLsin based

lentiviral vector system of gene delivery (right hand images showing "HIGH" gene transduction efficiency for each cell type). Images provided from our lab by (Chen et al., 2003). FACS analysis was used to determine gene transduction efficiency and reported by the USC core facility as a percentage of all cells showing GFP fluorescence following transfection.

Demonstration of GFP production following the lentiviral transduction procedure was assumed to be representative of successful transduction of this gene and of the additional genes transduced into separated keratinocyte subcultures at the same time by the same technique. (GFP expressing cells were not assayed during the experiments presented in this thesis). The procedural control achieved by performing, in parallel, the simultaneous transduction of the vector pRRLsin-GFP and the vectors containing the genes to be studied, pRRLsin-xxx, provided only circumstantial evidence as to the successful completion of the process for studying genes. More recent work has achieved dual constructs and their expression in keratinocytes allowing for direct visualization and quantification of a successful gene transduction, but this was not possible at the time of these experiments.

Subsequent Western blot was used as further evidence of successful (or otherwise) transduction and of expression of the desired experimental transgene. Each transgene incorporated into the host genome during the transduction process was typically expressed at a level significantly above those of the normal cellular gene, due to promoters present in the lentiviral vector. Competition between the cellular gene that is expressed at normal or reduced levels (due to feedback inhibition), and the over-expressed transgene, resulted in the effect of the transgene dominating. Keratinocytes transduced with the pRRLsin vector alone or vector plus functional ("wild-type") transgene of interest were used as controls in migration assays. Following transduction of the vector alone or vector plus functional ("wild-type") gene, it was hoped the effects on cellular function were negligible, although undoubtedly there is alteration in the stoichiometry within the cell that may have consequences other than those intended. Transduction of the inactive form and its subsequent over-expression should lead to lack of gene function. (In the case where the transgene has a negative effect, it is referred to as a "dominant negative"). Potential cytotoxicity caused by over-expression of the transgene was assessed by Trypan blue exclusion assay and cell proliferation studies. These assays were performed following completion of the gene transduction process in keratinocytes prepared and handled identically to those undergoing the migration and wound assays. By either assessment,

cytotoxicity did not occur following any of the gene transductions performed during these experiments.

2.7.2.1 Lentiviral constructs (for transgenes of p38, MEK1, JNKK2, FAK and Src)

Lentiviral constructs were formed using established standardized techniques and protocols. Each transgene was provided pre-inserted in a known orientation in a known plasmid. Details of the transgene, any modifications and source of that modification, of its insertion sites and of the containing vector plasmid are provided here and in (Table 3).

(Table 3)

Cellular Gene (Reference to NCBI PubMed Core Nucleotide Submission)	Transgene ("Wild-Type")	Transgene ("Dominant Negative")	Dominant Negative functional deficit	Insertion sites in vector pRRLsin	Reference to Dominant Negative effect
p38α (Han et al., 1994)	p38α-Wt	p38α-Dn	Kinase inactive	SalI and SacII	(Han et al., 1996; Jiang et al., 1996)
MEK1 (Zheng and Guan, 1993)	MEK1-Wt	MEK1-Dn	Kinase inactive	SalI and EcoRI	(Catling et al., 1994)
JNKK2 (Lu et al., 1997)	JNKK2-Wt	JNKK2-Dn	Kinase inactive	XbaI and SalI	(Tang et al., 2001)
FAK (Hanks et al., 1992)	FAK-Wt	FAK-F397	Auto-phosphorylation site absent	EcoRV and SalI	(Schaller et al., 1994)
		FAK-R454	Kinase inactive	EcoRV and SalI	(Hildebrand et al., 1993).
c-Src (Weijland et al., 1983)	Src-Wt	Src-KM	Kinase inactive	BamHI and EcoRI	(Mukhopadhyay et al., 1995).

Table 3: Detail and reference information for the cellular genes and transgenes ("wild-type" and dominant negative) used in experimentation, along with the restriction endonuclease insertion sites in the pRRLsin lentiviral vector.

Two p38α transgenes were used: "Wild-type" p38 MAP kinase (p38α-Wt) and a dominant negative mutant of p38 MAP kinase (p38α-Dn), which blocks endogenous p38 MAP kinase signalling. NCBI Pub med Core Nucleotide reference for p38α-Wt is U10871: Mus musculus MAP kinase mRNA [Submitted: (Han et al., 1994)]. For both p38α and p38β, Thr188 and Tyr190 are phosphorylation sites involved in regulating the kinase activity. The existing dominant negative (p38α-Dn) supplied, was a double mutant created by substituting Thr188 with Ala188 and Tyr190 with Phe190, using site directed mutagenesis that resulted in p38α with absent kinase activity (Han et al., 1996; Jiang et al., 1996). This mutant has been widely used and was previously obtained as a gift to our lab from Professor J Han, Department of Immunology, The Scripps Research Institute, CA, USA. Both p38α-Wt and p38α-Dn used in these experiments were provided pre-inserted into pRRLsin at restriction sites SalI and SacII (Table 3).

Two MEK1 transgenes were used: "Wild-type" MEK1 (MEK1-Wt) and a dominant negative mutant of MEK1 (MEK1-Dn), which blocks endogenous MEK1 signalling. NCBI Pub med Core Nucleotide reference for MEK1-Wt is L11284: *Homo sapiens* Erk activator kinase (MEK1) mRNA [Submitted: (Zheng and Guan, 1993)]. In MEK1, Ser218 and Ser222 are phosphorylation sites involved in regulating kinase activity (normally they are phosphorylated by the proto-oncogene product Raf-1). The existing dominant negative (MEK1-Dn) supplied, was a double mutant created by substituting both serine residues at sites 218 and 222 with alanine residues using site directed mutagenesis that resulted in MEK1 with absent kinase activity (Catling et al., 1994). This mutant has been widely used and was previously obtained as a gift to our lab from Professor A Catling, University of Virginia, Charlotteville, VA, USA. Both MEK1-Wt and MEK1-Dn used in these experiments were provided pre-inserted into pRRLsin at restriction sites SalI and EcoRI (Table 3).

Two JNK MAP kinase kinase 2 isoforms (JNKK2) transgenes were used: "Wild-type" JNKK2 (JNKK2-Wt) and a dominant negative mutant of JNKK2 (JNKK2-Dn), which blocks endogenous JNKK2 signalling. NCBI Pub med Core Nucleotide reference for JNKK2-Wt is AF006689: *Homo sapiens* MAP kinase kinase JNKK2 mRNA [Submitted: (Lu et al., 1997)]. The existing dominant negative (JNKK2-Dn) supplied, was created by site-directed mutagenesis at a single site that substituted an existing lysine residue at position 149 with methionine (Tang et al., 2001). The resulting form of JNKK2 has absent

kinase activity. This dominant negative form JNKK2-Dn has been well documented to show this kinase deficient behaviour and has been widely used (Tang et al., 2001). It was previously obtained as a gift to our lab from Professor A Lin, University of Chicago, Ben May Institute for Cancer Research, Chicago, IL, USA. Both JNKK2-Wt and JNKK2-Dn used in these experiments were provided pre-inserted into pRRLsin at restriction sites XbaI and SalI (Table 3).

Three FAK transgenes were studied: "Wild-type" FAK (FAK-Wt) and two dominant negative mutants; FAK-F397, and FAK-R454. NCBI Pub med Core Nucleotide reference for FAK-Wt is M95408: Mouse focal adhesion kinase mRNA [Submitted: (Hanks et al., 1992)]. The plasmids (pcDNA3.1) containing the three FAK genes (FAK-Wt -F397, and -R454) were provided as a gift from Professor D Schlapfer, Department of Immunology, The Scripps Research Institute, CA, USA. The tyrosine residue at position 397 is the primary autophosphorylation site of FAK; it is positioned at the junction between the kinase domain and an N-terminal region referred to as the FERM domain. This site is rapidly phosphorylated when FAK becomes bound to integrins within focal adhesions. When this has occurred, the conformation adopted presents a Src homology 2 (SH2) binding region that has been shown to be the major site for the interaction between FAK and the Src family of protein tyrosine kinases. The lysine residue at positions 454 lies within the kinase domain of FAK. The dominant negative (FAK-F397) supplied was previously created by site directed mutagenesis that changed the tyrosine residue at 397 to phenylalanine. This change abolished the autophosphorylation site and prevented subsequent formation of the SH2 site, which in turn prevented interactions with Src family kinases. The activity of this dominant negative form of FAK, FAK-F397 has been widely studied (Schaller et al., 1994). The dominant negative (FAK-F454) supplied, was previously created by site directed mutagenesis and was used to change the lysine residue at position 454 to an arginine. This resulted in a dominant negative form of FAK, FAK-R454. This form has been well documented to show a significantly reduced kinase function and has been widely used (Hildebrand et al., 1993) (Table 3).

Each FAK gene was provided to me inserted in the plasmid vector pcDNA3.1 at the same restriction endonuclease sites, KpnI and XbaI. Consideration of restriction endonuclease sites within the FAK genes and those within the two vectors, pcDNA3.1 and pRRLsin, showed it was not possible to directly transfer the FAK genes from one vector to another.

An intermediate vector was used pSP73 with more favourable restriction endonuclease sites was used. All three FAK genes were sequentially transferred to pSP73 at restriction endonuclease sites KpnI and XbaI. The new vector was then digested with EcoRV and SalI and the fragments were transferred to those same sites present that were present in pRRLsin. The methods used in this procedure are described below.

Two c-Src transgenes were studied: "Wild-type" c-Src (Src-Wt) and a dominant negative mutant; (Src-K295M also referred to as Src-KM). NCBI Pub med Core Nucleotide reference for Src-Wt is V00402: *Gallus gallus* gene c-Src producing protein pp60-c-Src. This gene is homologous to the rous sarcoma virus gene v-Src [Submitted: (Weijland et al., 1983)]. The plasmids (pRK7) containing the two c-Src genes were provided as a gift from Professor D Schlapfer, Department of Immunology, The Scripps Research Institute, CA, USA. The lysine residue at position 295 lies within the kinase domain. The dominant negative (Src-KM) supplied was previously created by site directed mutagenesis that change the lysine residue at 295 to a methionine, a mutation that yielded the kinase deficient dominant negative. This form dominant negative form of c-Src has been well documented to be kinase inactive and has been widely used (Mukhopadhyay et al., 1995) (Table 3).

Each c-Src gene was provided to me inserted in the vector pRK7 at the restriction endonuclease sites BamHI and EcoRI. Consideration of restriction endonuclease sites within the c-Src genes and those within the two vectors, pRK7 and pRRLsin, showed it was possible to directly transfer the c-Src genes from one vector to another at the same restriction endonuclease sites, BamHI and EcoRI. The methods used in this procedure are described below.

The GFP gene had previously been cloned into pRRLsin at restriction endonuclease sites SalI and XbaI.

2.7.3 Restriction endonuclease digestion and DNA separation

All reagents unless otherwise specified are from New England Biolabs or USC stock lab solutions with standard common formulations. During a restriction endonuclease digest, reagents were mixed in an Eppendorf tube (1.5ml) and made up to a total volume of 20μl. The reagents present comprised; the DNA sample (volume was varied according to concentration of sample typically 2-5μl); the restriction enzymes (typically two enzymes,

1µl of each); NEBuffer 10x (2µl ± BSA 1µl for each endonuclease: Each restriction endonuclease requires a specific buffer, NEBuffer, and some also require BSA. Each was prepared according to the manufacturer's guidelines); RNAase (to remove RNA, 1µl); Distilled water was added to make the total volume 20µl. This mixture was warmed to 37^{o}C and maintained at that temperature for 30min. with gentle swirling. Upon completion, 2µl of sample buffer was added to the sample digests and the total was loaded into wells corresponding to individual lanes of pre-prepared agarose DNA electrophoresis gels. 2µl of a 1kb ladder was loaded into one well for reference. (Agarose gels (0.8%) were made by mixing 0.8g of agarose with 100ml of 1xTris-acetate-EDTA (TAE) running buffer; a stock solution. The mixture was microwaved until the agarose was completely dissolved. It was intermittently swirled gently. 0.6µl of ethidium bromide was then added and the solution cooled to room temperature. The mixture was added to the electrophoresis moulds and solidified in 1-2 hours. Sample wells were formed within the mould. All used reagents were increased proportionally when more gels were needed). Separation was achieved by electrophoresis, migration under an electric field; during which the net negatively charged DNA migrated from cathode to anode. Speed of migration is determined by fragment size [number of nucleotides, base pairs, or kb (1000's of base pairs)], larger fragments moved more slowly. After separation by electrophoresis the DNA fragments were visualized under UV illumination (ultra violet radiation ≈200-300nm). UV radiation illumination caused the ethidium bromide, an intercalating agent, to fluoresce intensely when bound to nucleic acid, revealing the position of any DNA fragments present. A Polaroid photograph was taken of the pattern of fluorescence bands (DNA fragments) present and these images were digitally scanned prior to further analysis. DNA fragment size estimates were made by comparison of the position of the fluorescence bands in the image from the experimental DNA fragments, with the position of the fluorescence bands from fragments of known size that were present in the standard DNA ladder (that contains a series of DNA fragments of known size) and in some cases to the position of the fluorescence bands from additional control fragments of known size also present.

2.7.4 DNA extraction, purification and ligation

A mixture of DNA fragments (commonly obtained following restriction endonuclease digestion) was separated by electrophoresis in an agarose gel as described above. The QiaexII Gel Extraction kit (Qiagen cat#20021) was used to extract and purify the selected DNA fragments. This system is based upon solublisation of the agarose gel and subsequent

selective absorption of the nucleic acids onto silica particles in the presence of a chaotropic salt. After purification the DNA is eluted and reclaimed. All reagents were supplied with the kit. Under UV illumination the DNA fragments were visible (as fluorescence bands as described above) and the region of the gel containing the desired DNA fragment was excised with a clean razor blade (care was taken to avoid including more than a minimal amount of the agarose gel surrounding the gel, larger amounts negatively affected yield). All handling was done with clean gloves for safety and to avoid contamination of the DNA. The agarose sample containing the desired fragment of DNA was placed in a 1.5ml Eppendorf tube. The protocol was followed per the manufacturer's instructions for 0.8% agarose in TAE buffer. The purified DNA was eluted by the addition of 20µl of 10mM Tris·Cl, pH 8.5. Upon completion, 1µl of the purified DNA sample(s) obtained were added to 1µl of sample buffer and 8µl of de-ionized sterile water (total 10µl). Each similarly prepared sample was loaded into a well corresponding to individual lanes of pre-prepared 0.8% agarose gels. 2µl of a 1kb ladder (made up to 10µl with de-ionized water) was loaded into one well for reference. In some cases a second reference was used consisting of 1µl of a lab standard sample of known concentration (pcDNA3 vector at a concentration of 1µg/ml). This control was added to 1µl of sample buffer and 8µl of de-ionized sterile water (total 10µl) and was also loaded into another well. This reference standard was used to estimate the concentration of the purified DNA sample(s) by comparison of the relative intensity of the fluorescence band of the purified DNA sample in the digitally scanned image, compared to the intensity of the fluorescence band of the lab standard sample. (The intensity of ethidium bromide fluorescence when bound to DNA is proportional to the concentration of DNA and of ethidium bromide present).

The gel was "run" and the DNA fragment(s) present migrated under the influence of the electric field. Viewed under UV illumination a Polaroid image of the fluorescence bands corresponding to the position of the DNA fragments was taken and digitally scanned. Comparison of the fluorescence band position corresponding to the experimental fragments of DNA, with those bands from the fragments of known size on the 1kb ladder ensured the experimental DNA fragment bands obtained corresponded with their expected position (fragment size). All reagents for DNA ligation unless otherwise specified were from New England Biolabs or USC stock lab solutions with standard common formulations.

During DNA ligation (non-blunt end) reagents were mixed in an Eppendorf tube (1.5ml) and made up to a total volume of 10µl. The reagents present comprised; the purified, linearized DNA gene insert in water or elution buffer following QiaexII separation. (The volume of the gene insert used was varied according to its concentration, typically 3-6µl); the purified, linearized DNA vector. (The volume of the vector used was adjusted to remain in a 1:3 ratio, of 1 part vector to 3 parts gene by volume, typically 1-2µl); the enzyme T_4 DNA ligase (1µl); and DNA ligase buffer 10x (1µl). Deionised sterile water was added to make the total volume to 10µl (typically 2-4µl). This mixture was warmed to $16^{O}C$ and maintained at that temperature for a minimum of 30min typically overnight. Upon completion, 5µl of ligase reaction mixture was added to 1µl of sample buffer and 4µl of deionised sterile water. This was loaded into a well(s) corresponding to individual lanes of pre-prepared agarose DNA electrophoresis gels. 2µl of a 1kb ladder (made up to 10µl with de-ionized water) was loaded into one well for reference. In some cases the same second reference was used (pRRLsin vector at a known concentration of 0.4 or 1µg/ml) to estimate the concentration of the purified DNA sample(s) as described above. Additional reference lanes were loaded that could be used to judge success (or failure) of the ligase reaction if needed e.g. digested linearized unligated vector. Following electrophoresis the fragment size was determined (as described previously) and assuming that the ligation was successful the ligated vector and gene were excised and purified using the QiaexII Gel Extraction kit. The final plasmid (vector and gene) concentration was determined in the same manner as described previously against a lab standard of known concentration.

2.7.5 Plasmid transformation and Midiprep

Bacterial transformation was performed using X10-gold Ultra-competent cell kits (Stratagene cat#200314). These specialized cells were required, due to the large plasmid size of the ligated vector and gene (pRRLsin-xxx, 8.2-10.1kb). NZY^{+} broth, Lysogeny broth (LB) Medium, LB Agar, and LB-Ampicillin Agar Petri dishes, TENS solution, 3M Sodium Acetate, and Tris-EDTA solution, were provided by our laboratory core facility at USC. Two 14-ml BD Falcon polypropylene round-bottom test tubes (BD Biosciences Cat#352059) were placed on ice (one tube was used for the experimental plasmid transformation and one tube for the transformation of the pUC18 control plasmid supplied with kit) and the NZY^{+} broth was warmed to 42°C. The X10 gold cells were thawed slowly resting on ice and 100µl of cells was added to each of the chilled tubes. 4µl of β-mercaptoethanol (supplied with kit) was mixed gently with the cells in each tube by swirling

gently. The cells were then incubated on ice for 10 minutes, and swirled gently every 2 minutes. 2µl of the purified experimental plasmid was added to one tube and 1µl of the pUC18 control plasmid to another, swirling gently to mix. The tubes were then incubated on ice for 30 minutes followed by rapid exposure in a water bath at 42°C for 30 seconds before being returned to the ice for a further 2 minutes. 0.9 ml of the preheated (42°C) NZY^+ broth was added and the tubes then incubated at 37°C while shaking for one hour at 225-250 rpm. 200µl of the transformation mixture (5µl of the control mixture) was plated on LB-ampicillin agar plates and incubate overnight at 37°C. Successful control transformation produced approximately 250 colonies per dish. For the experimental DNA plasmid the number of colonies varied. Colonies were selected for Miniprep (typically 6 per transformation) individually transferred into 5ml of LB medium and incubated at 37°C while shaking at 225-250 rpm overnight. The cells were centrifuged to a pellet and the supernatant decanted, except for a small residual quantity into which they were re-suspended by vortexing at high speed. 300µl of TENS were added and the mixture was vortexed for 2-5 seconds until viscous. 150µl of 3M sodium acteate (pH5.2) was added and the vortex repeated for 5 seconds to mix thoroughly.

The cellular debris and chromosomal DNA was separated by centrifuging at high speed (forming an off-yellow pellet at the base of the tube). The supernatant containing the plasmid DNA was transferred to a fresh tube and the process (TENS, 3M sodium acetate, centrifuge) was repeated. Following the second transfer of the supernatant to a fresh Eppendorf tube, 0.9ml of 100% ethanol was added and the tube was centrifuged for 5 minutes. The plasmid DNA separated as a white pellet on the wall of the tube and the supernatant was discarded. The pellet was rinsed with 0.5ml of 70% ethanol, vortexed and re-pelleted by centrifuge for 5 minutes. The supernatant was removed and the pellet dried under a vacuum for 5 minutes. The dry pellet was re-suspended in 20µl of Tris-EDTA solution and 2µl of RNAse was added. The suspension was incubated for 1-hour at 37°C. Upon completion, 1µl of each of the suspended plasmids was added to 1µl of sample buffer and 8µl of deionised sterile water. Each 10µl was loaded into a well corresponding to an individual lane on a pre-prepared agarose DNA electrophoresis gel. 2µl of a 1kb ladder (made up to 10µl with de-ionized water) was loaded into one well for reference. In some cases the same second reference was used (pcDNA3 vector at a concentration of 1µg/ml) to estimate the concentration of the purified DNA sample(s) as described above. If necessary other controls for comparison were also loaded.

The gel was “run” and the DNA fragment(s) present migrated under the influence of the electric field. Viewed under UV illumination a Polaroid image of the fluorescence bands corresponding was taken and digitally scanned. Comparison of the fluorescence band positions corresponding to the experimental fragments of DNA, with those bands from the fragments of known size on the 1kb ladder ensured the experimental DNA fragment bands corresponded with their expected position (fragment size). Assuming that the ligation was successful (correctly sized fragments were obtained), the ligated vector and gene were excised and purified using the QiaexII Gel Extraction kit. The final plasmid (vector and gene) concentration was determined in the same manner as above against the lab standard. All reagents for DNA ligation unless otherwise specified were from New England Biolabs or USC stock lab solutions with standard common formulations.

Chapter 3. *In vitro* Wound Assay ("Scratch Assay")

3.1 Introduction

Throughout this chapter and those subsequent, two experimental models were used in which keratinocyte movement and behaviour could be modified and quantified: a "scratch assay" (*in vitro* wound assay) performed in confluent keratinocyte cultures migrating into a manufactured wound and migration on colloidal gold (colloidal gold migration assay) which allowed individual keratinocytes to be tracked and the distances they moved, measured. The *in vitro* wound assay was chosen to corroborate and complement the findings of the colloidal gold migration assay, and is reported in a variety of experimental protocols. While the *in vitro* wound assay when complete gives an immediate and clear impression of any migration that is occurring and allows for a simple assessment of conditions that affect migration by visual comparison to control conditions, when quantified this assay was found initially to produce considerable variability when comparing results obtained in experiments repeated under the same conditions. This was unlike quantification of migration demonstrated with the colloidal gold assay (that reliably reproduced consistent results).

It was necessary to identify one protocol of the *in vitro* wound assay (amongst those reported by other authors) for use in further experiments. Seven different protocols were identified. The methodology of each was optimized in an attempt to improve consistency of results. Four protocols could not be modified to produce consistent results and were excluded, they are not considered further. These efforts alone (which primarily included identification of particular experimental improvements in the assay that enhanced consistency) preselected protocols that showed less variability of results. Three experimental protocols of the *in vitro* assay were identified for further consideration.

Each of the three *in vitro* wound assay protocols selected continued to show more noticeable variability (comparing results obtained in experiments repeated under the same conditions) than the colloidal gold migration assay. In addition for each of the three protocols chosen it was recognized that there have been published instances in which the results obtained in an otherwise similar experiments appeared to be inconsistent when compared (discussed further below). An explanation for this discrepancy in outcome that has been suggested by prior authors was that the assay(s) were influenced by secretion of extracellular matrix

components by migrating cells during evaluation. In fact secretion of ECM has been suggested to contribute to both the variability of results (observed here with each protocol) and to inconsistencies in comparisons between results obtained from different protocols of the *in vitro* wound assay (and also with those from other assays). Secretion of ECM during experiments affected different protocols of the *in vitro* wound assay to differing degrees. It was acknowledged that the colloidal gold migration assays and the *in vitro* wound assay differ in the model of migration they represent and hence might have produced differing outcomes despite similar conditions. Nonetheless, the criterion for selection of the experimental protocol of the *in vitro* assay to be used in subsequent experiments was; the protocol (if any) that produced the most consistent results between repeated experiments and between the two assays in each case under the same conditions.

The *in vitro* wound assay was first described by (Matthay et al., 1993) and is shown schematically in (Figure 13). It has been modified subsequently (Zhang and Kramer, 1996; O'Toole, 1997).

(Figure 13)

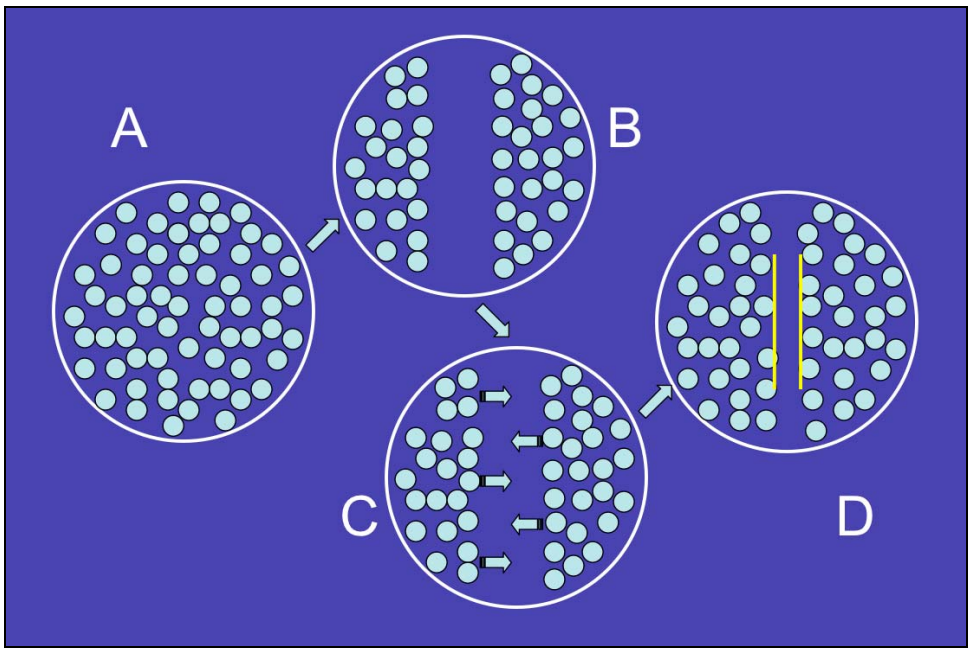


Figure 13: Schematic of the *in vitro* wound assay. A: Cells established and growing to high confluence 80-90% in a monolayer. B: A reproducible defined wound created in the monolayer of cells (width 2-3mm). C: Cells migrate for fixed time period into the wound under experimental conditions. D: Standardized visualization and quantification of the migration that has occurred.

The three protocols that were evaluated are commonly used and differ in the concentrations of extracellular matrix deposited, the manner and timing of the migratory substrate deposition in relation to the seeding of the cells, along with different approaches to the manufacture of the wound. For each protocol repeated experiments were performed under the same conditions; keratinocyte migration in the presence and absence of growth factors on pro-migratory type I collagen, non-migratory poly-L lysine, and on a "secreted-ECM" coating [prepared under conditions described in (Section 2.3.2)]. The results of three repeated experiments of each protocol were compared for variability and were compared to those results obtained by the same keratinocytes migrating under identical experimental conditions in the colloidal gold assay.

These preliminary experiments identified an experimental protocol of the *in vitro* wound assay used in subsequent evaluations. Additionally the results are used to demonstrate the statistical analysis performed in further experiments. A limited analysis of the extra cellular matrix secreted by keratinocytes during the *in vitro* wound assay is also presented.

The *in vitro* wound assay is a more physiologically representative model of an *in vivo* wound than the colloidal gold migration assay. During wound healing *in vivo*, a sheet(s) of cells migrates across the wound bed from the margins. The scratch assay creates an analogous situation with many similarities. *In vivo* and in the scratch assay, at the leading edge of the migrating sheet, keratinocytes are migrating into an "open space" with limited cell-cell contact in the direction of migration. *In vivo* leading edge keratinocytes migrate across the nascent extracellular matrix of the wound while in the scratch assay they migrate over the experimentally chosen and deposited migration substrate being evaluated. Both *in vivo* and *in vitro* cells in the migrating sheet have been shown to deposit extracellular matrix components as they move forwards. Migrating keratinocytes have been reported to produce limited amounts of proteins such as laminin-5 (Zhang and Kramer, 1996), fibronectin (O'Keefe et al., 1987), and to a lesser degree collagen IV (Amano et al., 2001; Jussila et al., 2002).

In a re-epithelializing wound *in vivo*, following behind the leading edge are many layers of trailing cells, which at their base are migrating over the nascent extracellular matrix that is progressively modified by secretion and deposition of (ECM) proteins and by other processes such as remodelling (Singer and Clarke, 1999). In the *in vitro* wound assay a near

single layer (monolayer) of cells migrates into the manufactured wound, but similarly to the situation *in vivo,* the experimental "ECM" over which they move is progressively modified by secretion and deposition of ECM proteins from the migrating cells. *In vitro* this process has been demonstrated repeatedly (Nguyen et al., 2000; Hintermann and Quaranta, 2004). Aside from the thickness of the migrating layer, in both cases the trailing cells are surrounded and in contact with other cells, including cells present in the direction of migration. (*In vivo* migrating cells are known to be proliferating at and near to the wound edge but not on the wound bed (Patel et al., 2006); proliferation was inhibited in the *in vitro* wound migration assay by the addition of Mitomycin-C).

During the *in vitro* wound assay, cells at the wound edge (the leading edge cells) and those trailing behind (trailing cells) inherently experience different environments, but this is a consistent feature of the experimental design in all experimental protocols of the assay. In the *in vitro* assay secreted ECM modifies primarily the environment local to the cell that is secreting. Additionally secreted ECM can also spread to more distant sites in the surrounding medium from where it can then be unevenly deposited onto the culture plate in exposed areas, including into the site of any wound that has been produced. When this occurs, the composition of the substrate on which the keratinocytes are migrating is altered and becomes to some extent unknown (that is the substrate on which migration is occurring may not be precisely that supposed in the experimental conditions and its composition is unknown with certainty). The effect of the alterations in ECM-cell interaction over time, particularly due to deposition at distant sites has been reported to be a confounding factor in comparison (Nguyen et al., 2000; Hintermann and Quaranta, 2004). "Contradictory" results concerning keratinocyte migration reported using this assay apparent in the literature include: Zhang et al (Zhang and Kramer, 1996) who demonstrated "Laminin-5 deposition promotes keratinocyte motility" using one experimental protocol of the *in vitro* wound migration assay, while O'Toole et al (O'Toole, 1997) demonstrated "Laminin-5 inhibits human keratinocyte migration" using a different protocol. Similar conflicting results have been reported with the use of different protocols in other cell types, including but not limited to; human dermal fibroblasts (Javelaud et al., 2003; Satish et al., 2003), murine embryonic fibroblasts (Nobes and Hall, 1999; Bladt et al., 2003; Cuevas et al., 2003; McAllister et al., 2003), human lung epithelial cells (Galiacy et al., 2003) and smooth muscle cells (Hsieh et al., 2003). "Contradictory" results relating to keratinocytes migration have also been reported when comparing results achieved using different migration assays

(the *in vitro* wound migration assay in some experimental protocol is often one) (Russell et al., 2003; Satish et al., 2003).

In comparison to the *in vivo* wound assay, during the colloidal gold migration assay cells migrate in isolation (without any cell-cell contact) over an experimental migratory substrate that likely remains essentially unmodified. While this assay is a less clearly analogous model of an *in vivo* wound, it represents a system that is more precisely definable and controllable *in vitro.* (The system may represent a better model for migration of the leading edge cells *in vivo* where cell-cell contact is more limited). In the colloidal gold assay any effects observed are likely due to those produced by the experimental conditions. Quantification in this case provided for a more unequivocal interpretation of results as all cells present experience the same environment (i.e. the same medium and the same experimental substrate).

3.2 Colloidal gold migration assay of keratinocyte migration of on pro-migratory type I collagen, non-migratory poly-L lysine, and on a secreted-ECM coating

To determine the protocol of the *in vitro* assay to be used subsequently, a comparison was made to result from the colloidal gold migration assay (as well as between repeated experiments). The preliminary experiments using the colloidal gold migration assay for this purpose are described here and the results are also used to demonstrate the statistical analysis that was used throughout later experimentation. In addition, and shown first experiments are described performed to demonstrate whether or not secretion and deposition of ECM into the surrounding medium was indeed occurring and to characterize what effect, if any, such activity might have on migration.

3.2.1 Results: Analysis of the ECM secreted into the medium and subsequently deposited during the *in vitro* wound assay

As described, it has been reported that alterations in ECM-cell interaction over time occur during an *in vitro* wound assay. This effect due is to deposition of secreted-ECM locally (adjacent to the cell) and at more distant sites (spreading via the media) and can lead to difficulty in comparisons between different types of assay and produce variability of results seen when using a single protocol of the *in vitro* assay and under similar conditions. This effect seems to vary in significance between different protocols of the *in vitro* assay.

Generally, such changes may lead to an average enhanced or inhibited migration or have no effect on migration at all.

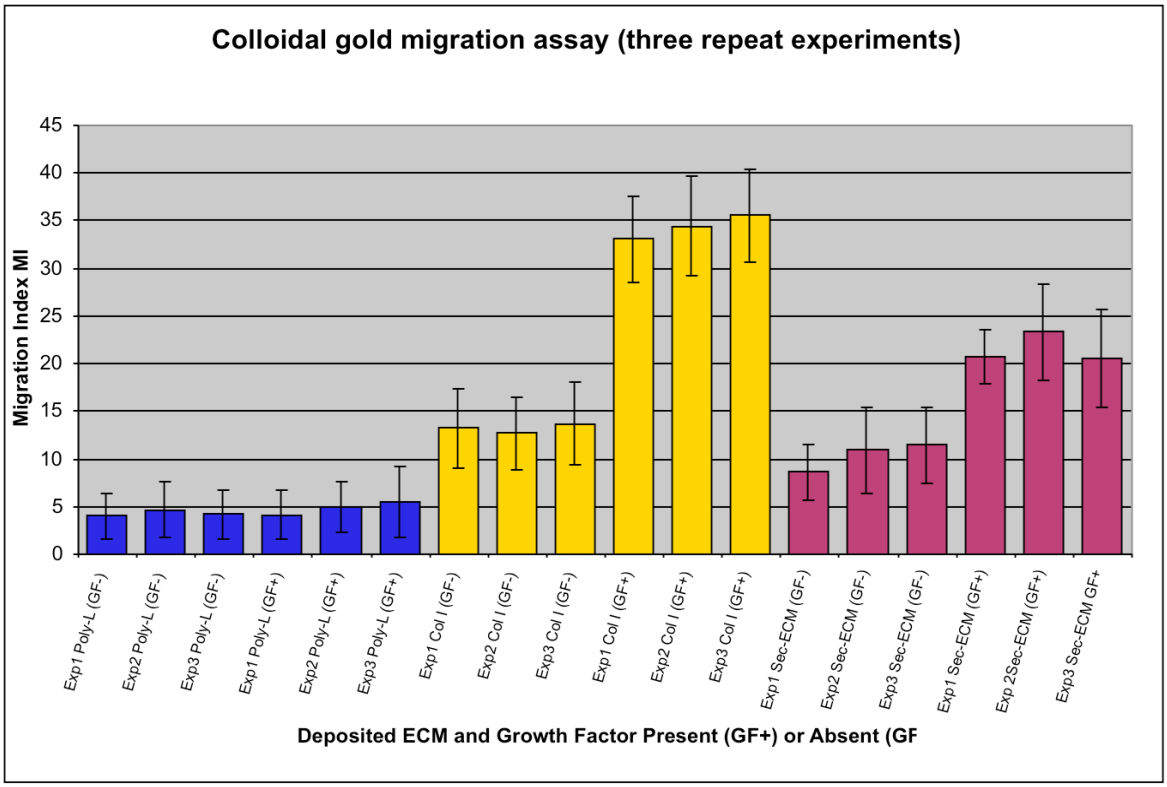
A limited preliminary analysis of the constituent(s) of this secreted-ECM was performed (data discussed, but not shown). A supposed "secreted-ECM" conditioned media was gathered by collecting the medium surrounding migrating keratinocytes under conditions as described in Section 2.3.2. This collected conditioned media (labelled "secreted-ECM") was used to coat glass cover slips according to the described experimental protocol (Section 2.3.1) for substrate deposition prior to an *in vitro* migration assay. After aspirating the remaining conditioned media (secreted-ECM containing medium) at the end of the coating interval, the surface of each glass slide was gently rinsed with PBS solution. Fluorescent antibodies to known substrates (type I collagen, type IV collagen, laminin-5, fibronectin and vitronectin) were added separately, and the presence or absence of fluorescence staining (presence or absence of each substrate) noted. This analysis, undertaken with antibody to laminin-5 and fibronectin, showed greater fluorescence, suggesting significant amounts of these substrates were present. Fluorescence staining of antibody to type IV collagen was weakly present, suggesting lower amounts of this substrate was present, while that with antibody to vitronectin and type I collagen was not observed. These observations are qualitatively consistent with previously reported findings (O'Keefe et al., 1987; Zhang and Kramer, 1996; Amano et al., 2001; Jussila et al., 2002).

3.2.2 Results: Preliminary colloidal gold migration assay of keratinocyte migration

In preparation for the colloidal gold migration assay, conditioned media (secreted-ECM containing medium) was gathered as described in Section 2.3.2. This collected media was then handled in the same manner as the solutions of experimental substrates (type I collagen and non-migratory control poly-L lysine) that were prepared from stock materials and were deposited at the concentrations listed in (Table 1). Preparation for the assay otherwise followed the usual experimental protocol for substrate deposition onto gold salt-coated glass cover slips prior to the colloidal gold migration assay. Colloidal gold migration assay of keratinocyte migration under the conditions described was performed in the three repeated independent experiments; the results are shown in (Figure 14). (The three independent experiments are identified in as Exp1, Exp2, and Exp3).

(Figure 14)

A



B

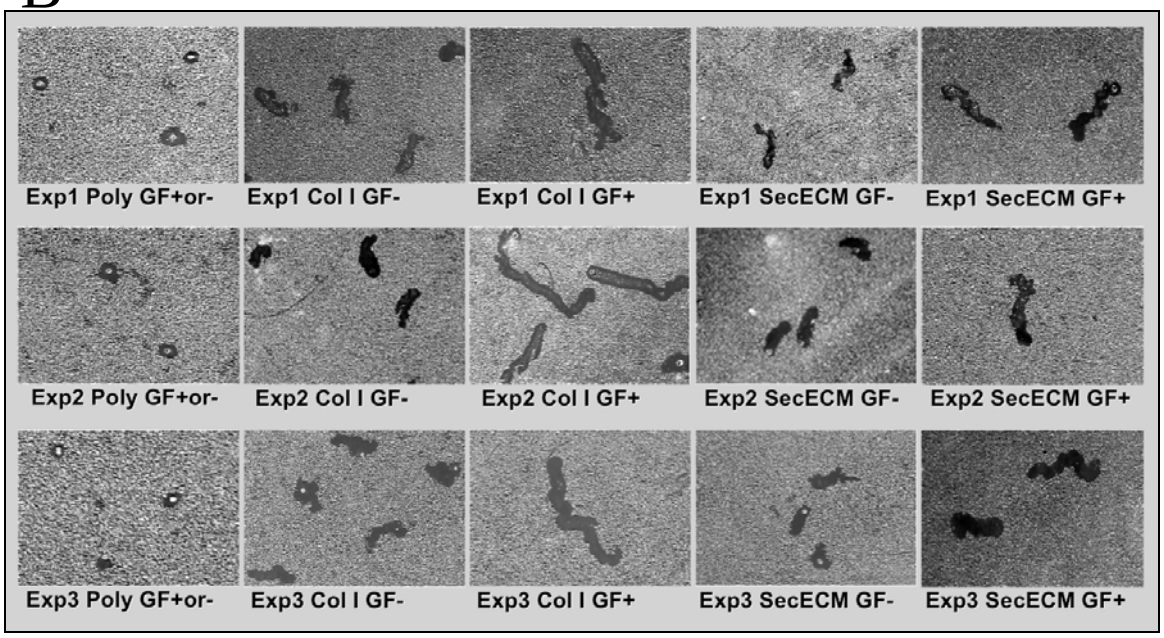


Figure 14: Colloidal gold migration assay of keratinocyte migration in the presence and absence of growth factors (GF+ and GF-) on non-migratory poly-L lysine (Poly-L), pro-migratory type I collagen (Col I), and substrate deposited from the conditioned media containing secreted-ECM (Sec-ECM). A: For each combination of substrate and growth factor exposure results (Migration Index MI_{CG}) from three independent experiments are shown (Exp1 Exp2 and Exp3). B: Visual depiction

of keratinocyte migration during the colloidal gold migration assay under the same conditions. In each case the images shown were captured the end of migration at 16-hours, and the conditions present are labelled at the base of each image. In each case the area of the dark track left by the migrating keratinocyte is proportional to the size of the migration index.

3.2.2.1 Statistical analysis of results

In this work all migration assays were repeated three times independently under identical conditions. As a means of demonstrating variability one-way analysis of variance (ANOVA: Single factor) was applied to the three data sets obtained (for example those identified in Figure 14 as; Exp1, Exp2, and Exp3). The ANOVA test determined if the three data sets and the mean migration index (MI) from each independent experiment likely represent values from a single population. The null hypothesis for the ANOVA test was chosen as: There are no differences among the three (or more) mean MI_{CG}. The alpha level for the ANOVA was chosen as 0.05. The null hypothesis was rejected if $F\text{-}F_{crit} \geq 0$ from the ANOVA test. (See note below for details of the statistic $F\text{-}F_{crit}$). The results of the ANOVA test for the colloidal gold migration assay under each set of conditions (poly-L lysine, type I collagen, and secreted-ECM in the presence and absence of growth factor) are shown in (Table 4).

(Table 4)

	Poly-L Lysine GF Present	Type I Collagen GF Present	Secreted-ECM GF Present
$F\text{-}F_{crit}$ Value	-2.08	-2.85	-0.33
	Poly-L Lysine GF Absent	**Type I Collagen GF Absent**	**Secreted-ECM GF Absent**
$F\text{-}F_{crit}$ Value	-2.86	-1.89	-0.05

Table 4: Results of a one-way ANOVA (Single factor) analysis of the three data sets obtained from three independent experiments performed under each set of experimental conditions listed. Note: As described previously: F ratios, are the statistic derived from the ANOVA test, they are determined from variance estimates. The significance level, or alpha level, of the test was chosen as 0.05, which

is analogous to the choice of $p<0.05$ as being significant. A null hypothesis for comparison can be rejected (or accepted) by comparison of the F ratio calculated (F) for one data set and that calculated (F_{crit}) for the whole at the chosen level of significance. The null hypothesis is then rejected if $F-F_{crit}\geq 0$. When comparing results from migration assays accepting the null hypothesis was equivalent to the statement that there is no significant difference between the three groups compared, (the data likely represent that from a single population).

The results in (Table 4) shows that under all conditions the null hypothesis was accepted - independent experiments performed gave: $F-F_{crit}<0$. That is, the data from each three similar experiments, likely represented values from the same population and there was no significant difference between the mean MI values obtained ($p<0.05$).

Interpretation of this finding was that the independent experiments (performed under otherwise identical conditions) using the colloidal gold migration assay show consistent of results (minimal variability). Additionally following an ANOVA test (and under the circumstance that the null hypothesis was not rejected), data from all three experiments was combined to calculate overall average MI_{CG}'s and their standard deviation. These overall average values of MI_{CG} are displayed for each set of distinct experimental conditions (in the presence and absence of growth factors, on non-migratory poly-L lysine, pro-migratory type I collagen, and substrate deposited from the conditioned media containing secreted-ECM) in Figure 15.

(Figure 15)

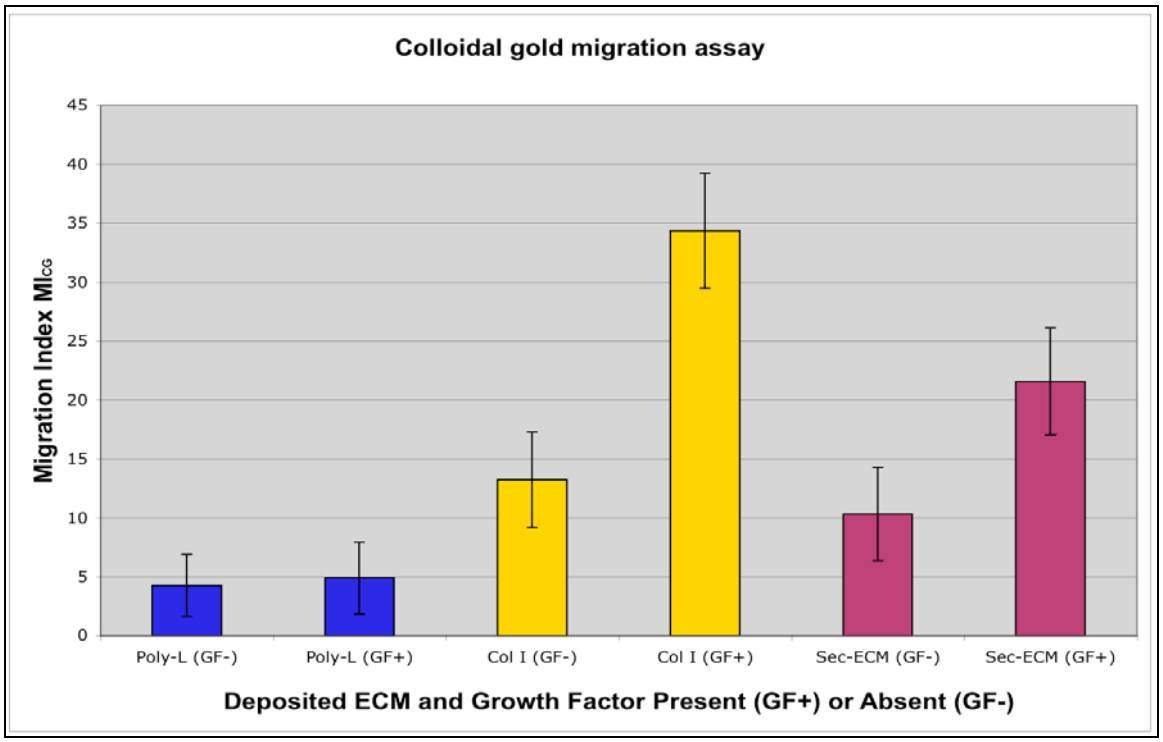


Figure 15: Combined raw data from the three independent colloidal gold migration assays of keratinocyte migration in the presence and absence of growth factors (GF+ and GF-) on non-migratory poly-L lysine (Poly-L), pro-migratory type I collagen (Col I), and substrate deposited from the conditioned media containing secreted-ECM (Sec-ECM).

Further statistical analysis was performed comparing the migration indices achieved under different experimental conditions; different substrates and in the presence and absence of growth factors. Comparisons were made using Welch's (unpaired) t-test. (Note: Welch's (unpaired) t-test was chosen as this data represents small numbers of normally distributed continuous variable samples with differing variances that are not paired). The null hypothesis chosen for the Welch's t-test was chosen as: The average MI's obtained under the control and the experimental conditions do not differ (the alternate hypothesis states that there are significant differences between average MI's) with a level of significance of 0.05.

Several comparisons were made using Welch's t-test. In the presence of growth factors migration of keratinocytes on type I collagen and on substrate deposited from conditioned media containing secreted-ECM was compared to migration on non-migratory poly-L lysine. These comparisons were repeated in the absence of growth factors. Significantly

greater migration of keratinocytes was observed on type I collagen and substrate deposited by the conditioned media ($p<0.05$ in all cases). Additionally, migration of keratinocytes on type I collagen was compared to their migration on substrate deposited by the conditioned media in the presence and absence of growth factors. In the absence of growth factors there was no significant difference in migration of keratinocytes ($p>0.05$). However, in the presence of growth factors, keratinocytes migrated significantly ($p<0.05$) further on type I collagen than on substrate deposited by the conditioned media (containing secreted-ECM). Migration indices are shown in Figure 15 and the t-test findings Table 5.

(Table 5)

Colloidal Gold Migration Assay	Type I Collagen vs. Poly-L lysine	Secreted-ECM vs. Poly-L lysine	Type I Collagen vs. Secreted-ECM
p-value (GF Present)	< 0.001	0.001	0.001
p-value (GF Absent)	0.001	0.017	0.066

Table 5: Comparison of keratinocyte migration indices MI_{CG} observed in the colloidal gold migration assay using Welch's (unpaired) t-test under different experimental conditions. "p" values shown in red indicate the comparison did not achieve statistical significance.

Note: Data from the three repeated independent experiments under identical experimental conditions (such as that shown in Figure 14), the subsequent ANOVA test results (such as the F-F_{crit} values shown in Table 4) and the tabulated results of any t-test comparisons (such as that shown in Table 5) are not presented further unless relevant, only overall average data and relevant p-values are presented.

The interpretation of these results is that the conditioned medium collected from the preliminary subculture of keratinocytes contains one or more secreted-ECM substrates that facilitate keratinocyte migration. The substrate(s) deposited by the conditioned media (secreted-ECM containing media) significantly enhanced migration compared to the control poly-L lysine, both in the presence of growth factors and in their absence. However, the

substrate(s) deposited by the conditioned media are not as effective a pro-migratory substrate as type I collagen in the presence of growth factors. In the absence of growth factors this experiment could not distinguish a difference in migration between the pro-migratory effects of the substrate(s) deposited by the conditioned media and type I collagen. These results and those discussed in Section 3.2.1 circumstantially confirm observations made by authors (O'Keefe et al., 1987; Zhang and Kramer, 1996; Amano et al., 2001; Jussila et al., 2002) that keratinocytes secrete and deposit extracellular matrix components and also shows that whatever the precise composition of those substrates, on balance they favour an enhanced keratinocyte migration. These results also provide a mechanism (secretion of extracellular matrix components by migrating keratinocytes) by which some of the differences observed between results using the *in vitro* wound migration assay, could be explained.

3.3 *In vitro* wound assay (protocols 1, 2, and 3) of keratinocyte migration on pro-migratory type I collagen, non-migratory poly-L lysine, and on a secreted-ECM coating

Secreted-ECM spread to distant sites via the media has been demonstrated here and reported previously. Three commonly used protocols of the *in vitro* wound assay were chosen following pre-experimental optimization and were analyzed more thoroughly. The experiments performed in this analysis were under the same conditions as those used in the colloidal gold migration assay (in Section 3.2), keratinocyte migration on pro-migratory type I collagen, non-migratory poly-L lysine and on substrate deposited by the conditioned media (containing secreted-ECM) in the presence and absence of growth factors. Results obtained were compared to those obtained from the colloidal gold migration assay under the same conditions. The three experimental protocols of the *in vitro* wound assay analyzed are described below:

1. *In vitro* wound migration assay: Protocol 1. The significant feature of this experimental protocol is substrate deposition prior to plating of cells and prior to creation of the wound. [The experimental methods used with this protocol are described previously (Section 2.3.1)].

2. *In vitro* wound migration assay. Protocol 2. The significant feature of this experimental protocol is substrate deposition that followed plating of the cells and creation of the wound.

(In protocol 2, experimental substrate is deposited onto the exposed areas of the base of the well between cells, and the wounded area).

3. *In vitro* wound migration assay. Protocol 3. The significant feature of this experimental protocol is that substrate deposition is performed twice, firstly prior to plating of cells (similarly to protocol 1) and again after wounding of the cell layer (similarly to protocol 2). It has been suggested (in personal communication) that this would repair any damage to the substrate coating that might have occurred during production of the wound.

3.3.1 Results: Choice of non-migratory control surface for the *in vitro* wound assay

Preliminary identification of the three protocols of the *in vitro* assay for further evaluation also identified an additional factor which appeared to improved consistency of results between repeat experiments; that was the use of poly-L lysine as the non-migratory control surface in favour of uncoated well used by some authors. (Some studies have used the uncoated plastic base of the wells of cell culture plates as the non-migratory control surface). Preliminary migration experiments with each protocol (1, 2, 3) separately were performed comparing keratinocyte migration on type I collagen to migration on both poly-L lysine and to that achieved on uncoated wells (data not shown). Data from three repeat experiments were analyzed by the ANOVA test. When using uncoated wells, as the non-migratory control the three data sets obtained typically did not satisfied the null hypothesis of the ANOVA test; that is they appear to be data from a different population. Use of poly-L lysine coating, rather than uncoated wells showed consistent results (satisfying the ANOVA test) between experiments performed under identical conditions for all three protocols. Poly-L lysine was used in all subsequent assays as the non-migratory control surface (in both the *in vitro* and colloidal gold migration assays).

3.3.2 Results: Preliminary *in vitro* wound assay of keratinocyte migration using protocols 1, 2, 3

Results of experiments evaluating keratinocyte migration on type I collagen compared to that on poly-L lysine and on a secreted-ECM coating using the *in vitro* wound assay experimental protocols 1, 2, and 3 are shown in Figure 16. [These are average values for the migration index (MI_{IV}) calculated from all data following successful application of the ANOVA test as described in Section 3.2.2.1].

(Figure 16)

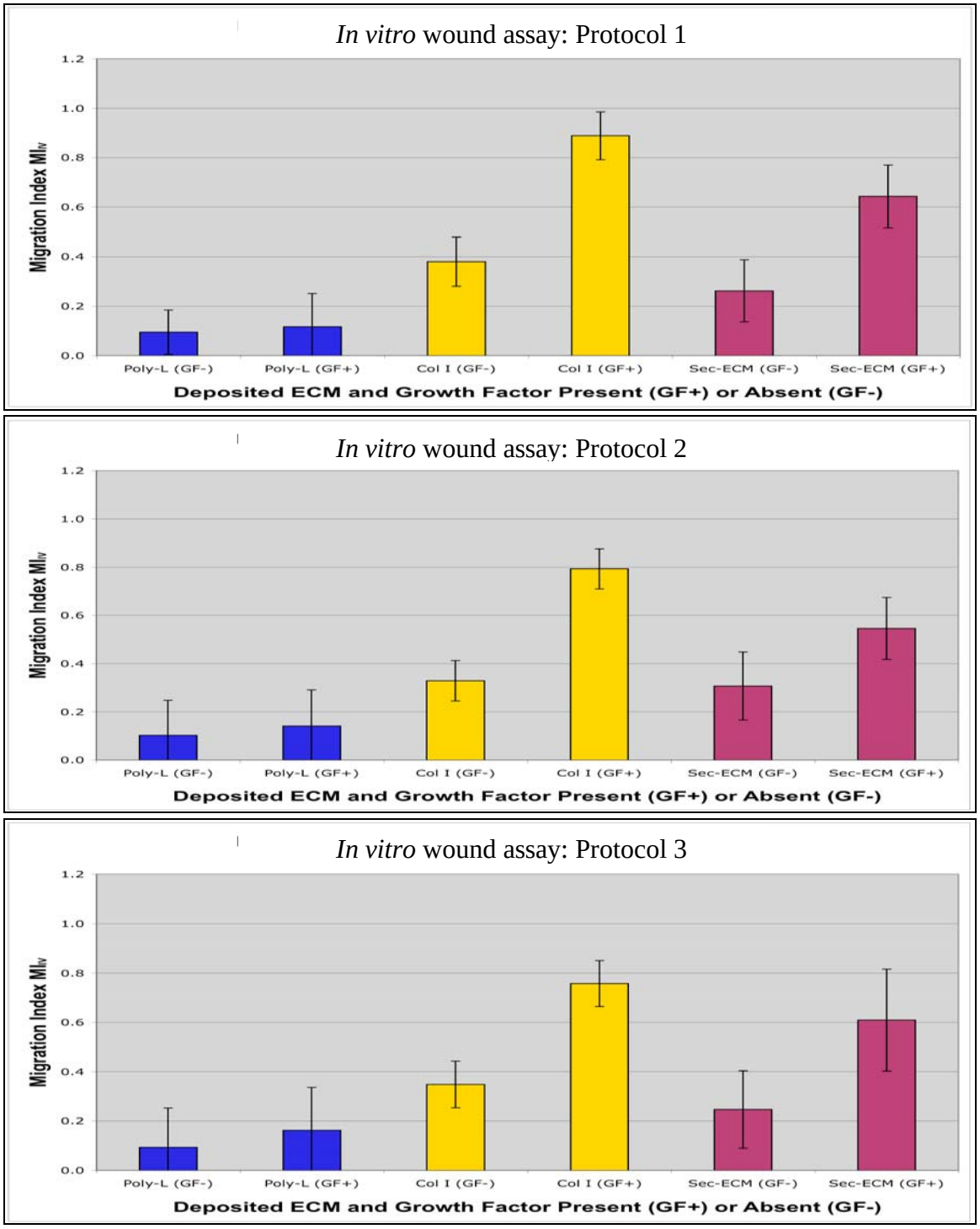


Figure 16: *In vitro* wound assays (protocols 1, 2, and 3) of keratinocyte migration in the presence and absence of growth factors (GF+ and GF-) on poly-L lysine (Poly-L), type I collagen (Col I), and substrate deposited from the conditioned media containing secreted-ECM (Sec-ECM). Average values for MI_{IV} are displayed for each combination of substrate and growth factor exposure.

Further statistical analysis was performed to compare migration indices achieved under different experimental conditions. Several comparisons, identical to those made in the analysis of data from the colloidal gold migration assay (Table 5), were made using Welch's unpaired t-test as described in Section 3.2.2.1. The results for the three experimental protocols of the *in vitro* migration assay are summarized in Table 6.

(Table 6)

A *In vitro* **wound assay Protocol 1**	**Type I Collagen vs. Poly-L lysine**	**Secreted-ECM vs. Poly-L lysine**	**Type I Collagen vs. Secreted-ECM**
p-value (GF Present)	**<0.001**	**<0.001**	**0.004**
p-value (GF Absent)	**<0.001**	**0.048**	**0.134**
B *In vitro* **wound assay Protocol 2**	**Type I Collagen vs. Poly-L lysine**	**Secreted-ECM vs. Poly-L lysine**	**Type I Collagen vs. Secreted-ECM**
p-value (GF Present)	**<0.001**	**0.003**	**0.008**
p-value (GF Absent)	**0.011**	**0.057**	**0.752**
C *In vitro* **wound assay Protocol 3**	**Type I Collagen vs. Poly-L lysine**	**Secreted-ECM vs. Poly-L lysine**	**Type I Collagen vs. Secreted-ECM**
p-value (GF Present)	**0.004**	**0.012**	**0.046**
p-value (GF Absent)	**0.021**	**0.061**	**0.147**

Table 6: Comparison of keratinocyte migration indices observed during the *in vitro* wound assay experimental protocols A: Protocol 1 B: Protocol 2 C: Protocol 3 using Welch's (unpaired) t-test

under different experimental conditions. p-values that did not reach statistical significance (that is $p>0.05$) are shown in red.

3.4 Discussion

The *in vitro* wound migration assay was chosen as a second assay to corroborate and complement the findings of the colloidal gold migration assay, and is widely used in a variety of experimental protocols. It was necessary to identify one protocol for use in further experiments.

When quantifying migration, the colloidal gold migration assay produced the most consistent results between repeated experiments under similar conditions. The data from repeated experiments analyzed using the ANOVA (single factor) test was reliably not statistically different (shown here in Figure 14 and Table 4). This was not typically the case for the *in vitro* wound assay; compared to the colloidal gold migration assay greater variability in results was observed between repeat experiments. Amongst the different protocols of this assay that have been described and have been reported; seven protocols were selected and three of these were identified as producing more consistent results. During these preliminary experiments it was identified that using poly-L lysine as a non-migratory substrate (as opposed to uncoated wells used by some authors) significantly improved reproducibility in the *in vitro* assay (Section 3.3.1), and will be used in further experiments.

An important consideration in the choice between the remaining three protocols of the *in vitro* wound assay was the effects of secreted extracellular matrix components on the results obtained. Secreted extracellular matrix components are thought to contribute to variability between results following repeated experiments using the same protocol of the *in vitro* wound assay and also to be responsible, in part, for the discrepancy between results that have been reported by other authors when using different protocols (the effect of secreted extracellular matrix components on this seems to vary in significance between different experimental protocols). Here the substrate deposited by the conditioned media (secreted-ECM containing media) that is extracellular matrix components spreading to distant sites via the media was demonstrated and was shown to enhance migration (Section 3.2.2). The findings here were consistent with those previously reported (O'Keefe et al., 1987; Zhang

and Kramer, 1996; Amano et al., 2001; Jussila et al., 2002). In general both assays, colloidal gold and the three protocols of the *in vitro* wound assay chosen produced superficially very similar migration results under the same conditions (Figure 15 and Figure 16). Differences were most apparent in t-test comparisons made between results. Significance was reported differently in some instances for comparisons made from the results of colloidal gold migration assay and those from protocols 2 and 3 of the *in vitro* assay (which can be seen by comparing the results shown in Table 5 and Table 6). Differences in significance present in results from some of the protocols used and not others suggests these differences are most likely due to effects inherent to the protocol, rather than being due to intrinsic differences in the model of migration for the two assays represented.

Protocol 1 of the *in vitro* wound assay produced the least variability between repeated experiments (that is the data from repeated experiments analyzed using the ANOVA test was most reliably not statistically different). In addition results from protocol 1 were identical to those from the colloidal gold migration assay (which can be seen by comparing Figure 15 and Figure 16, and Table 5 and Table 6). While the colloidal gold migration assays and the *in vitro* wound assay differ in the model of migration they represent and hence, may have produced differing outcomes despite similar conditions, the criterion for selection of the experimental protocol of the scratch assay used in subsequent experiments was; the protocol that produced the most consistent results between repeated experiments and between the two assays in each case under the same conditions in these preliminary experiments. Experimental protocol 1 of the *in vitro* wound assay met this criterion and was used in subsequent experiments.

Chapter 4 Initiation and augmentation of human keratinocyte migration on a type I collagen substrate.

4.1 Introduction

The coordinated balance of changing extracellular matrix components, soluble factors, and cellular elements accomplishes wound healing by influencing its progress over time. Each cell that is recruited and activated begins a functional role in the process of wound healing and adds its own contribution to the local environment. In this chapter migration of keratinocytes will be studied under conditions chosen to reflect those present at times during the healing of an acute *in vivo* wound. The effects of different extracellular matrix substrates and growth factors will be evaluated in the initiation and continued migration of human keratinocytes.

In review, early in wound healing, keratinocytes and fibroblasts begin to migrate into the wound from surviving adnexal structures and from adjacent wound edges. As healing progresses, clot (platelets embedded into a mesh of cross-linked fibrin with small amounts of fibronectin and vitronectin) is replaced by granulation tissue. Granulation tissue is produced primarily by fibroblasts, with contributions from epithelial and vascular endothelial cells. At this time the extracellular matrix over which keratinocytes migrate into the wound, is a mixture of fibronectin, type I collagen, fibrin, and to a lesser extent vitronectin and hyaluronic acid (Clark et al., 1982). With time (of the order of a few days and while migration is still occurring) this provisional extracellular matrix becomes progressively more collagenous (Nguyen et al., 2000; Hintermann and Quaranta, 2004), containing increasing amounts of type I collagen, due to the activity of fibroblasts within the wound (a transition under the influence of TGFβ1 and regulated by TGFβ3) (Welch et al., 1990; Bandyopadhyay et al., 2006). In addition the matrix is modified by migrating keratinocytes themselves that have been shown here and reported to produce limited amounts of proteins such as laminin-5 (Zhang and Kramer, 1996), fibronectin (O'Keefe et al., 1987) and to a lesser degree collagen IV (Amano et al., 2001; Jussila et al., 2002). The progress of keratinocyte migration appears to be determined by the array of integrins expressed on their cell membranes (Clark et al., 1996). In general the pattern of integrin expression observed on migratory keratinocytes favours movement on type I collagen and laminin-5 and to a lesser extent fibronectin and vitronectin. Completion of re-

epithelialization (when migration ceases) is accompanied by synthesis of a new basement membrane including synthesis of type IV collagen, and laminin-1, reformation of keratinocyte adhesions such as hemidesmosomes in basal cells, and a return to a pattern of proliferation and differentiation of keratinocytes usually seen in unwounded epithelium. At the same time within the wound there is a reduction in levels of type I collagen with remodelling, in the number of cells present, such as fibroblasts, by apoptosis.

The soluble factors (including growth factors) present during wound healing also vary over time. In the early phase of wound healing the combination of soluble factors present stimulates migration of local parenchymal cells (keratinocytes, fibroblasts, and endothelial cells) adjacent to the wound and in surviving adnexal structures. At the same time soluble cytokines and chemotactic factors recruit inflammatory cells such as neutrophils and macrophages. Platelets incorporated into the clot act as a reservoir for many soluble mediators which they release as the wound heals. Such factors include platelet derived growth factor (PDGF), epidermal growth factor (EGF), and transforming growth factor β (TGFβ1 and 2) (Singer and Clarke, 1999). Serum is also a component of the milieu during the early phase of acute wound healing. It contains many of these same soluble mediators and others that have particular functional importance including proteases such as serum-derived plasmin (important in remodelling and facilitating migration), growth factors such as transforming growth factor α (TGFα), and regulatory factors such as TGFβ3; involved in coordinating several of the events that are occurring (Bandyopadhyay et al., 2006). Inflammatory cells recruited early to the site of wound healing, macrophages and neutrophils, secrete an impressive array of soluble factors including cytokines such as interleukin-1 (IL-1), and tumour necrosis factor α (TNFα) (Hubner et al., 1996). They also maintain production of many of the soluble factors initially derived from platelets and serum such as PDGF, TGFα, and TGFβ1,2, and 3 and secrete some additional factors such vascular endothelial growth factor (VEGF) and fibroblast growth factor 1 (FGF1) (Figure 1) (Rappolee et al., 1988). The parenchymal cells (fibroblasts, keratinocytes, and vascular endothelial cells) also secrete soluble factors, promote progress in wound healing and facilitate each other's actions (Coulombe, 2003).

Fibroblast, endothelial cell, and keratinocyte migration are important components of wound healing. Fibroblast migration has been studied most extensively and these cells have been

shown to migrate effectively on a number of extracellular matrix components in two-dimensional assays including the colloidal gold migration assay, the *in vitro* wound assay, and others such as the Boyden chamber haptotaxis assay (Sieg et al., 1998). Previous work on fibroblast migration using the colloidal gold and *in vitro* wound assays (Li et al., 2004a) investigated effects due to soluble growth factors, extracellular matrix components, and potential interactions between them. It demonstrated a number of relevant positive and negative findings for those cells. Migration of human keratinocytes has been evaluated in a more limited number of studies. In a similar manner to the previous work on fibroblasts, the following experiments were performed to evaluate migration of human keratinocytes on various extracellular substrates and the effects on that migration due to growth factors, both considered alone and in combination.

The experiments presented here were conceived to test the hypothesis that initiation (and continued) migration of keratinocytes would depend on the presence of certain substrates (such as type I collagen and others known to be present in the *in vivo* healing wound matrix). It was also hypothesized that in the presence of type I collagen (and possibly other similar substrates shown to be pro-migratory) migration of keratinocytes would occur with or without the addition of soluble growth factors, and in addition the presence of soluble factors would significantly augment (enhance) keratinocyte migration.

Components of the interstitial extracellular matrix and basement membrane matrix were evaluated as substrates for migration. The interstitial extracellular matrixes chosen for these assays reflect those components shown to be present in the early matrix of a healing wound *in vivo* (as described above), and included type I collagen, fibronectin, and vitronectin. Substrates secreted by keratinocytes during *in vivo* wound healing such as laminin-5, and type IV collagen, (as well as fibronectin) were also evaluated, as was laminin-1 a component of the epidermal basement membrane (along with type IV collagen). Morphology of keratinocytes migrating on these same matrix components was visualized using fluorescence microscopy.

In addition and presented first, a limited number of experiments were performed to determine the time frame over which keratinocyte migration occurs in the two assays. Identified, this time frame was used in subsequent experiments. Also presented but studied only briefly, was the effect of keratinocyte migration on type I collagen in relation to

changes in the concentration of the solution used to deposit this substrate and similarly in relation to changes in concentration of supplemental growth factors and serum provided in the medium. Migration in the presence of serum versus plasma on type I collagen was also briefly evaluated. As reviewed above, levels of type I collagen in the extracellular matrix of an *in vivo* wound vary, generally they increase during the early phase of wound healing when keratinocytes are migrating. Similarly composition and levels of growth factors present during the early phase of acute *in vivo* wound healing also vary and serum is a component of this milieu. (Here an analysis of the effects of varying the concentration of individual growth factors was not performed, nor were detailed measurements of the deposited concentration of type I collagen at the migration surface undertaken).

4.2 Time frame of the colloidal gold migration and *in vitro* wound assays

Various cellular processes including proliferation, differentiation, and migration involve regulation by intermediate-early genes and their products, immediate-early proteins. An immediate-early response follows a wide variety of cellular stimuli and occurs over a time frame of the order of several minutes up to a few hours. In cellular processes such as those mentioned, activation of the immediate-early response in a resting cell begins with signal transduction of external stimuli such as growth factors into the cell, across the cytoplasm, and into the nucleus. Within the nucleus immediate-early genes show a rapid and transient expression occurring in the absence of de novo protein synthesis. They represent the first round of transcription response to transduced stimuli. They are distinct from "late response" genes, which can only be activated following protein synthesis (“late response” gene synthesis and expression are in a variety of ways initiated by the immediate early response genes and their products). Many immediate-early genes were first identified as homologues to retroviral oncogenes and they include proteins such as c-jun that are the end stage products (signals) of the MAP Kinase pathways. As well as transcription factors and DNA binding regulatory proteins, other important classes of immediate early gene products include secreted proteins (e.g. soluble factors), cytoskeletal proteins, and growth factor receptor subunits. Many of these immediate early gene products are directly involved in the process of cellular migration.

4.2.1 Results: Time frame of the colloidal gold migration and *in vitro* wound assays

A qualitative impression of migration of keratinocytes during the course of the experiment is most easily demonstrated in the setting of the *in vitro* wound assay and is shown in (Figure 17: A). These images and similar images taken at fixed time points (0, 2, 4, 8, 16, 24 hours) were then used to quantify the migration occurring and calculate the migration indices MI_{IV} (Figure 17: B).

(Figure 17)

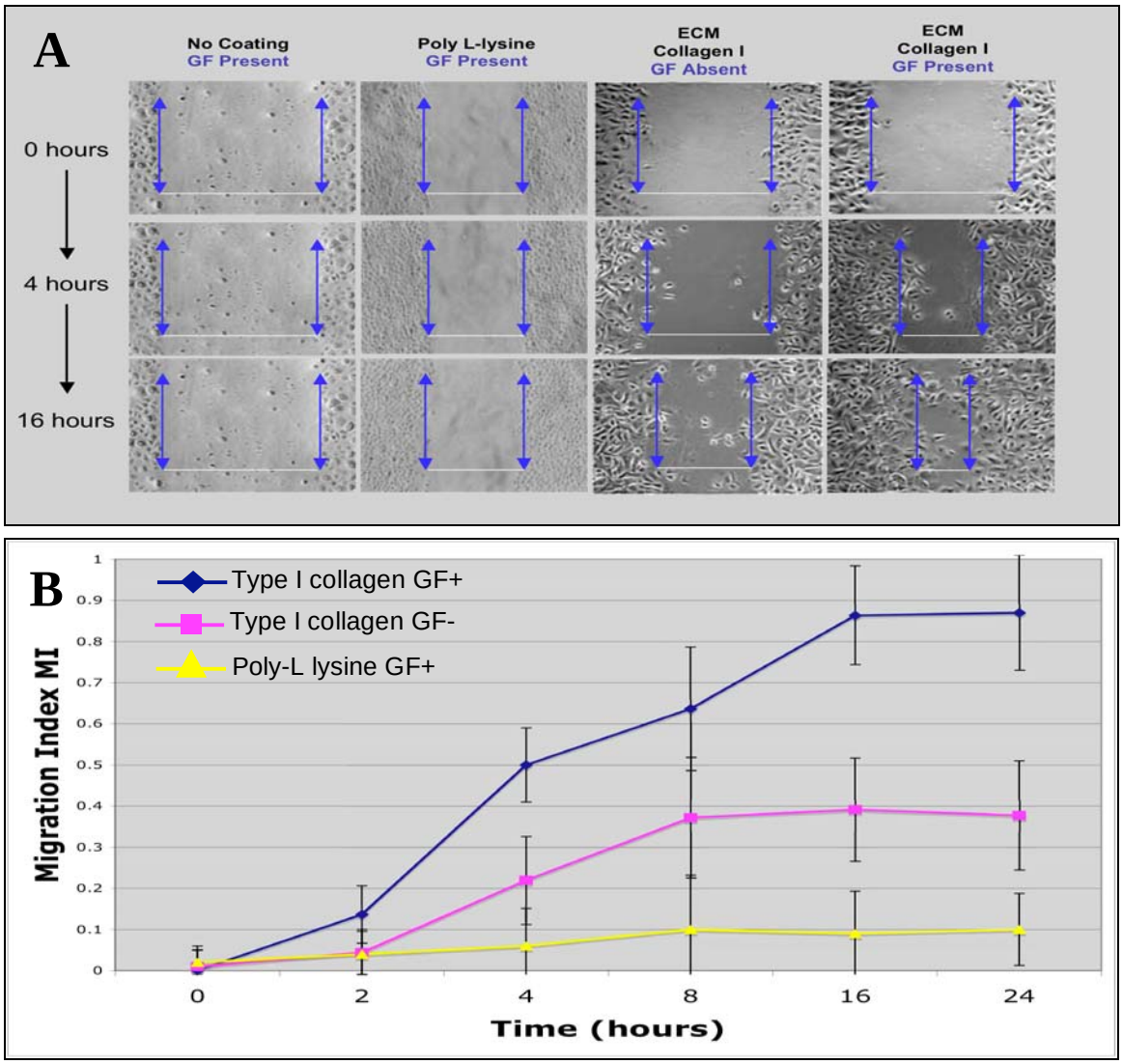


Figure 17: *In vitro* wound assay demonstrating keratinocyte migration over time. A: Representative images of keratinocyte migration captured at 0, 4 and 16 hours during *in vitro* wound assays. Substrates were: two non-migratory surfaces; uncoated wells and poly-L lysine in the presence of growth factor (first and second columns respectively), and one pro-migratory substrate; type I collagen in both the absence and presence of growth factors (third and fourth columns respectively). Superimposed on the images, the blue arrows shown are aligned with the advancing

borders of the migrating keratinocyte sheets and the white lines indicate the distance between them. The length of the white lines was used to calculate the migration index MI_{IV}. (Additional images were taken at 2 and 24 hours, these are not shown. Images taken at 24-hours were unchanged from those taken at 16-hours). B: Migration indices MI_{IV} calculated at time points 0, 2, 4, 8, 16, and 24-hours under the same conditions of keratinocyte migration on non-migratory poly-L lysine and pro-migratory type I collagen in the presence of growth factors, and type I collagen in its absence (data for uncoated wells is not shown). Note: These experiments reproduced and confirmed unpublished work performed previously in our Lab (by Dr. Jinhua Fan).

These findings demonstrate that maximum migration occurs at approximately 16-hours during the *in vitro* wound assay under these conditions. Similar experiments using the colloidal gold migration assay confirmed the same finding (data not shown).

Based on these findings all subsequent migration assays were performed over a 16-hour period. By confining the duration of migration experiments to this time frame, a time frame that encompassed the period during which the immediate-early gene response occurs, it was hoped that effects on migration that were secondary to *de-novo* protein expression, including any effects of the "late response" genes, would be minimized. In addition assays performed over a 16-hour period allowed for maximum migration while minimizing effects from any cellular proliferation that might occur– this time frame is significantly less than the doubling time for keratinocytes in cell culture.

Note: An important factor identified earlier (in Section 3.3.1) as contributing to improved consistency of results between repeated experiments during the *in vitro* wound assay was the use of poly-L lysine as the non-migratory control surface. Poly-L lysine has been shown to promote cell adhesion, but not migration. Images of keratinocyte migration on both non-migratory surfaces (poly-L lysine and uncoated cell culture plates) are shown here for reference only (Figure 17: A). Uncoated cell culture plates or uncoated gold salt as non-migratory controls were not used further.

4.3 Limited evaluation of the effects of soluble factors at various concentrations on keratinocyte migration on type I collagen

The composition of soluble factors (including growth factors) present during wound healing varies over time, as does the concentration of each factor present. As described previously, during wound healing the combination of soluble factors released from sources including platelets and inflammatory cells and also present in serum, stimulates migration of local parenchymal cells (keratinocytes, fibroblasts, and vascular endothelial cells) from sites adjacent to the wound and surviving adnexal structures (Rappolee et al., 1988; Hubner et al., 1996; Singer and Clarke, 1999; Bandyopadhyay et al., 2006).

4.3.1 Results: Limited evaluation of the effects of growth factor supplement concentration on keratinocyte migration on type I collagen

Here examined briefly are the effects of varying concentrations of growth factor supplementation, (supplements supplied with the purchased culture medium EpiLife medium kit), on keratinocyte migration on pro-migratory type I collagen and non-migratory poly-L lysine substrates in the colloidal gold migration and *in vitro* wound assays. Normally reconstituted medium (supplemented as per the manufactures guidelines) was termed "1xGF", and contained pro-migratory factors at the following concentrations: BPE 0.2% v/v (1:500 dilution), Insulin (Bovine) 5μg/ml, and Human Epidermal Growth Factor 0.2ng/ml. Concentrations of these soluble factors were varied from the 1x level in multiples of the concentrations present at the 1xGF level; that is 0x, 0.5x, 1x, 5x, and 10x (e.g. 5x = BPE 1% v/v (1:100 dilution), Insulin (Bovine) 25μg/ml, and Human Epidermal Growth Factor 1.0ng/ml). Note: additionally present, but in fixed amounts (that were not varied) was calcium chloride 0.06M, Hydrocortisone 0.18μg/ml, Transferrin (Bovine) 5μg/ml, 1xPSA (penicillin, streptomycin, amphotericin B). Potential cytotoxicity caused by elevated soluble factors was assessed by Trypan blue exclusion assay, and cell proliferation studies. These assays were performed following exposure of a subset of keratinocytes prepared and handled identically to those undergoing the migration assays (particularly, including the same time frame of any exposure to the medium). Cytotoxicity by either assessment did not occur at concentrations of supplementation 0x, 0.5x, 1x, and 5x. At 10x low-level cytotoxicity was observed. Results for the colloidal gold migration assay are shown in Figure 18. (Results for *in vitro* wound assay are not shown, but demonstrated the same findings).

(Figure 18)

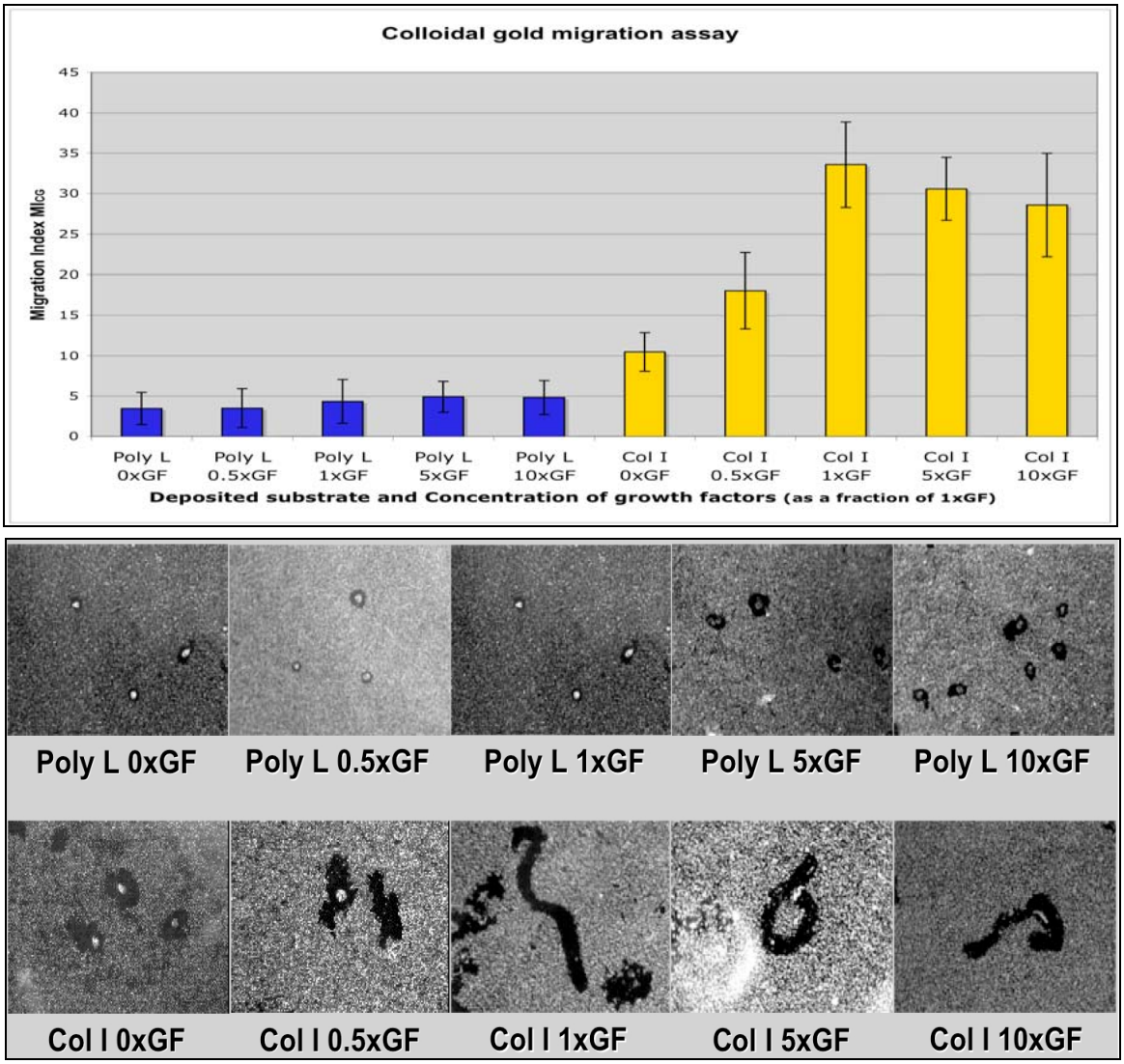


Figure 18: Effects of supplementation with various concentrations of growth factors (GF) on keratinocyte migration on type I collagen (Col I) and poly-L lysine (Poly L) substrates in the colloidal gold migration assay. Concentration of growth factor supplementation is expressed a multiples of 1x media; that is 0x, 0.5x, 1x, 5x, and 10x. 1x growth factor media contained supplements added according to manufacturer's guidelines for the EpiLife kit used (see text for constituents).

These results demonstrated that media reconstituted according to the manufacturers guidelines (1xGF) produced maximal migration of keratinocyte on a type I collagen matrix in the two assays used. Increasing the concentration of growth factor supplements above that recommended produced no further increase in migration and at very high concentrations of growth factor supplementation (10xGF), cytotoxicity was observed and

migration was significantly decreased (MI_{CG} 1xGF compared with 10xGF, $p<0.05$). Decreasing the concentration of growth factors below that recommended (0.5xGF and 0xGF) was associated with a progressive reduction in migration. Across concentrations (below levels associated with cytotoxicity i.e. 10x) apparent dose-dependent changes in migration are seen.

As early as 1992 it was shown that human epidermal keratinocyte migration occurs maximally when all pro-migratory components of the kit are present (BPE, EGF, and insulin), as specified by the manufacturers' guidelines (Sarret et al., 1992b). This finding was confirmed here. Few studies since have considered the effect on keratinocyte migration of varying either the concentration of several components of growth factor supplement kits together as was shown here, or the concentration of elements individually (Henry et al., 2003; Li et al., 2004b; Li et al., 2006). Frequently, migration experiments have used the growth factor supplements supplied with the purchased culture medium. This has been considered to represent less physiological exposure (stimulus), but until more detail is available on the physiological relevance and importance of specific soluble factors in keratinocyte migration, the manufacturer's supplementation kit provides a reproducible controlled environment for study.

4.3.2 Results: Limited evaluation of the effects of human serum and plasma at various concentrations on keratinocyte migration on type I collagen

Similar to the effects of growth factor supplementation, the effects of various concentrations of human serum and plasma on keratinocyte migration on pro-migratory type I collagen and non-migratory poly-l lysine substrates in the colloidal gold migration and *in vitro* wound assays were evaluated. Results for the colloidal gold migration assay are shown (Figure 19), results for *in vitro* wound assay are not shown, but demonstrated the same findings.

(Figure 19)

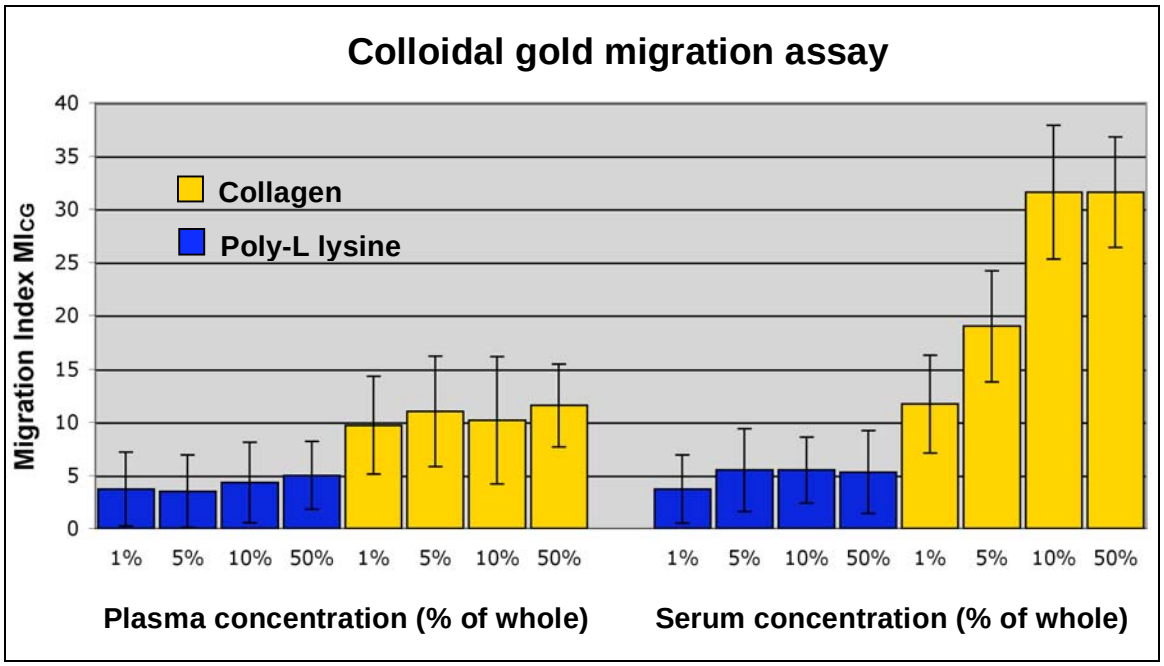


Figure 19: Effects of various concentrations of human serum and plasma on keratinocyte migration on type I collagen and poly-L lysine substrates in the colloidal gold migration assay. Concentration of both serum (pooled human serum, separated from clotted human blood, sterile-filtered) and plasma (pooled human plasma separated from citrate anticoagulated blood) are expressed as a percentage by volume of whole. Note: These experiments reproduced, complimented and confirmed published work performed previously in our Lab (Henry et al., 2003).

The apparent dose-dependent increases in migration on type I collagen observed in the presence of serum but not plasma, strongly suggest the presence of one or more factors within serum that regulate or directly promote keratinocyte migration on a type I collagen substrate. One previous report has demonstrated that serum, but not plasma was pro-migratory for keratinocytes on type I collagen in the colloidal gold migration assay (Henry et al., 2003) and a further analysis has begun to determine individual pro-migratory and regulatory factors present in serum (Bandyopadhyay et al., 2006; Li et al., 2006). The findings here confirm the earlier observations that serum, but not plasma, enhanced keratinocyte migration in this setting. Some authors have proposed that serum may represent a more physiological medium to be used in migration assays in favour of the growth factor supplements provided with purchased medium. Despite the appeal of a more physiological system, many of the factors present in serum that may affect keratinocyte migration are not known and not all of those identified have been well characterized.

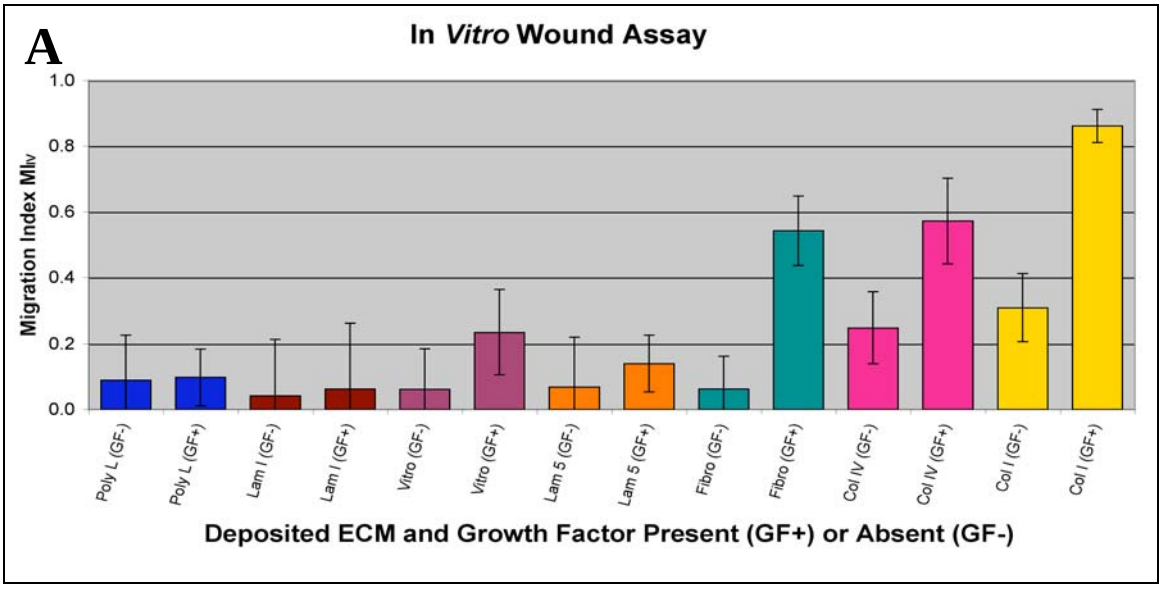
Furthermore, it is likely that the composition of the reagents (serum and plasma) varies to some extent between preparations. While such experimental construction may relate more readily to *in vivo* response (and as such represent a better model in certain circumstances) these factors make detailed analysis and interpretation more difficult.

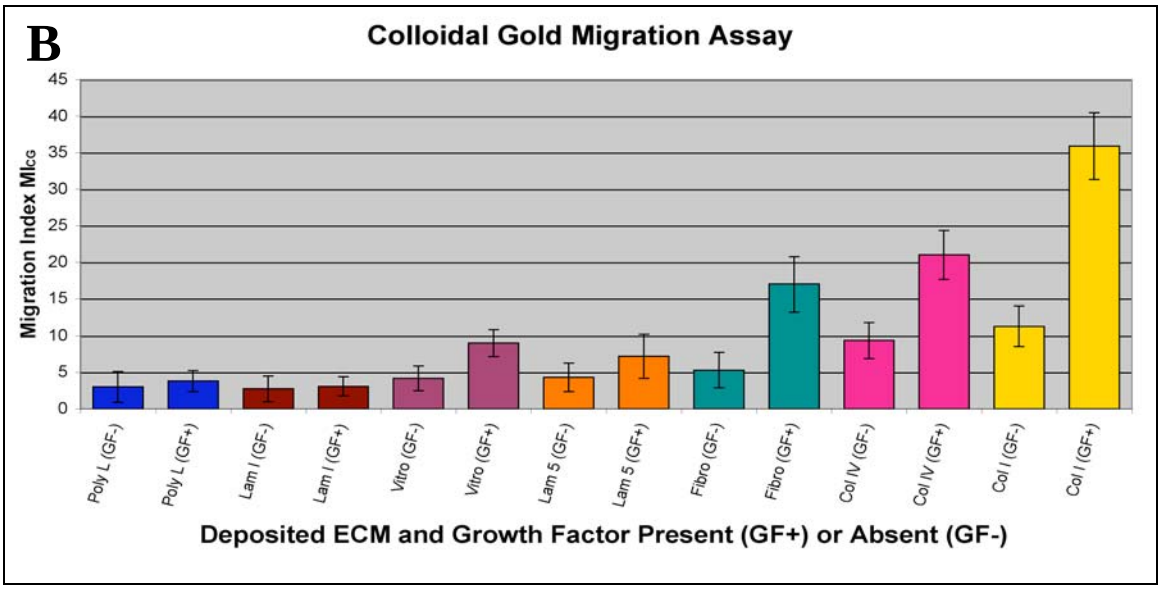
4.4 Results: Evaluation of keratinocyte migration on selected extracellular matrix components and other substrates present in the *in vivo* wound matrix

Experiments were performed using the *in vitro* wound and colloidal gold migration assays to evaluate migration of keratinocytes on selected extracellular matrix components and other substrates. The extracellular matrix components chosen for these assays reflect those components present in a healing wound *in vivo* and included type I collagen, fibronectin, and vitronectin that are components of the early extracellular matrix of a wound. Laminin-5 and type IV collagen are basement membrane components that are also secreted by migrating keratinocytes during *in vivo* wound healing. These substrates were included along with laminin-1, another basement membrane component not normally seen in a healing wound until migration of keratinocyte ceases and the new basement membrane is being formed. Poly-L lysine was included as a non-migratory control substrate. Note: The concentrations of solution used to deposit the selected substrates were chosen following a limited number of preliminary experiments. These preliminary experiments evaluated keratinocyte migration on each substrate varying the concentration of the solution used for its deposition. Results of these experiments varying the concentration of type I collagen depositing solution are shown later, in Section 4.6 and were performed (repeated) by the candidate. Similar experiments (unpublished) for each of the other substrates were performed by investigators in our lab (Dr. Jinhua Fan and Dr. Edel O'Tool, but not by the candidate) and this data is not shown. Deposition concentrations used in later experiments (unless noted) were the lowest that produced maximal migration and are listed in (Table 1).

The results of the *in vitro* wound and colloidal gold migration assays demonstrating keratinocyte migration on selected extracellular matrix components are shown in (Figure 20: A and B). Also shown are representative images of migration captured during the colloidal gold migration assays (Figure 20: C).

(Figure 20)


A
In Vitro Wound Assay
Migration Index MIiv
1.0
0.8
0.6
0.4
0.2
0.0
Poly L (GF-)
Poly L (GF+)
Lam I (GF-)
Lam I (GF+)
Vitro (GF-)
Vitro (GF+)
Lam 5 (GF-)
Lam 5 (GF+)
Fibro (GF-)
Fibro (GF+)
Col IV (GF-)
Col IV (GF+)
Col I (GF-)
Col I (GF+)
Deposited ECM and Growth Factor Present (GF+) or Absent (GF-)


B
Colloidal Gold Migration Assay
Migration Index MIcg
45
40
35
30
25
20
15
10
5
0
Poly L (GF-)
Poly L (GF+)
Lam I (GF-)
Lam I (GF+)
Vitro (GF-)
Vitro (GF+)
Lam 5 (GF-)
Lam 5 (GF+)
Fibro (GF-)
Fibro (GF+)
Col IV (GF-)
Col IV (GF+)
Col I (GF-)
Col I (GF+)
Deposited ECM and Growth Factor Present (GF+) or Absent (GF-)

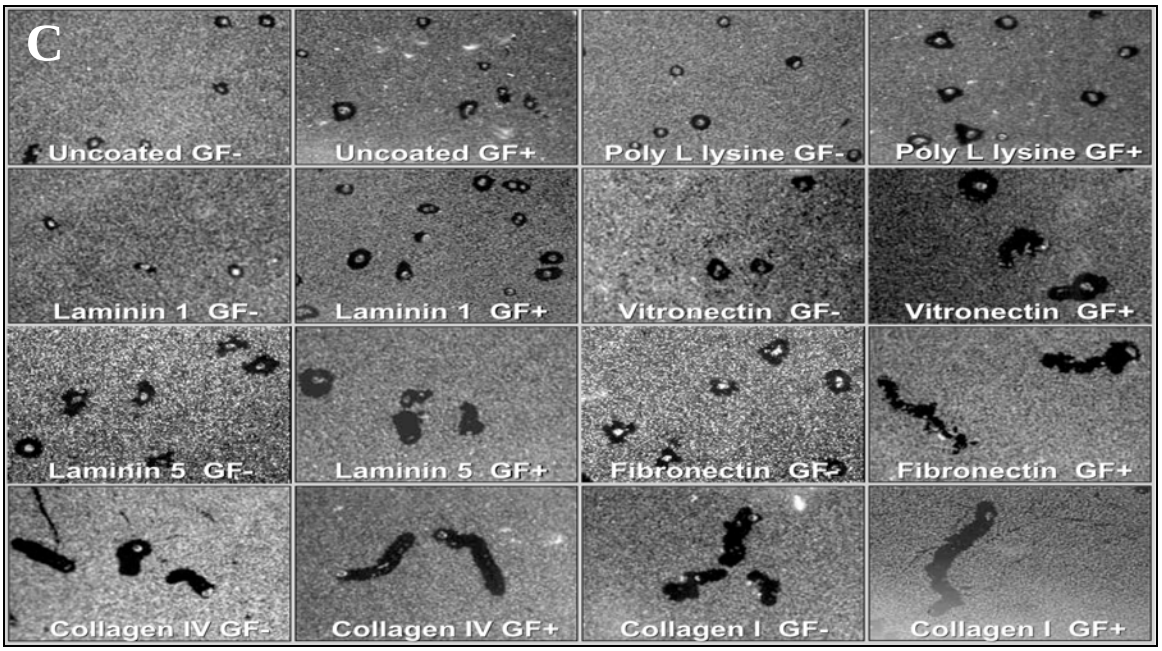

C
Uncoated GF-
Uncoated GF+
Poly L lysine GF-
Poly L lysine GF+
Laminin 1 GF-
Laminin 1 GF+
Vitronectin GF-
Vitronectin GF+
Laminin 5 GF-
Laminin 5 GF+
Fibronectin GF-
Fibronectin GF+
Collagen IV GF-
Collagen IV GF+
Collagen I GF-
Collagen I GF+

Figure 20: *In vitro* wound and colloidal gold migration assays demonstrating keratinocyte migration on selected substrates. A: MI_{IV} indices from the *in vitro* wound assay for keratinocyte migration on non-migratory control poly-L lysine and substrates, laminin-1 (Lam 1), vitronectin (Vitro), laminin-5 (Lam 5), fibronectin (Fibro), type IV collagen (Col IV), and type I collagen (Col I) both on the presence (GF+) and absence (GF+) of growth factors. B: MI_{CG} indices from the colloidal gold migration assay for keratinocyte migration under the same conditions as shown in A. [All migration indices (MI_{CG} and MI_{IV}) shown here and in subsequent experiments are calculated from all data from three repeated experiments following application of the ANOVA test as described in Section 3.2.2.1]. C: Visual depiction of keratinocyte migration during the colloidal gold migration assay on various extracellular matrix substrates and on non-migratory controls. In each case the images shown were captured the end of migration at 16-hours, and the substrate present is labelled at the base of each image. Images of keratinocyte migration occurring on two non-migratory surfaces are shown; uncoated gold salt (top left images) and poly-L lysine (top right images). (Neither, MI_{IV} or MI_{CG} data for uncoated gold salt migration is shown). Keratinocyte migration occurring on the extracellular matrix components and non-migratory controls in the absence of growth factor (GF-) is shown in the first and third columns and in the presence of growth factors (GF+) in the second and fourth columns. In each case the area of the dark track left by the migrating keratinocyte is proportional to the size of the migration index.

For the substrates that are known ECM components, type I collagen, fibronectin, and vitronectin and for the basement membrane component type IV collagen, there was a significant difference in migration indices (MI_{CG} and MI_{IV}) in the presence of growth factor compared with their absence ($p<0.05$ in each case). A laminin-5 substrate showed a significant difference in migration index in the presence of growth factor compared to its absence only on the colloidal gold migration assay and not in the *in vitro* wound assay (MI_{CG} $p<0.05$ and MI_{IV} $p>0.05$). However, the absolute values with the colloidal gold migration assay were similar in the presence and absence of growth factors. In all cases showing a significant difference, migration in the presence of growth factors was greater. (Note: In general statistical significance of comparisons between the results of two or more identical conditions for each assay considered separately, were in agreement between assays. Any relevant difference in significance obtained between the two assays will be specifically identified). The substrate laminin-1 and the non-migratory control substrate poly-L lysine, which showed no significantly difference ($p>0.05$ in both assays) in migration in the presence and in the absence of growth factors. Migration indices for both

of these substrates were low, approximately MI_{CG}≈3.8-4.8 and MI_{IV}≈0.04-0.1, in the colloidal gold and *in vitro* assays respectively. Migration of keratinocytes on laminin-5 and on vitronectin in the absence of growth factors was not statistically different or different to that achieved on the non-migratory control poly-L lysine ($p>0.05$ in each case). Migration indices for vitronectin, in the presence of growth factors occurred at an intermediate level, approximately MI_{CG}≈9.3 and MI_{IV}≈0.24 in the colloidal gold and *in vitro* assays respectively. Both of these indices were significantly greater ($p<0.05$) than those for keratinocyte migration on the non-migratory control in the presence of growth factors. Migration of keratinocytes on laminin-5 compared to the non-migratory control in the presence of growth factors showed a differing significance between the two assays; migration was not significantly different from keratinocyte migration on the non-migratory control in the *in vitro* wound assay ($p>0.05$), while on the colloidal gold assay a statistically different migration was observed ($p<0.05$). However, the absolute values with the colloidal gold migration assay were similar to those of the non-migratory control in the presence (and absence) of growth factors. Migration of keratinocytes on type I collagen, type IV collagen, and fibronectin in the presence of growth factors was significantly greater when compared to that seen on laminin-5, vitronectin, laminin-1, and on non-migratory poly lysine ($p<0.05$). Amongst these substrates, type I collagen, type IV collagen, and fibronectin, in the presence of growth factors, the greatest migration in both assays was seen on type I collagen (MI_{CG}≈35 and MI_{IV}≈0.86), compared to type IV collagen (MI_{CG}≈21 and MI_{IV}≈0.57) ($p<0.05$) and fibronectin (MI_{CG}≈16.5 and MI_{IV}≈0.54) ($p<0.05$). In the absence of growth factors migration seen on type I collagen (MI_{CG}≈11 and MI_{IV}≈0.31) was the same as that with type IV collagen (MI_{CG}≈9.3 and MI_{IV}≈0.28) ($p>0.05$), but greater than that seen on fibronectin (MI_{CG}≈5.3 and MI_{IV}≈0.06) ($p<0.05$). Also apparent in the presence of growth factors (but not in their absence) is that migration of keratinocytes on type IV collagen compared to fibronectin showed a difference in statistical significance between results obtained from the colloidal gold migration assay, in which the migration was significantly different ($p<0.05$), compared to the *in vitro* wound assay in which migration was the same ($p>0.05$).

These results were consistent with the hypothesis that initiation (and continued) migration of human keratinocyte migration would depend on the presence of certain substrates such as type I collagen and others present in the *in vivo* wound matrix. Migration was favoured, to varying degrees, by all of the extracellular matrix elements present *in vivo* in the nascent

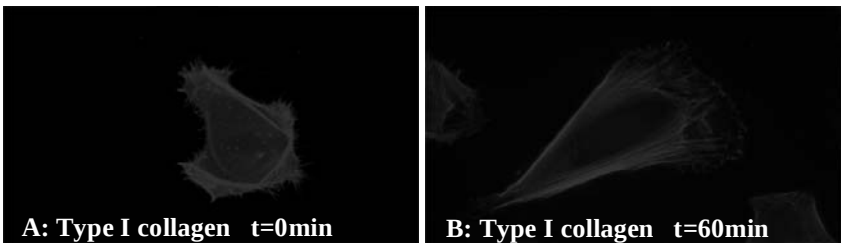
extracellular matrix of a healing wound, type I collagen, fibronectin, and vitronectin. *In vivo,* the pattern of integrin expression on migratory keratinocytes favours movement on type I collagen (both at the leading edge of the healing wound and in the cells migrating behind) and to a lesser extent fibronectin and vitronectin (Kiosses et al., 2001; Laukaitis et al., 2001; Ridley et al., 2003). Over time in an *in vivo* wound the provisional extracellular matrix becomes progressively more collagenous (Nguyen et al., 2000; Hintermann and Quaranta, 2004), containing increasing amounts of type I collagen due primarily to the activity of fibroblasts. It is reassuring and not surprising to find here that the extracellular matrix component type I collagen, produced by far the greatest migration in these assays and migration on fibronectin and vitronectin was also favoured (above that of the non-migratory control), but to a lesser extent, and only in the presence of growth factors. Previous reports regarding the migration of keratinocytes on laminin-5 suggest this substrate inhibits migration (Woodley et al., 1988; O'Toole, 1997) and here migration on this substrate was comparable to that on the non-migratory control. As described, keratinocytes migrating *in vivo* and *in vitro* are known to secrete laminin-5 (Zhang and Kramer, 1996) and also to express integrin receptors for laminin-5. Further, it has also been reported that deposition of laminin-5 by keratinocytes during migration can enhance migration (Zhang and Kramer, 1996; Nguyen et al., 2000). The findings here regarding keratinocyte migration on laminin-5 are equivocal and deferred under some conditions from prior reports; this may be explicable by consideration of effects relating to differences between the two assays, effects of substrate deposition during migration and or by effects due to the concentration of laminin-5 deposited that is also know to be a factor in keratinocyte migration on laminin-5 (O'Toole, 1997). However, further work is required to provide detailed explanation for the differences observed. *In vivo* laminin-1, a component of the basement membrane, is only deposited later during wound healing, at a time when migration of keratinocytes is complete (Singer and Clarke, 1999). Absence of migration on this substrate is consistent with this role and might reasonably be expected from such observations. Interestingly, type IV collagen also a ubiquitous component of the basement membrane resulted in significant migration (at a level second only to that seen on type I collagen). Only type I and type IV collagen produced significant migration (compared to non-migratory controls) in the absence of growth factors ($p<0.05$). Type IV collagen has been shown to be a substrate that supports cell adhesion, spreading, and migration of a number of cells including neoplastic cell lines such as melanoma (Chelberg et al., 1989), and also primary cell lines including keratinocytes (Sarret et al., 1992a), the latter result

being confirmed again here. These same authors also report that in the colloidal gold migration assay type IV collagen supports keratinocyte migration at a level greater than that seen with the extracellular matrix component fibronectin (Sarret et al., 1992a), again a result confirmed here. This later finding was not seen in the *in vitro* assay. The difference in the significance of results determined between the two assays in this case may be due to interactions between type IV collagen (but not with fibronectin) and secreted laminin, both of which are components of the basement membrane. Secretion of laminin-5 in the *in vitro* assay was shown in (Section 3.2.1) and has been widely reported. An interaction between collagen IV and secreted laminin has already been reported to influence migration (and metastasis) of hepatocellular carcinoma cells (a response thought to reflect differing patterns of integrin expression particularly the β_1 subunit which is also important in keratinocyte migration) (Torimura et al., 2001).

4.5 Results: Evaluation of keratinocyte morphology during migration on selected extracellular matrix components and other substrates present in the *in vivo* wound matrix

To complement the observations of the two migration assays, experiments were performed to demonstrate the cellular morphology adopted by keratinocytes when plated on the various substrates (poly-L lysine, laminin-1, vitronectin, laminin-5, fibronectin, type IV collagen and type I collagen) used in the two migration assays with the same exposure to growth factor. Cells were initially plated onto the respective substrates in medium containing no growth factors. 4-hours later the medium was replaced with one containing growth factors. This change was referred to as time point t=0. Keratinocytes were then visualized by staining of F-actin with Rhodamine-conjugated phalloidin under fluorescence microscopy (as described in Section 2.6). Images were taken immediately (time t=0) and again after 60 minutes (time t=60 min), representative images are shown in (Figure 21).

(Figure 21)



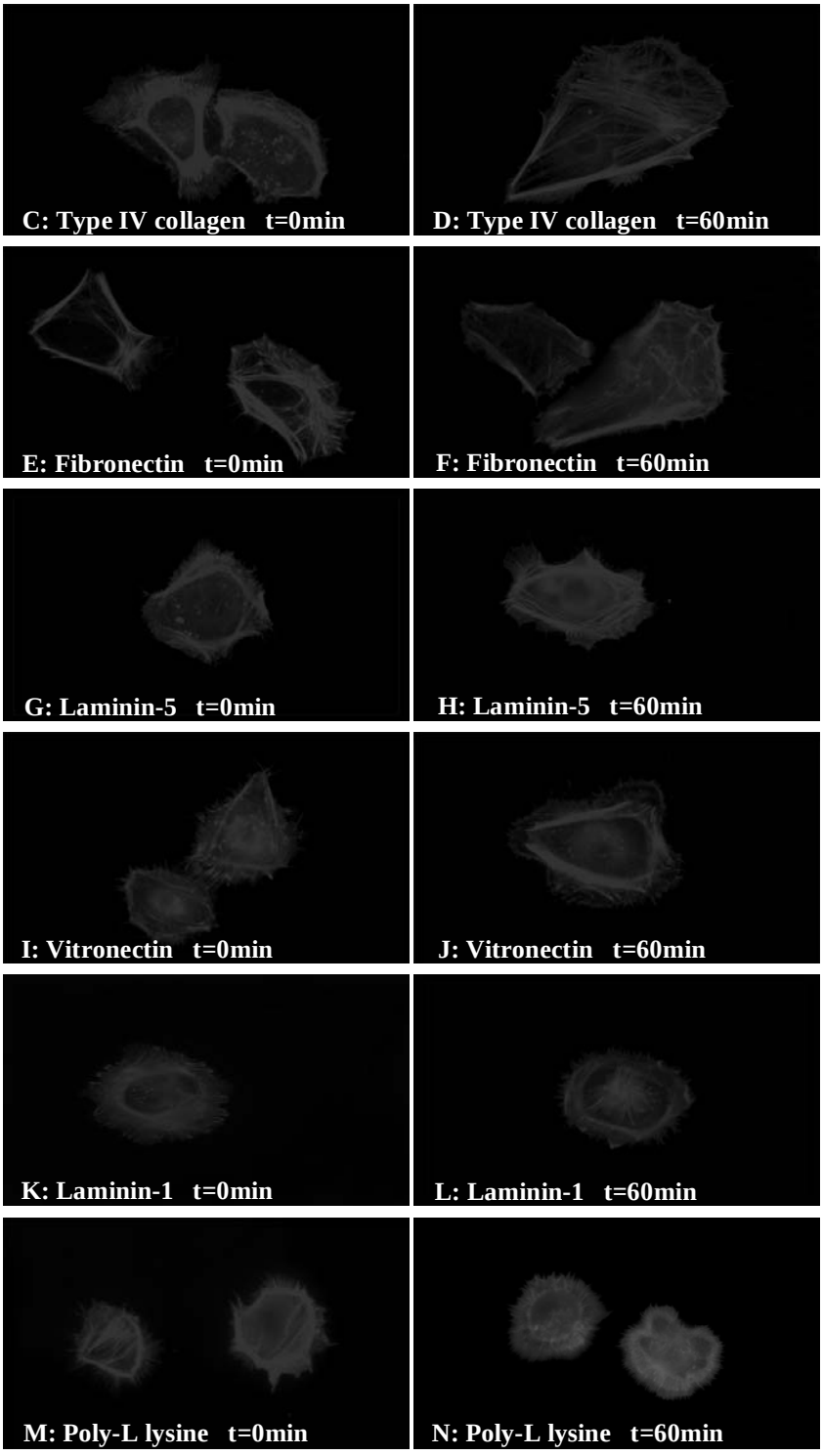


Figure 21: Representative images of human keratinocytes during migration on selected extracellular matrix components and other substrates present in the *in vivo* connective tissue. Type I collagen (A: t=0 and B: t=60min), Fibronectin (E: t=0 and F: t=60min), and Vitronectin (I: t=0 and J: t=60min) that are components of the early extracellular matrix of a wound. Laminin-5 (G: t=0 and H: t=60min) and Type IV collagen (C: t=0 and J: D=60min) are basement membrane components and that are also secreted by migrating keratinocytes during *in vivo* wound healing. Laminin-1 (K: t=0 and L: t=60min) another basement membrane component not normally seen in a healing wound until

migration of keratinocytes ceases with completion of e-epithelialization. Poly-L lysine (M: t=0 and N: t=60min) was included as a non-migratory control substrate.

Cellular morphology is known to show characteristic features associated with adhesion, spreading and during migration. These features were observed here and can be generalized as follows: Adherent cells that were not significantly spreading or migrating (Figure 20: B MI_{CG}≈3-5) remained small (retaining approximately the same mean diameter or becoming slightly smaller over time compared to when initially plated). They also showed few and poorly developed membrane protrusions (frequently seen were large numbers of small fine filopodia-like structures visible around the circumference of the cell). Adhesion occurred alone or in associated with cell spreading and migration; in all cases cells formed attachments with the substrate. Spreading cells became enlarged with a greater mean diameter than when initially plated. Extending from their periphery were substantial membrane protrusions that were clearly visible. These protrusions included both broad flat lamellipodia and fine finger or hair-like filopodia. [Within lamellipodia, actin filaments form a branching "dendritic" network, whereas in filopodia they are organized into long parallel bundles, but this detail was not clearly visible in the images taken (Ridley et al., 2003)]. In instances when cell spreading was not associated with significant migration (determined in the migration assays) the membrane protrusions occurred on average in all directions (an average considered across a number of similar cells) and there was little discernable polarization of the assembled actin structure. The level of migration in such cells was limited (Figure 20: B MI_{CG}≈10-12) and occurred apparently in a more random manner in relation to direction (as shown in comparable images from the colloidal gold migration assay Figure 20: C). With greater migration (Figure 20: B MI_{CG}≈30-32), cells became physically polarized. In this case both lamellipodia and filopodia were produced in greatest density in one direction, the presumed direction of migration and polarized actin assembly was seen. These migrating cells became elongated along an axis in the direction of migration and frequently visible trailing behind the cell body was a cytoplasmic extension or “tail” (Figure 22). Comparable images of these cells migrating during the colloidal gold migration assay (Figure 20: C) showed in a number of cases long tracks of more “directional” migration.

(Figure 22)

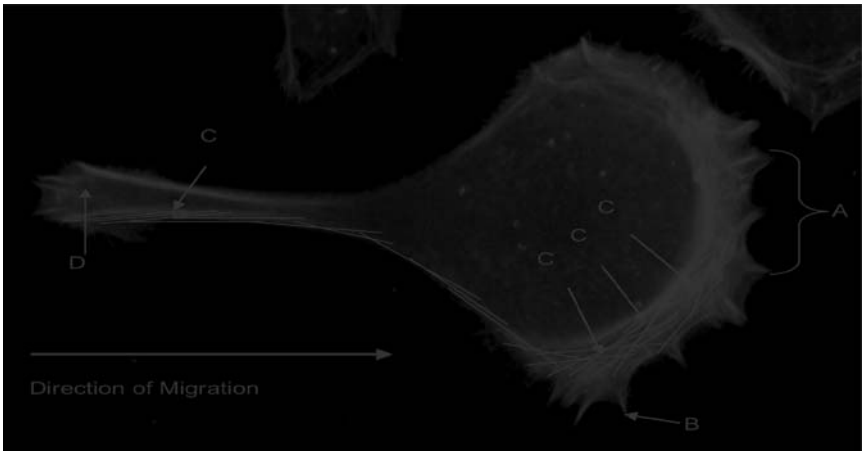


Figure 22: Directional migration of a human keratinocyte, visualized by staining of F-actin with Rhodamine-conjugated phalloidin under fluorescent microscopy. [Image provided from our lab (taken by Dr. Jinhua Fan)] A: Broad lamellipodia (actin filaments form a branching "dendritic" network). B: Thin filopodia (actin filaments organized into long parallel bundles). Both lamellipodia and filopodia are produced in greatest density in the direction of migration. C: Polarized actin assembly of the cytoskeleton. D: Retracting cellular “tail.”

For each substrate up to 100 cells (range 50-100) were imaged by fluorescence microscopy at each time point. A visual inspection of the images was performed to identify features of cellular spreading and migration (as described and defined above and in Section 2.6). The number of spreading and polarized cells was counted and is presented in (Table 7). Note: Images of keratinocytes plated on poly-L lysine, a substrate known cause attachment but not spreading or polarization, were used as controls for the visual comparisons with cells on other substrates.

(Table 7)

Percentage of cells showing Spreading (Sp) and Polarization (Po)														
	Type I collagen		**Fibronectin**		**Type IV collagen**		**Vitronectin**		**Laminin-5**		**Laminin-1**		**Poly L-Lysine**	
t=0 min	Sp	Po	Sp	Po	Sp	Po	Sp	Po	Sp	Po	Sp	Po	Sp	Po
	71	9	7	3	70	7	8	3	5	5	5	0	4	0
Total = (Sp+Po)	80		10		77		11		10		5		4	
t=60 min	Sp	Po	Sp	Po	Sp	Po	Sp	Po	Sp	Po	Sp	Po	Sp	Po
	20	69	40	45	41	42	60	22	49	8	7	0	2	0
Total = (Sp+Po)	89		85		82		82		57		7		2	

Table 7: Percentages of cells showing morphological features of spreading (Sp) or polarization (Po) at time t=0 and one hour later time t=60 min during keratinocyte migration on selected substrates. Additionally percentages of cells showing either feature (Total=Sp+Po) under each condition and at each time point are also shown.

Keratinocytes plated on poly-L lysine are known to attach, but not to spread or migrate (polarize) significantly. This was confirmed here and as stated images of cells on this substrate were used as controls for comparison with keratinocytes on other substrates (Figure 20: M and N). Cells at t=0 on poly-L lysine all show a rounded morphology with minimal evidence of spreading or polarization (Table 7). Lamellipodia are absent and small filopodia-like protrusions are visible extending in all directions (Figure 21: M). (Note: t=0 corresponds to a condition following exposure of keratinocytes to the substrate but effectively not to growth factor, growth factor was added at t=0, 4-hours after the cells were plated onto the substrate). This pattern remained despite the presence of growth factors at t=60min (Figure 21: N), although the cells appeared slightly larger. These same findings were also seen in cells plated on the basement membrane substrate laminin-1, (Table 7), (Figure 21: K and L). Cells at t=0 on fibronectin (Figure 21: E), laminin-5 (Figure 21: G), and vitronectin (Figure 21: I) exhibited these same features with limited evidence of spreading and polarization. The numbers of cells showing spreading and polarization was approximately twice that of the poly-L lysine controls, but they were still very few in number. The total (Sp+Po) cells showing spreading and polarization was 5 for the control poly-L lysine and 10-11 for the substrates fibronectin, laminin-5, and vitronectin (Table 7).

At t=60min cells on laminin-5 (Figure 21: H), and vitronectin (Figure 21: J) showed noticeable changes visually, the majority of cells exhibited spreading (Table 7: Sp=49 on laminin-5 and Sp=60 on vitronectin) and to a lesser extent polarization was also visible (Table 7: Po= 8 on laminin-5 and Po=22 on vitronectin).

At t=0 cells plated on type I (Figure 20: A) and type IV (Figure 21: C) collagen showed a spreading morphology (Table 7: Sp= 70 on type I collagen and Sp=70 on type IV collagen) with additional limited numbers of cells being polarized (Table 7: Po=9 on type I collagen and Po=7 on type IV collagen). At t=60min cells plated on type I collagen (Figure 21: B), type IV collagen (Figure 21: D), and fibronectin (Figure 21: F) showed marked increases in the presence of a polarized morphology (Table 7: Po=69 on type I collagen, Po=42 on type IV collagen, and Po=45 on fibronectin). This was the most common pattern of cell morphology seen in each case, and in each case the vast majority of cells showed spreading or polarization (Table 7: Sp+Po=89 on type I collagen, Sp+Po=82 on type IV collagen, and Sp+Po=85 on fibronectin).

These results demonstrating the morphological features of migration (Table 7 and Figure 21) were in complete agreement with quantification of migration on all of the substrates considered, in both the colloidal gold migration assay and *in vitro* wound assay (Figure 20: A and B). Furthermore, as previously indicated was the case examining the images of cells migrating during the colloidal gold assay (Figure 20: C) it is noteworthy that conditions associated with negligible migration ($MI_{CG}\approx$3-5.3 and $MI_{IV}\approx$0.04-0.1) such as poly-L lysine and laminin-1 (with or without growth factors), and vitronectin, laminin-5, and fibronectin (without growth factors) all show the same pattern of gold salt phagocytosis (roughly circular areas of darkness around the cell body). Similarly the images of cells migrating at an intermediate level such as on type I collagen ($MI_{CG}\approx$11 and $MI_{IV}\approx$0.31) and type IV collagen ($MI_{CG}\approx$9.3 and $MI_{IV}\approx$0.28) (in the absence of growth factors) and vitronectin (both $MI_{CG}\approx$9.3 and $MI_{IV}\approx$0.24) (in the presence of growth factors), again all show approximately the same pattern of gold salt removal (Figure 20: C). Under these conditions the "phagokinetic tracks" left in the gold salt extend beyond an area adjacent to the cell body, are relatively broad and convoluted. These conditions were primarily associated with morphological features of cell spreading (membrane protrusions occurring on average in all directions) and less so with discernable polarization (Table 7). Such cells appear to be migrating, but in a limited manner and apparently in a more random pattern (in relation to

direction). Finally, the images of cells showing the greatest migration such as those on type I collagen (MI_{CG}≈36 and MI_{IV}≈0.86), type IV collagen (MI_{CG}≈21 and MI_{IV}≈0.57) and fibronectin (MI_{CG}≈16.5 and MI_{IV}≈0.54) in each case in the presence of growth factors, again all show approximately the same pattern of gold salt phagocytosis (Figure 21: C). The "tracks" left in the gold salt extend beyond an area adjacent to the cell body, are relatively narrow and show long stretches of darkness where the cell migrated linearly. In these conditions morphologically cells were polarized (Table 7). Such cells are migrating in a far more linear manner (with "directional" migration, cells becomes physically polarized as was seen here).

4.6 Results: Keratinocyte migration in relation to concentration of deposited type I collagen

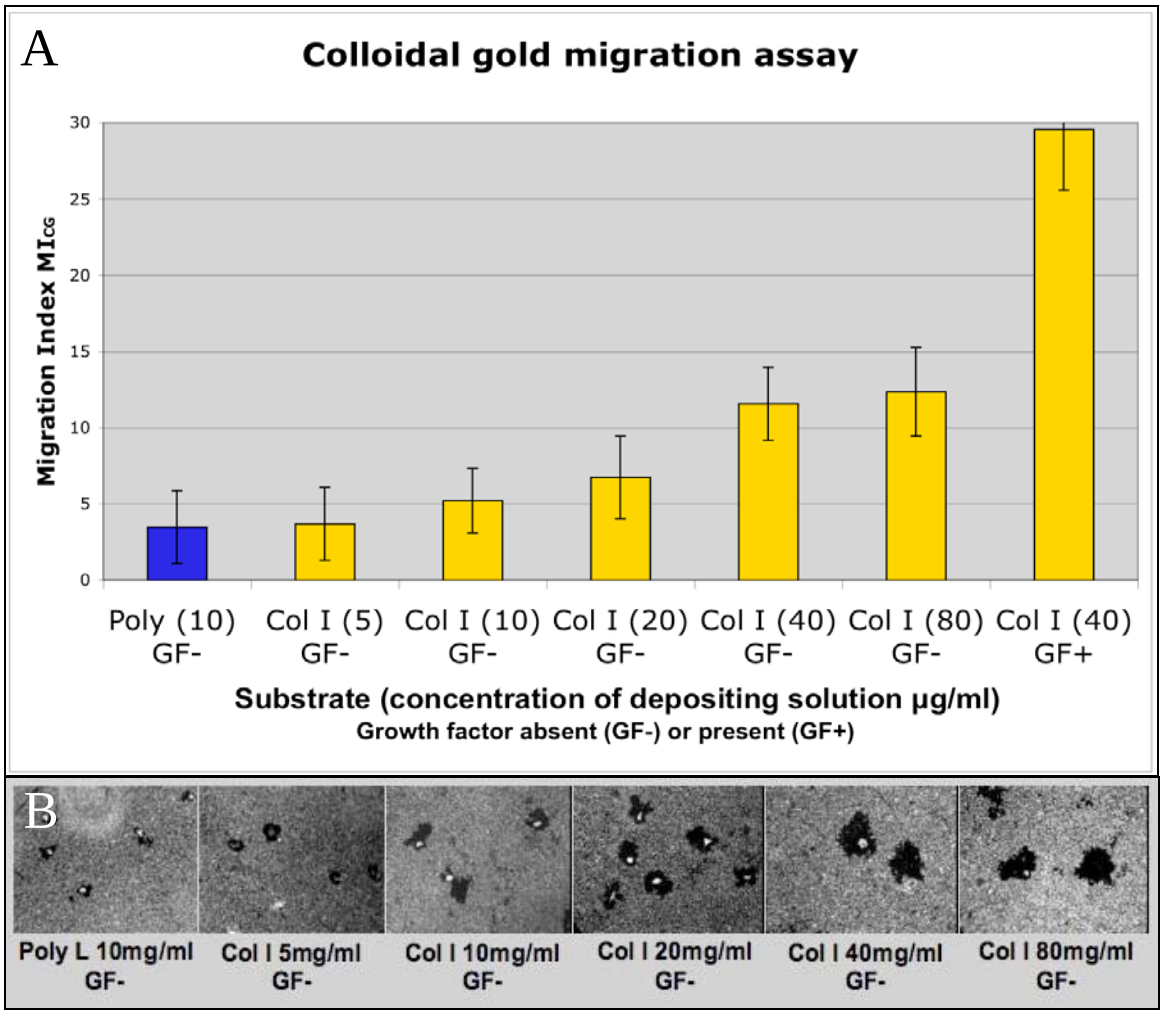
In a healing *in vivo* wound, during the period when keratinocytes are migrating, levels of type I collagen in the extracellular matrix progressively increase, primarily due to the activity of fibroblasts. To further investigate migration of keratinocytes on type I collagen, various concentrations of the substrate solution were used to deposit a type I collagen surface on which the cells then migrated, in the presence and absence of growth factors. It was anticipated that varying the concentration of the deposition solution would be reflected in changes in the concentration of type I collagen present on the surface over which the keratinocytes migrated, analogous to changes occurring *in vivo*. The protocol, including timing of deposition, for each solution was identical.

Note: A direct measurement of type I collagen concentration at the migration surface that resulted from deposition using each of the different concentrations of type I collagen (5, 10, 20, 40 and 80μg/ml) was considered desirable. However, it proved impractical to directly and reliably make such a measurement. Attempts were made to observe these changes indirectly using fluorescent antibody staining of deposited type I collagen. Fluorescent antibody specific to type I collagen was added to wells prepared using the same protocol for each concentration of type I collagen solution and an average intensity across the wells was measured. This technique did demonstrate subjectively an increasing average intensity of fluorescence with increasing concentration (5, 10, 20, and 40μg/ml) of depositing solution, suggesting an increasing concentration of substrate present at the surface on which migration was occurring. At higher concentrations (40 and 80μg/ml), fluorescence intensity

appeared identical. Further, significant variation between repeat experiments was observed and at lower concentrations of depositing solution (5 and 10µg/ml) there was noticeable variability in intensity across the surface of each well. (This data is not shown).

The results of the colloidal gold migration assays demonstrating keratinocyte migration on type I collagen substrate deposited with solutions of varied concentration (5, 10, 20, 40 and 80µg/ml) are shown in (Figure 23). (Results for *in vitro* wound assay are not shown, but demonstrated the same findings).

(Figure 23)



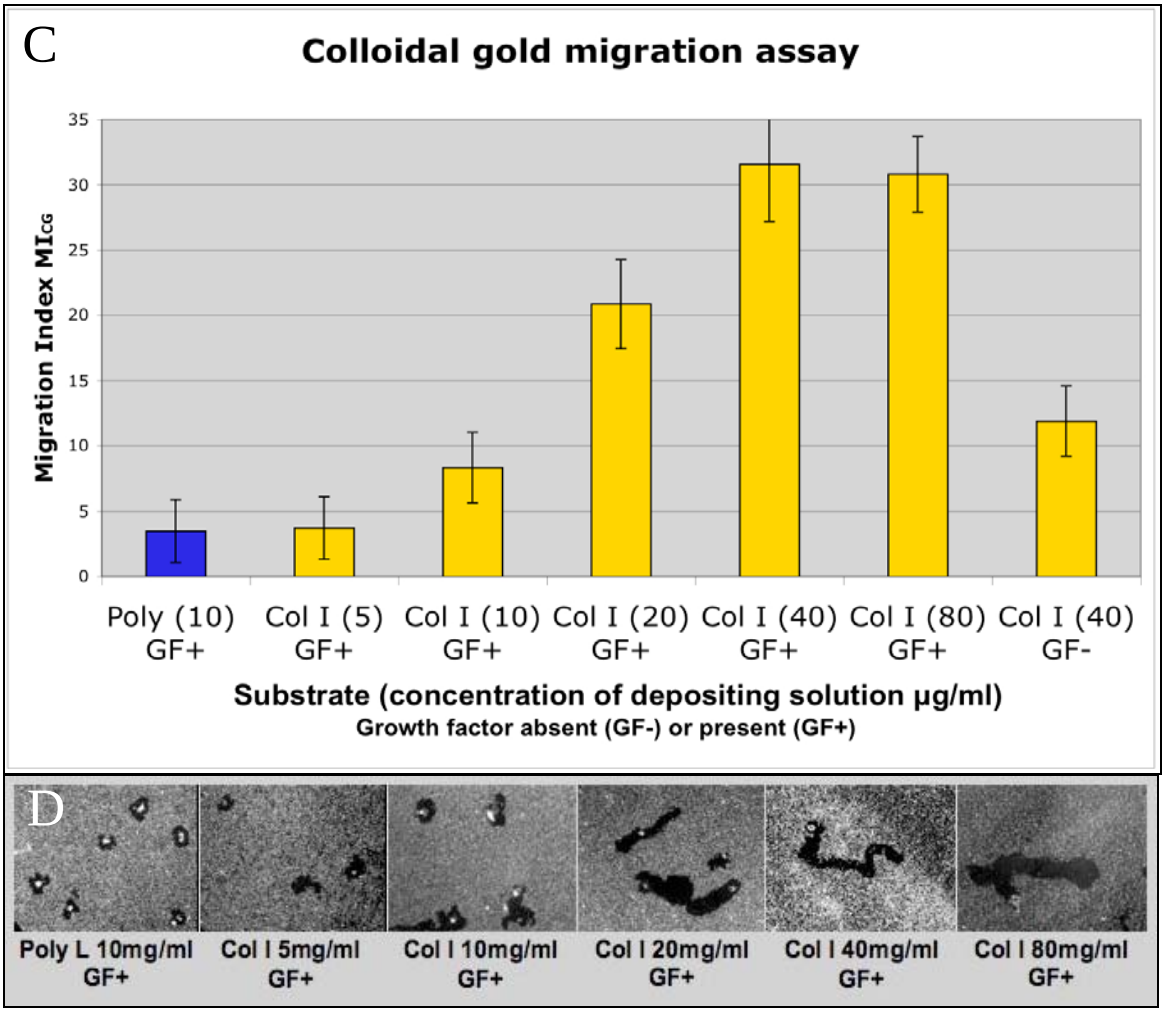


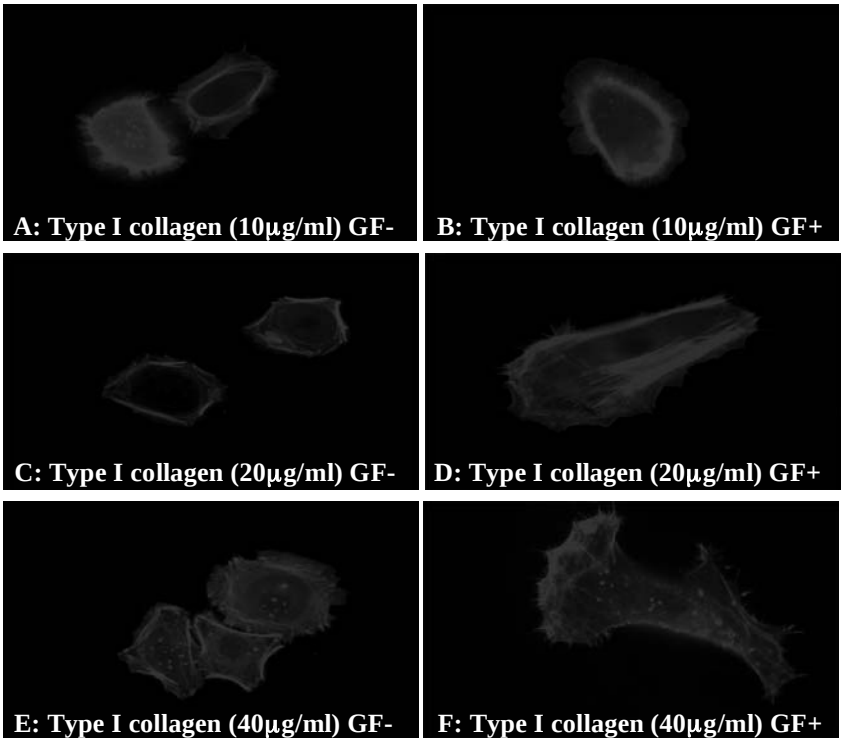
Figure 23: Effects of varying concentrations of solution used to deposit type I collagen substrate on keratinocyte migration in the colloidal gold migration assay. A: Shows MI_{IV} indices obtained in the absence of growth factors (GF-) on type I collagen (Col I) substrate deposited from solution at concentrations 5, 10, 20, 40 and 80µg/ml. Non-migratory poly-L lysine (Poly L) substrate deposited at 10µg/ml was used as a negative control (shown in the left hand column) and a second control, migration on type I collagen deposited at concentration 40µg/ml with growth factor (GF+) exposure is also shown (right hand column). B: Shows representative images of migrating keratinocytes captured during the colloidal gold migration assay under the same conditions (Note: Images are not shown for the second control). C: Shows MI_{IV} indices obtained on type I collagen substrate deposited from solution at concentrations 5, 10, 20, 40 and 80µg/ml in the presence of growth factors. Again non-migratory poly-L lysine substrate deposited at 10µg/ml was used as a negative control (left hand column) and a second control, migration on type I collagen deposited at 40µg/ml without growth factor exposure is also shown (right hand column). D: Shows representative images of migration captured during the colloidal gold migration assay under the same conditions (again images are not shown for the second control). Note: These experiments expanded upon and

confirmed published (Li et al., 2004b) and unpublished work performed previously in our Lab (by Dr. Jinhua Fan).

4.6.1 Results: Keratinocyte morphology during migration in relation to deposition concentration of type I collagen

To complement the observations of the two migration assays, experiments were performed to demonstrate the cellular morphology adopted by keratinocytes when migrating on type I collagen deposited from solutions at the same concentrations (5, 10, 20, 40 and 80μg/ml) analyzed in the colloidal gold and *in vi*tro wound assays. These experiments were performed as described previously in Section 4.5 and all images here were taken after 60 minutes (time t=60 min). Representative images are shown in (Figure 24). (Note: Images of cells migrating on type I collagen at deposition concentrations 10, 20, and 40μg/ml are shown. Images at 5μg/ml were not significantly different from those at 10μg/ml, and similarly images at 80μg/ml were not significantly different from those at 40μg/ml. The images from concentrations 5μg/ml and 80μg/ml are not shown).

(Figure 24)



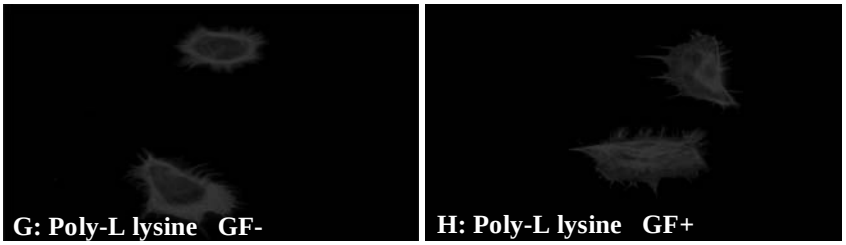


Figure 24: Representative images of cellular morphology adopted by human keratinocytes migrating in the presence (right hand images) and absence (left hand images) of growth factors on type I collagen deposited from solutions at the same concentrations analyzed in the colloidal gold and *in vitro* wound assays. A non-migratory poly-L lysine substrate deposited at 10μg/ml was used as a control (G: growth factors absent, H: growth factors present).

Up to 100 cells (range 50-100) were stained and imaged under fluorescence microscopy, at each deposition concentration of type I collagen and the poly-L lysine control. Visual inspection of the images was performed to identify features of cellular spreading and migration (as described previously). Keratinocytes plated on poly-L lysine were used as controls for comparison with cells on type I collagen. The number of spreading and polarized cells was counted and is presented in (Table 8).

(Table 8)

Percentage of cells showing Spreading (Sp) and Polarization (Po)								
	Poly-L lysine		**Type I collagen (10μg/ml)**		**Type I collagen (20μg/ml)**		**Type I collagen (40μg/ml)**	
GF Absent	Sp	Po	Sp	Po	Sp	Po	Sp	Po
	5	0	10	2	49	13	61	13
Total = (Sp+Po)	5		12		52		74	
GF Present	Sp	Po	Sp	Po	Sp	Po	Sp	Po
	5	0	40	7	31	41	25	62
Total = (Sp+Po)	5		47		72		87	

Table 8: Percentages of cells showing morphological features of spreading (Sp) or polarization (Po) in the absence of growth factors (GF absent) and the presence of growth factors (GF present) during

keratinocyte migration on type I collagen deposited by solutions of various concentrations (10, 20, and 40μg/ml). Additionally percentages of cells showing either feature (Total=Sp+Po) under each condition are also shown.

In both the presence and absence of growth factors, keratinocyte migration on type 1 collagen showed dose-dependent increases in migration indices in relation to increasing concentration (from 5 to 40μg/ml) of depositing solution; a deposition concentration of 5μg/ml showed no significant difference in migration index from the poly-L lysine control cells (MI_{IV}≈4-5) and maximum migration was achieve at 40μg/ml. As expected maximum migration was significantly greater in the presence of growth factors MI_{IV}≈30 compared to MI_{IV}≈11.5 in their absence ($p<0.05$). (Note: Statistical significance of comparisons of results, comparing two or more identical conditions for each assay considered separately, were in agreement between assays). Above this concentration (at deposition concentration 80μg/ml) no further increase in migration index was seen in either the presence or absence of growth factors.

Keratinocytes on poly-L lysine showed a rounded morphology with minimal evidence of spreading or polarization (Table 8), no lamellipodia, and small filopodia-like protrusions extending in all directions (Figure 24: G and H). In the absence of growth factors, increasing deposition concentration of type I collagen was associated with dose-dependent increases in migration and marked increase in the number of cells showing evidence of spreading (Table 8: Sp=10 at deposition concentration 10μg/ml to Sp=61 at 40μg/ml, compared to 5 on poly-L lysine). This was visible in enlargement of the migration tracks (Figure 23: B) and cellular morphology under fluorescence microscopy (Figure 24: A, C, and E). [In the absence of growth factors only a limited number of cells showed a polarized morphology, the maximum number (Po=13) being seen equally at 20 and 40μg/ml (Table 8)]. In the presence of growth factors increasing deposition concentration of type I collagen was again associated with dose-dependent increases in migration and marked increases in numbers of cells showing evidence of spreading and polarization (Table 8: Total (Po+Sp)=47 at deposition concentration 10μg/ml to Total (Po+Sp)=87 at 40μg/ml, compared to Total (Po+Sp)=5 on poly=L lysine). An increase in the number of cells showing a polarized morphology was particularly noticeable (Table 8: Po=7 at deposition concentration 10μg/ml to Po=62 at 40μg/ml, compared to Po=0 on poly=L lysine). As was

the case in the absence of growth factors, these changes were visible in enlargement of the migration tracks (Figure 23: D) and cellular morphology under fluorescence microscopy (Figure 24: B, D, and F).

The results of Figure 23 and 24, and Table 8 are in agreement. Furthermore, as shown previously (Figure 20 and 21, and Table 7) here conditions supporting negligible migration (MI_{CG}≈4-5) such as poly-L lysine and low deposition concentrations of collagen-1 with or without growth factors, all show the same pattern of gold salt phagocytosis (roughly circular areas of darkness around the cell body). In the absence of growth factors and at deposition concentration 40μg/ml (MI_{CG} up to 10-12) the "tracks" left in the gold salt extend beyond an area adjacent to the cell body, appear relatively broad and are convoluted (lacking linear segments). Morphological features of these cells were primarily those of spreading (Table 8). Similarly in the presence of growth factors and at a lower deposition concentration (10μg/ml) (MI_{CG} ≈8) these same features were observed. However as deposition concentration increased (20 and 40μg/ml) cells showed relatively narrow "tracks" that include long stretches where the cell migrated linearly. Under these conditions cells were morphologically polarized (Table 8). Such cells are migrating in a far more linear manner (with "directional" migration cells becoming physically polarized, as seen here).

Migrating keratinocytes during wound healing *in vivo* experience an environment in which there is an increasing level of type I collagen in the extracellular matrix, primarily due to the activity of fibroblasts. These findings confirm the importance of type I collagen as a pro-migratory substrate and demonstrate that early wound migration would be progressively enhanced by changes occurring in the extracellular matrix.

4.7 Discussion

In summary, the experiments presented confirm the hypothesis that in the presence of type I collagen, migration of keratinocytes would occur with or without the addition of soluble growth factors, and in addition the presence of soluble factors would significantly augment (enhance) keratinocyte migration. Also confirmed was the hypothesis that initiation and continued migration of keratinocytes depend on the presence of certain substrates (such as type I collagen and others known to be present in the *in vivo* healing wound matrix).

In Section 4.4 it was demonstrated that significant keratinocyte migration occurred in both assays on various substrates such as type I collagen, type IV collagen and fibronectin and at a lower levels on vitronectin in the presence of growth factors. Significant migration was also seen in the absence of growth factors on the substrates type I and type IV collagen (Figure 20). In Section 4.5 these findings were corroborated and supplemented by morphological studies (Figure 21) and (Table 7). In the case of laminin-1 a basement membrane component migration did not occur. It appears that in the absence of integrin-ECM stimulation from a suitable substrate (e.g. on poly-L lysine or laminin-1) growth factors could not induce migration of keratinocytes. Additionally, neither increasing concentrations of growth factor supplementation or high concentrations of serum could cause keratinocytes to migrate on poly-L lysine (Figure 18) and (Figure 19) respectively. As was hypothesized, it was shown that growth factors significantly enhance migration, but alone (without integrin-ECM stimulation) at any level they appear unable to initiate migration in the absence of a pro-migratory substrate.

Migration in the absence of growth factors (as well as their presence) has been demonstrated in a number of cell types including fibroblasts (Li et al., 2004a) but not previously in keratinocytes. It seems likely that there are several pathways leading to keratinocyte migration on type I collagen, initiated and continued by integrin-ECM signalling and linked to growth factors and their receptors. Downstream signalling that occurs within the keratinocyte as a result of integrin-ECM interactions will be considered further in Chapter 6, and in combination with downstream signalling by growth factor stimulation, will be considered next in Chapter 5.

One explanation that might link the differences in migration observed here to the substrate present is the pattern of integrin expression on keratinocytes. Integrins have a limited specificity to bind extracellular matrix components (Holly et al., 2000; Hynes, 2002; Miranti and Brugge, 2002; Gilcrease, 2007) and different patterns of integrin expression are known to be present in migratory compared to non-migratory keratinocytes. Expression of certain integrins or not, and expression of certain patterns of integrin favours an interaction with particular extracellular matrix proteins. The expression of the integrin β_1 subunit is increased in keratinocytes participating in healing an acute wound *in vivo* and *in vitro* (both in cells migrating at the leading edge of the wound and in those trailing behind). The β_1-subunit can associated with a number of different α-subunits, such as the α_2-subunit, an

arrangement that primarily mediates the integrin interaction with type I collagen, (but also with other collagens such as the basement membrane component type IV collagen) (Ridley et al., 1995). The β_1 in association with the α_5 subunit in migrating keratinocytes and mediates integrin-fibronectin interaction ($\beta_1\alpha_5$ is highly up-regulated in migrating fibroblasts) (Laukaitis et al., 2001; Gilcrease, 2007). Migrating keratinocytes in an acute healing *in vivo* wound also express α_v integrins that are not present in the unwounded epithelium (Clark et al., 1996). α_v in association with β_6-subunit mediates integrin-fibronectin interaction, and α_v in association with β_3-subunit is know to mediate integrin-vitronectin interaction (Kiosses et al., 2001).

Keratinocyte migration on laminin-5 observed here occurred at a level of $MI_{CG}\approx$7-8 in the presence of growth factors. Keratinocytes *in vivo* express several integrins with specificity for laminin-5 and integrin interaction with this substrate seems to play an important role in both facilitating and regulating migration (Goldfinger et al., 1999; Nguyen et al., 2000; Frank and Carter, 2004; Gilcrease, 2007). The β_1-subunit associated with α_3 and the β_4-subunit associated with α_6 mediate integrin interaction with laminin-5. Both α_3 and α_6 are upgraded in the migrating phenotype. A redistribution of $\alpha_3\beta_1$ and $\alpha_6\beta_4$ integrins, between migratory and non-migratory keratinocytes has been shown to occur, and is thought to play an important regulatory role (Goldfinger et al., 1999; Nguyen et al., 2001). Secretion of laminin-5 by keratinocytes as they migrate (Zhang and Kramer, 1996), can produce differences in migration (as was demonstrated in Chapter 3) and might also contribute to the differences in migration of keratinocytes on the two laminins studied here, laminin-5 and laminin-1, and also the differences in significance observed comparing migration in the presence and absence of growth factors using the colloidal gold migration and *in vitro* wound assays and during the colloidal gold migration assay in the presence of growth factors in comparison with the non-migratory control poly-L lysine.

Significant migration was not seen on the synthetic non-migratory control poly-L lysine, as expected, and on the basement membrane component laminin-1; in both of these cases there was no effect of growth factors. Laminin-1, a component of the basement membrane, is not secreted by migrating keratinocytes, but is deposited later during wound healing, at a time when migration ceases (Singer and Clarke, 1999). All keratinocytes (migratory and non-migratory) express the $\alpha_6\beta_4$ integrin which mediates integrin interaction with laminin-1 (and

other laminins). However, redistribution of this integrin ($\alpha_6\beta_4$) to the cell-cell regions and away from the cell-substrate region occurs during migration, a pattern that does not favour migration on laminin-1 (Goldfinger et al., 1999). Reversal of this pattern occurs upon completion of re-epithelialization when migration ceases and new basement membrane is formed that includes laminin-1 (Niessen et al., 1994). Absence of migration on laminin-1 demonstrated here might reasonably have been expected from these observations; it is certainly consistent with its role in the basement membrane and seems likely to be absent from its interactions with integrins positioned on the basal surface of non-migratory keratinocytes.

By far the most significant migration seen occurred when keratinocytes migrated on a type I collagen matrix in the presence of soluble growth factors. In Section 4.6 dose-dependent increases in keratinocyte migration occurred with increasing levels of type I collagen in the deposition solution (and probable increasing concentration on type I collagen on the migrating surface), in both the presence and absence of growth factors (Figure 23). These findings were corroborated and supplemented by morphological studies (Figure 24) and (Table 8) and may have a physiological counterpart with the changes occurring during wound healing. Early in wound healing *in vivo* during the period when keratinocytes are migrating, the provisional matrix is continuously modified by the action of the cells present, notably deposition of laminins by migrating keratinocytes, and deposition and catabolism of type I collagen by fibroblasts and an active proteolytic system (that results in an increasing proportion of type I collagen over time). Given the findings presented here (increasing concentration of type I collagen deposition solution produced increasing keratinocyte migration), it seems reasonable that these *in vivo* changes in the extracellular matrix support keratinocyte migration. The mechanism by increasing migration is stimulated by increasing type I collagen could relate to changes in integrin-extracellular matrix binding affinity and to integrin-ECM initiated downstream signalling activity (Gilcrease, 2007). Integrin-ECM binding leads to integrin clustering and focal adhesion formation that links the extracellular matrix via integrins firmly to the cellular cytoskeleton. Integrin-ECM binding also initiates downstream migratory signals through a large number of signalling molecules that are translocated to the intracellular surface of focal adhesions, notably FAK and Src family members. Changes in integrin-ECM binding and signalling concomitant with alterations in type I collagen concentration seen here and as occurs *in vivo*, might reasonably affect migration in a manner leading to the enhance keratinocyte migration that was observed with

increasing type I collagen deposition concentration and similarly supporting keratinocyte migration during wound healing.

Interestingly, type IV collagen, also a ubiquitous component of the basement membrane, resulted in significant migration (at a level second only to that seen on type I collagen). The type IV collagen interaction with integrins during migration in these assays is likely to be through the same subunits as with type I collagen, (e.g. β_1-subunit in association with the α_2 subunit). Type IV collagen has been shown to be a substrate that supports cell adhesion, spreading, and migration of a number of cells including neoplastic cell lines such as melanoma (Chelberg et al., 1989), and also primary cell lines including keratinocytes (Sarret et al., 1992a), the latter result being confirmed again here. However, type IV collagen is not normally found in significant quantities in the healing wound *in vivo* until a later stage (with remodelling and at a time when keratinocyte migration in complete). The experimental arrangement using this substrate as a surface for keratinocyte migration is therefore not reflective of the *in vivo* circumstance during wound healing, but these findings likely do reflect activity in the same pathways that are involved during migration on type IV collagen as with other collagens such as type I.

As a final note, a limited analysis (in Section 4.3.1) of the effects of concentration of growth factor supplementation presented here (proportionally varying together bovine pituitary extract, insulin, and human epidermal growth factor in Section 4.3.1) showed migration increased with increasing supplementation to a maximum occurring in medium reconstituted according to the manufacturer's guidelines. Above this level of supplementation, no further increases in migration was observed and at very high levels of supplementation, ten times the levels suggested by the manufacturer, cytotoxicity was seen and migration appeared to be decreasing though not significantly. It was also demonstrated (in Section 4.3.2) that serum, but not plasma enhanced keratinocyte migration on type I collagen in a dose-dependent manner. Above a 10% concentration of serum, no further increase in migration was seen. Serum and plasma demonstrated significantly different effects on keratinocyte migration that strongly suggests the presence of one or more factors within serum, acting alone or in combination, that control or directly promote keratinocyte migration on a type I collagen in these assays. In the absence of a wound, skin cells are exposed to an ultrafiltrate of plasma. Following wounding, during haemostasis and the formation of the fibrin clot protein, several protein cascades are activated (notably,

including the coagulation cascades). The resulting fluid (serum) contains a different protein composition compared to the original plasma. Serum is a component of the milieu experienced by keratinocytes during the early phase of acute wound healing and has been shown to contain many of the same soluble mediators as those produced by the cells present in the wound and others that may have a particular functional importance (such as serum-derived plasmin). Analyzing the putative pro-migratory factors in serum has shown that a variety of the components present in serum, but not in plasma are stimulators of migration (Werner et al., 1994; Li et al., 2006). The mixture of soluble factors present in serum seem likely to be significant in facilitating and promoting migration during wound healing (Li et al., 2006).

Chapter 5 Downstream signalling of soluble mediators and the MAP kinase pathway in keratinocyte migration

5.1 Introduction

A diverse range of soluble factors and mediators are involved in regulating the complex events involved in wound healing. These include growth factors, cytokines, proteases and their inhibitors, and possibly neuropeptides, hormones and small molecules such as vitamins A and C. The importance of soluble mediators in wound healing is attested by the multiple functions they have been shown to perform *in vitro* in wound healing assays and their documented presence in the right place at the right time *in vivo* where they likely serve the same functions. During the process of wound healing, the profile of soluble factors present varies over time. The cells present in the wound are responsible for production and release of most of the more abundant soluble factors. There is a complex interchange and mutual relationship between the cellular activity and the action of soluble factors at any moment. At each phase, those factors (and cells) present influence the events that are occurring and also guide progression towards the next phase and ultimately a completely healed wound. Cells both secrete and respond to soluble factors. Serum is also a component of the milieu during the early phase of acute wound healing. It contains many of the same soluble mediators as those produced by cells present in the wound as well as others that may have a particular functional importance (such as serum-derived plasmin).

To effectively influence a response such as keratinocyte migration; the presence of a soluble factor must be transmitted to the cell. This occurs via signal transduction systems consisting initially of cell surface receptors with specificity for one or a limited number of soluble factors. Once bound to the receptor the signal is transmitted into the cell and further to target effector systems in the cytosol and nucleus that produce the desired response. Transduction of the signal across the cell membrane has been discussed in Chapter 1 and it is most directly achieved by conformational changes in the receptor protein leading to altered binding site affinity for adaptor proteins and or the kinase activity of the receptor. Indirectly, though no less importantly, other elements may play an immediate role such as activation of G-proteins (heterotrimeric GTPases), adenylate cyclase (and the cyclic-AMP system which is frequently linked to G-protein activation), and effects of the balance

between phospholipases and lipid kinases.

Within the cell the products of signal transduction across the membrane are elaborated, amplified, and the signal is directed onwards. This enhancement is referred to as "downstream signalling" and refines the effect of the initial stimulus - the mediators of downstream signalling are commonly referred to as "second messengers". Refinements that occur with downstream signalling include interaction or "cross-talk" between signals derived from multiple signalling systems. Some processes are transduced to downstream effectors within the cytosol. Others are transduced across the cytosol and into the nucleus where they commonly lead to immediate early gene (IEG) activation. IEGs are effectors in response to a wide variety of cellular stimuli (relevant here they include c-Myc, c-jun.). Within the nucleus, IEGs represent the first round of transcription response to cellular stimuli that occurs before any new proteins are synthesized. "Late response" genes are activated following protein synthesis (in response to stimuli), the immediate early response genes and their products typically initiate them. Important components of the second messenger systems within keratinocytes are adapter proteins, non-receptor tyrosine kinases, lipid kinases and phosphatases, SMAD proteins, small GTPases, and the MAP kinase pathways (these are all reviewed in Chapter 1).

In this chapter experiments are presented that explore the role of soluble growth factor signalling during keratinocyte migration on type I collagen. In particular involvement of the MAP kinase pathways (Erk, p38 and JNK, reviewed below) as downstream mediators are considered. In order to begin to understand the relationship between activities in these pathways during keratinocyte migration, the simplest initial questions to be answered were which, if any, of the three well-characterized MAP kinase pathways are active during keratinocyte migration and, if active, which pathways appear to be necessary. The presence or absence of activity in each pathway was investigated in keratinocytes migrating on pro-migratory type I collagen and non-migratory poly-L lysine, in the presence and absence of soluble growth factors. In addition, under the same conditions, the role of various members of the MAP kinase pathways in keratinocyte migration were investigated by blocking their activity either with selected dominant negative genes or with use of chemical inhibitors (both chosen for inhibition of elements of the MAP kinase pathways). Using both dominant negative inhibitors and chemical inhibition, it was hoped that complementary and corroborative data on the effects identified would be provided. Prior to this, an assessment

was made of basal levels of expression of the MAP kinase gene products in their active and inactive forms. This provided reference data allowing for interpretation of activity in the MAP kinase pathways under the conditions subsequently evaluated in the two assays.

While the role of MAP kinases has been studied extensively in cellular functions including cellular migration, in many different cell lines, very little work has looked at their role during the migration of human keratinocytes. From review of the current information available on MAP kinase activity during migration across all cell types, it seemed reasonable to hypothesize that during human keratinocyte migration the Erk MAP kinase pathway would be active and would have a direct effect on migration of keratinocytes, this is hypothesized to prove to be essential both in the presence and absence of growth factors. The situation regarding the SAPK pathways, JNK and p38, was less clear. They too share links to many of the same effectors as the Erk MAP kinase pathway, but are primarily mediators of the cellular response to stress. Links to migration-related pathways have been demonstrated but occur over a longer time frame than that of assays performed here. Therefore, it was unclear whether or not these pathways would be involved during keratinocyte migration. Nonetheless, it is hypothesized that the augmented migration proposed to follow growth factor stimulation will depend on the presence of an intact Erk MAP kinase pathway and possibly the p38, but not on the JNK MAP kinase pathways. Prior to evaluating these hypotheses and detailing the results of experiments a review of the MAP kinase pathways in relation to soluble factor signalling during migration is provided.

5.2 The MAP kinase pathways

Mitogen-activated protein (MAP) kinase pathways represent one of the major downstream signal systems used by eukaryotic cells to transduce extracellular signals into cellular responses. The pathways are elaborated by a family of protein kinases (non-receptor serine/threonine kinases) that utilize sequential kinase activation to regulate various cellular processes including cell division, migration, differentiation, and cellular response to physical stress. Various cell-cell and cell-environment stimuli including many soluble factors and physical stress are transduced through this system. Initiation of the MAP kinase pathway can result directly from receptor stimulation by ligand, such as interaction of EGF and the EGFR, or indirectly, following stimulation of other receptors and mechanisms such as a lipid kinases (e.g. PI3 kinase), G-protein linked signals and the cumulative effects of

adaptor proteins and non-receptor tyrosine kinases. Cellular processes with the same outcomes often share elements of the same downstream pathways and can enhance and elaborate their signals by activating the MAP kinase pathways in whole or in part (Lin et al., 1993; Johnson and Lapadat, 2002). The cumulative effect of activation of the MAP kinase pathway is the phosphorylation and regulation of many molecules, including cell surface proteins, cytoskeletal components and membrane-bound and cytoplasmic kinases. In addition and importantly, there is activation of intra-nuclear transcription by downstream effectors of activated MAP kinase pathways that can migrate into the nucleus (Lin et al., 1993; Johnson and Lapadat, 2002). These cellular changes lead to the intended cellular response following the initial stimulus.

Three principle MAP kinase subgroups have been identified in humans: Erk (extracellular signal-regulated protein kinase) and two pathways previously labelled as stress-activated protein kinase (SAPK) pathways: JNK (c-Jun N-terminal kinase) and p38. All members of the MAP Kinase pathway contain the motif, Threonine-“xxx”-Tyrosine within a region of the protein referred to as the activation loop in the kinase domain. “xxx” is single amino acid residue such as, glutamine, alanine, or proline. Members of a cascade with similar “xxx” are grouped together into linked pathways to help with classification. The Thr-Glu-Tyr motif is associated with the ERK pathway, the Thr-Pro-Tyr motif is associated with the JNK and the Thr-Ala-Tyr motif is associated with the p38 kinase pathway. Each member of a cascade is activated by phosphorylation of the threonine and tyrosine residues within the activation loop (Johnson and Lapadat, 2002). When this occurs, the phosphorylated form gains enhanced enzymatic activity (enhanced kinase activity). Schematically, subsequent events in each cascade follow a pattern with identifiable members at each level; there is activation of MAP kinase kinase kinase (MAPKKK) that in turn activates MAP kinase kinase (MAPKK) and finally the MAP kinase itself (after which the pathway is usually named) (Cano and Mahadevan, 1995) (Figure 25: A).

(Figure 25)

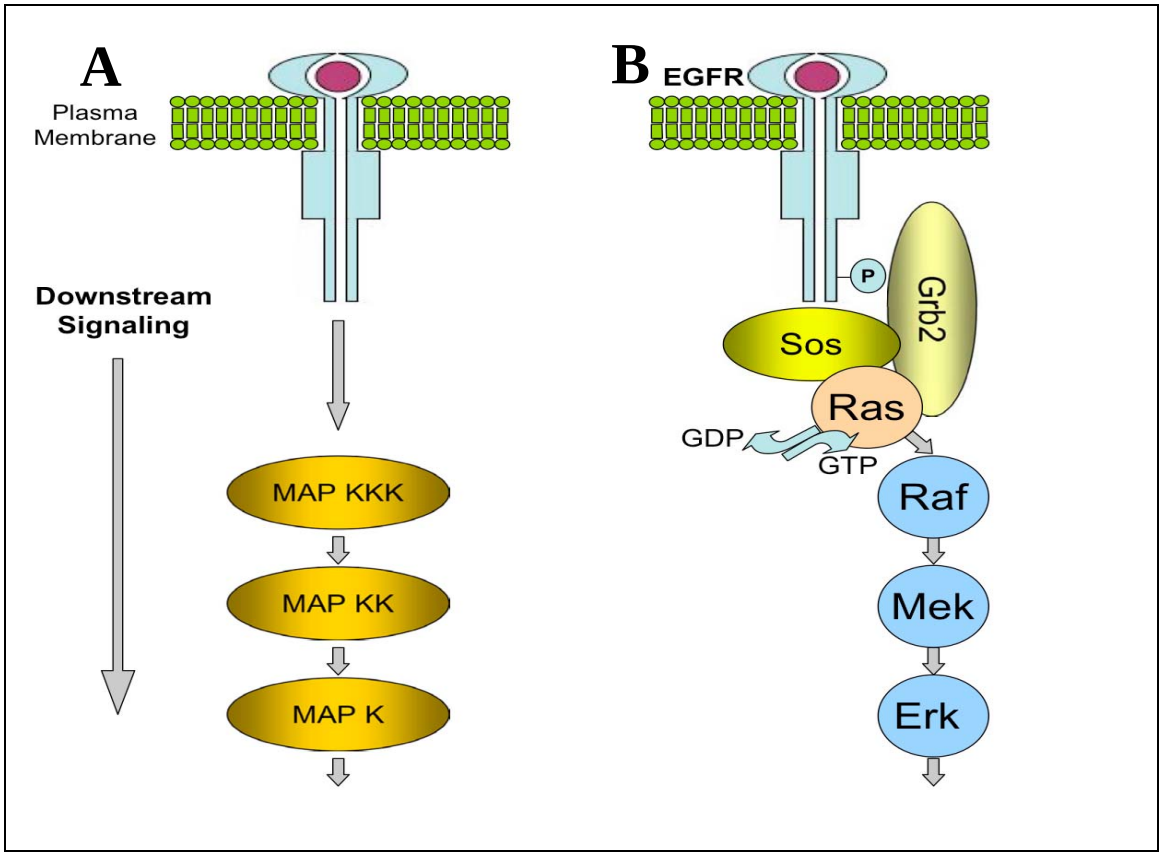


Figure 25: Schematic representations of a generic MAP kinase (mitogen-activated protein kinase) pathway (A) and significant elements of the EGFR mediated link to the Erk MAP kinase pathway (B). The details of the pathways are discussed in the text. A: Generic MAP kinase pathway (MAP KKK: MAP kinase kinase kinase, MAP KK: MAP kinase kinase, MAP K: MAP kinase). B: EGFR (epidermal growth factor receptor) mediated link to the Erk MAP kinase pathway (Grb2: an adapter protein, SOS: a GDP-GTP exchange protein or Ras-GEF (guanine nucleotide exchange factor), Ras, Raf: the MAP KKK, MEK: the MAP KK and Erk: the MAP K. GTP: guanosine triphosphate and GDP guanosine diphosphate (formed by hydrolysis of GTP).

5.2.1 Erk MAP kinase signalling during migration

The Erk MAP kinases are the most extensively studied subfamily of all MAP kinases. There are two isoforms - p44 (Erk-1) and p42 (Erk-2) - both contain a Thr-Glu-Tyr motif within the activation loop of the kinase domain. A wide variety of soluble growth factors and mitogens can stimulate activity in this pathway serving a number of cellular functions including wound healing (Johnson and Lapadat, 2002). Soluble factors such as EGF, TGFα, and HB-EGF binding to the EGF receptor and KGF binding to the fibroblast growth factor receptor 2 (FGFR2), activate the Ras-Raf1-MEK1/2-Erk1/2 signalling pathway (the

Erk MAP kinase pathway). Ras, a membrane-associate small GTPase, is activated by interactions with the receptor such as binding of the Grb2-SOS complex. This complex, in association with the EGF receptor, has been shown to activate Ras. (The receptor Grb2-SOS complex acts as a GDP-GTP exchange system activating Ras at the membrane) (Figure 25: B) (Seger and Krebs, 1995).

Activated Ras recruits the MAP kinase kinase kinase, Raf (also known as Raf-1), to the membrane, where Raf is activated by other protein kinases. It is believed that phosphorylation of Raf bound to Ras locks Raf into an activated conformation which is then independent of continued binding to Ras for its continued activity. Several kinases in the MAP kinase pathway have been suggested to be important for phosphorylation of Raf (bound to Ras), allowing for signal exchange between pathways as well as positive feedback phosphorylation by activated Erk itself. Downstream in the cascade, Raf specifically phosphorylates and activates the MAP kinase kinases MEK-1 and MEK-2, which in turn phosphorylates the Thr and Tyr residues within the Thr-Glu-Tyr motif of Erk-1 and Erk-2 (Seger and Krebs, 1995).

Significant focal aggregation of Ras, Raf-l, Grb2, SOS, MEK kinase (MEKK), MEK1, and Erk1/2 (along with other important regulators such as the Rho GTPase family members) can be demonstrated experimentally to occur following integrin-ECM interaction (e.g. fibroblasts on fibronectin) (Miyamoto et al., 1995). This does not occur on a poly-L lysine substrate.

Cellular effectors activated (phosphorylated) by the Erk MAP kinase pathway include MNK (MAP kinase interacting kinase), c-Myc, RSK (Ribosomal protein S6 kinase), MLCK, (myosin light chain kinase), FAK, paxillin, and calpain (calpain-2, also known as m-calpain). Briefly, MNK (MNK1, also known as (MKNK1 or MAP kinase interacting serine/threonine kinase) is a protein kinase that is directly phosphorylated and activated following interaction of ligand with EGFR leading to ERK activation (Chang et al., 2006). Phosphorylated MNK is implicated in the regulation of protein synthesis. MNK following phosphorylation by Erk and c-Myc directly, enter the nucleus and alter transcription of various genes. RSK phosphorylates ribosomal protein S6 that leads to altered translation of mRNA to proteins. More widely studied mediators of migration that include MLCK, FAK, paxillin, and calpain-2 as discussed in more detail below.

During migration it is believed that the force transmitted to focal adhesions is derived from the interaction of myosin II and the actin filaments. Myosin II activity is enhanced by MLCK and a Rho downstream effector protein Rho-associated coiled-coil containing protein kinase 1 (ROCK1). Myosin II activity is decreased by myosin light chain (MLC) phosphatase (which is also inhibited by ROCK1) (Ridley et al., 2003). Formation of lamellipodia at the leading edge of migrating cells and the turnover of focal adhesions may also depend on the action of MLCK (Webb et al., 2004).

Erk (and other members of the Erk MAP kinase pathway) have been shown to localize to focal adhesions as they are elaborated after formation at the leading edge of migrating cells (Miyamoto et al., 1995). Once activated at this site, it is known that activated Erk phosphorylates FAK on serine residues, such as Ser-910 within the FAT domain of FAK, both *in vivo* and *in vitro* (Hunger-Glaser et al., 2003). The effect of this Ser-910 phosphorylation is to inhibit the interaction between FAK and paxillin, an interaction central to migration. (This will be discussed in more detail in Chapter 6, briefly this pathway may proceed as: Src mediated phosphorylation of FAK at Tyr-925 and Shc at Tyr-317 results in Grb2 binding to SOS (in association with the EGF receptor) and subsequent Erk activation as described above. Activated Erk feeds back via Ser-910 phosphorylation to promote dissociation of FAK from paxillin).

Erk has also been shown to bind to paxillin and following activation to subsequently phosphorylate it (Erk can bind to paxillin at Tyr-118 and has been shown to phosphorylate a number of sites including Tyr-118 , Tyr-31 and others). This effect enhances the interaction between FAK and paxillin (Liu et al., 2002). Briefly, this pathway may proceed as: Erk activation by EGFR stimulation by growth factor (and or integrin-ECM stimulation) is followed by binding to paxillin and phosphorylation of paxillin by Erk. This pattern of binding and phosphorylation facilitates association of FAK and paxillin.

These intriguing complementary observations suggest that during migration, there may be a sophisticated regulation of the Src-FAK-paxillin complex by Erk. Activated Erk might at different times (and sites) promote complex-disassembly by phosphorylation of FAK (at Ser-910) and promote assembly by phosphorylation of paxillin, facilitating the cyclical changes that occur in focal adhesion assembly and disassembly during migration, but the precise mechanism remains to be clarified.

Calpains are a family of calcium-activated proteolytic enzymes that are involved in cell migration (Dourdin et al., 2001). Erk has been shown to phosphorylate calpain-2 (m-calpain) both *in vitro* and *in vivo*. This effect enhances calpain-2's activation and leads to degradation of cytoskeletal proteins and adhesion disassembly (Cuevas et al., 2003; Glading et al., 2004). Calpain-2 is recruited to focal adhesions by association with the N terminus of activated FAK (Carragher et al., 2003) and the authors too demonstrate a role for Erk MAP kinase-mediated activation of calpains in promoting focal-adhesion turnover during migration.

The Erk MAP kinase pathway is predominantly activated by mitogenic factors and is closely linked to activities relating to migration. The JNK and p38 pathways are preferentially activated by stress-inducing stimuli such as UV light, hypoxia, heat shock, and pro-inflammatory cytokines (Robinson and Cobb, 1997). Both JNK and p38 have been shown to be critical for transcription-dependent keratinocyte migration. Pathways such as the Rho-ROCK1-MEKK1-JNK pathway and the MEKK1-p38 pathway mediate this response. Both pathways have also been shown to exert transcription independent functions in migration; e.g. a Rho to JNK pathway induces the formation of actin stress fibres, a characteristic of keratinocyte migration (See below).

5.2.2 JNK MAP kinase signalling during migration

Jun N-terminal kinase (JNK), which has a Thr-Pro-Tyr motif within the activation loop, has multiple isoforms resulting from three genes JNK1 (e.g. -p46), JNK2 (e.g. -p54), which are the most widely distributed and JNK3. This pathway was first identified as being activated as a cellular response to various environmental stressors such as UV radiation, and hypoxia. Subsequently, this pathway was also shown to be activated by soluble factors including EGF, TNFα, PDGF, TNFβ and agents such as lysophosphatidic acid (Zhang et al., 2005). The JNK MAP kinase pathway serves many functions in the inflammatory response, cellular differentiation and apoptosis. It has also been shown to play an essential role in cell migration (e.g. in fibroblasts) and cytoskeleton reorganization. The receptors and mechanisms transducing environmental stressors into the cell are not discussed further here, but likely converge on the same downstream proteins as those from soluble factors.

Signals from soluble factors are mediated via their respective receptors initially and link intracellularly to the small GTPases of the Rho family and adaptor proteins/non-receptor

tyrosine kinases such as FAK and Src (particularly in relation to outcomes leading to migration) (Almeida et al., 2000). The FAK-Src complex and Rac GTPase have been shown to activate MEKK1 and MLK (both MAP kinase kinase kinases). MEKK1 in turn phosphorylates MKK4 and MKK7 (also known as JNKK1 and JNKK2 which are MAP kinase kinases). These MAPKKs then phosphorylate the threonine and tyrosine residues in the activation loop of the MAP kinase JNK1/2.

Activated JNK localizes in the cytoplasm and nucleus and has functions at both sites. Cellular effectors activated (phosphorylated) by the JNK MAP kinase include c-Jun, RSK, paxillin, Spir [a member of the Wiscott-Aldritch syndrome protein (WASP) homology domain 2 (WH2) family that is involved in actin binding and reorganization (Otto et al., 2000)], DCX [a microtubule associated proteins (MAP) that stabilizes microtubules (Horesh et al., 1999)], and other MAPs.

Transcription/translation dependent pathways leading to keratinocyte migration include the Rho-ROCK1-MEKK1-JNK pathway and the MEKK1-p38. Interactions such as those with c-Jun and RSK ultimately mediate these responses. Transcription independent migration functions of JNK are often regulatory and frequently involve the formation of actin stress fibres, a characteristic of keratinocyte migration (Huang et al., 2003). Some interactions with c-Jun are important in this regard also (Javelaud et al., 2003). However, most often interactions linked to these events are with structural proteins such as paxillin or regulatory proteins such as Spir. JNK phosphorylates paxillin *in vitro* and *in vivo* at Ser-178 (Huang et al., 2003). As mentioned above, paxillin interacts in a complex manner with FAK and Src to influence focal adhesion turnover and cell migration. The present belief is that, in this case, JNK phosphorylation of paxillin contributes to degradation of focal adhesions (via subsequent effects on FAK) and facilitates migration (Huang et al., 2004b). JNK has also been suggested to alter actin dynamics via Spir activation, which would promote cell migration (Otto et al., 2000). p38 and JNK are often activated in parallel, but independent activation of each also has been observed (Raingeaud et al., 1995).

5.2.3 p38 MAP kinase signalling during migration

p38 MAP Kinase has a Thr-Ala-Tyr motif within the activation loop, and like the JNK MAP Kinase has multiple isoforms including; p38α, p38β, p38γ, and p38δ (also known as SAPK 2a, 2b, 3, and 4 respectively). The p38 MAP kinase isoforms appear to have distinct tissue

distributions: p38α and p38β are widely expressed while p38γ and p38δ have a more limited distribution (Jiang et al., 1996). Also, like the JNK MAP kinase, this pathway was first identified as being activated as a cellular response to various environmental stressors, only subsequently were soluble factors also shown to be able to elicit a response. The two pathways serve many of the same cellular functions (Ono and Han, 2000).

Signals from soluble factors are initially mediated via their respective receptors and link intracellularly to the small GTPases of the Rho family GTPases notably, Rac, and Cdc42 (Mackay and Hall, 1998) and the cytoplasmic kinase Pak1 (Bagrodia et al., 1995). The MAP kinase kinase kinases activated by this means include MLK3, DLK, and TAK1. These in turn phosphorylate the MAP kinase kinases MKK6 and MKK3 whose immediate downstream effectors are the p38 MAP kinases.

Several protein kinases, such as MAPK-activated protein kinase 2/3 (MAPKAPK2/3) (McLaughlin et al., 1996), caldesmon (Goncharova et al., 2002) and paxillin (Hubner et al., 1996) as well as several transcription factors are downstream effector targets of the p38 MAP kinases that are thought to be important in its functions during cellular migration. Much less is known regarding the underlying mechanism behind p38 MAP kinases influence on migration than those of the other MAP Kinase pathways. Activation of MAPKAPK2/3 by p38 and consequent phosphorylation of heat shock protein 27 (HSP27) is crucial for the migration of certain cells including endothelial cells and may be important for directionality of migration (Rousseau et al., 1997). p38 phosphorylates paxillin at Ser-83 *in vitro* and in some tumour cell lines (Huang et al., 2004a). The p38 pathway is also implicated in regulating focal adhesions, but it is unknown whether phosphorylation of paxillin by p38 is essential for cell migration.

5.3 Results: Demonstration of transgene expression following lentiviral transfection of keratinocytes

The dominant negative transgenes were delivered by means of lentiviral gene transduction system detailed in (Section 2.7.2). In addition to the GFP control of the transfection process (performed during the lentiviral transfection procedure for production of cells containing the required transgenes) Western blot analysis was performed to demonstrate that all of the intended transgenes were subsequently expressed. The dominant negative genes used in

this evaluation were p38α-Dn (derived from the p38α MAP kinase), MEK1-Dn (derived from the Erk MAP kinase kinase MEK1), and JNKK2-Dn (derived from the JNK MAP kinase kinase JNKK2). Comparable "wild-type" genes p38α-Wt, MEK1-Wt and JNKK2-Wt (that are unaltered and with normal function) were also transduced for use as controls within the migration assays. Cells were starved overnight in GF- media, trypsinized and prepared as if for a migration assay. Cells were re-suspended in GF- media and re-plated onto type I collagen at 40-50% confluence. After 30 minutes all cells were adherent and the GF- media was changed to GF+. After a further 10 minutes the cells were lysed, the lysates normalized by spectrophotometric protein assay and 50μg total protein was resolved on SDS-PAGE gel before blotting (Figure 26).

(Figure 26)

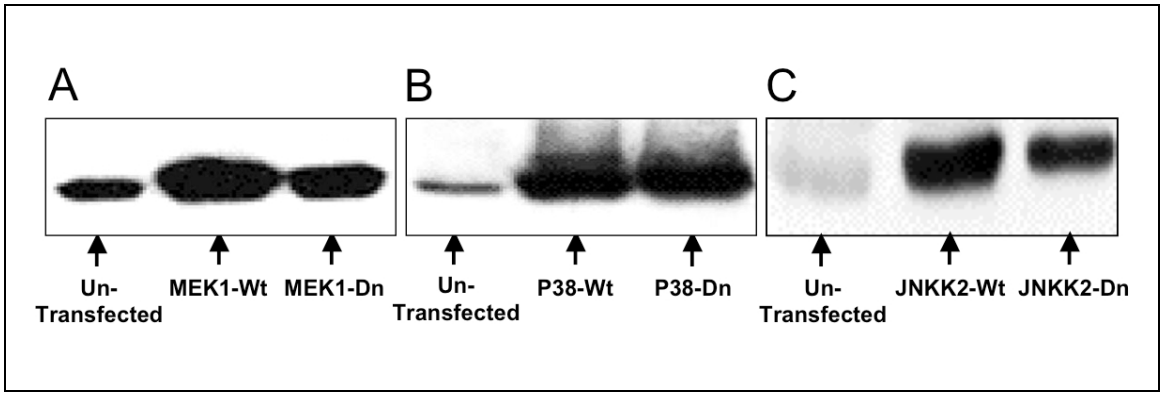


Figure 26: Western blots showing expression of the MEK1, p38, and JNKK2 gene products following transfection and in un-transfected keratinocytes. Using the lentiviral system of transgene delivery, keratinocytes were transfected with "wild-type" (-Wt) and dominant negative (-Dn) transgenes for elements of the MAP kinase pathway. A: MEK1 (an Erk MAP kinase kinase) blots are visualized with anti-MEK1 and show a 7.1-fold increase in MEK1-Wt and 6.1-fold increase in MEK1-Dn expression. B: p38α (the p38 MAP kinase) blots are visualized with anti-p38α and show 8.4-fold increase in p38-Wt and 8.3-fold increase in p38-Dn expression. C: JNKK2 (the JNK MAP kinase kinase) blots are visualized with anti-JNKK2 and show 8.2-fold increase in JNKK2-Wt and 5.9-fold increase JNKK2-Dn expression. In each case expression of the same gene products in un-transfected keratinocytes were used as control and reference to calculate increased expression.

These Western blots demonstrate over-expression (from between 5.9-8.4 fold increase above levels in un-transfected keratinocytes) for each of the transgenes. This finding was

confirmed on three repeated experiments and the increases were each statistically significant (at $p<0.05$).

5.4 Results: Basal expression of MAP kinase gene products in their phosphorylated and un-phosphorylated forms, evaluated by Western blot

Basal levels of expression of the MAP kinase gene products (Erk1/2, p38, and JNK) in their phosphorylated and un-phosphorylated forms were evaluated by Western blot. Demonstration of these levels provided useful reference information for interpretation of activity in the MAP kinase pathways under the conditions subsequently evaluated in the assays of keratinocyte migration on type I collagen and non-migratory poly-L lysine in the presence and absence of growth factors.

Cells were starved overnight in GF- media, trypsinized and prepared as if for a migration assay. The cells were re-suspended in GF- media and re-plated at 40-50% confluence onto either poly-L lysine (Poly) or type I collagen (Col I). Pairs of plates were prepared for each substrate to be used in the presence (GF+) and absence (GF-) of growth factor supplementation [4 pairs for each gene: Col I (GF+), Col I (GF-), Poly (GF+), Poly (GF-)]. After 30 minutes all cells were adherent and the GF- media was changed to GF+. One plate from each pair was taken immediately for Western blot and after a further 15 minutes the second plate of each pair was taken. In each case, the cells were lysed and the lysates normalized after spectrophotometric protein assay; 50μg total protein was resolved on SDS-PAGE gel before blotting (Figure 27).

(Figure 27)

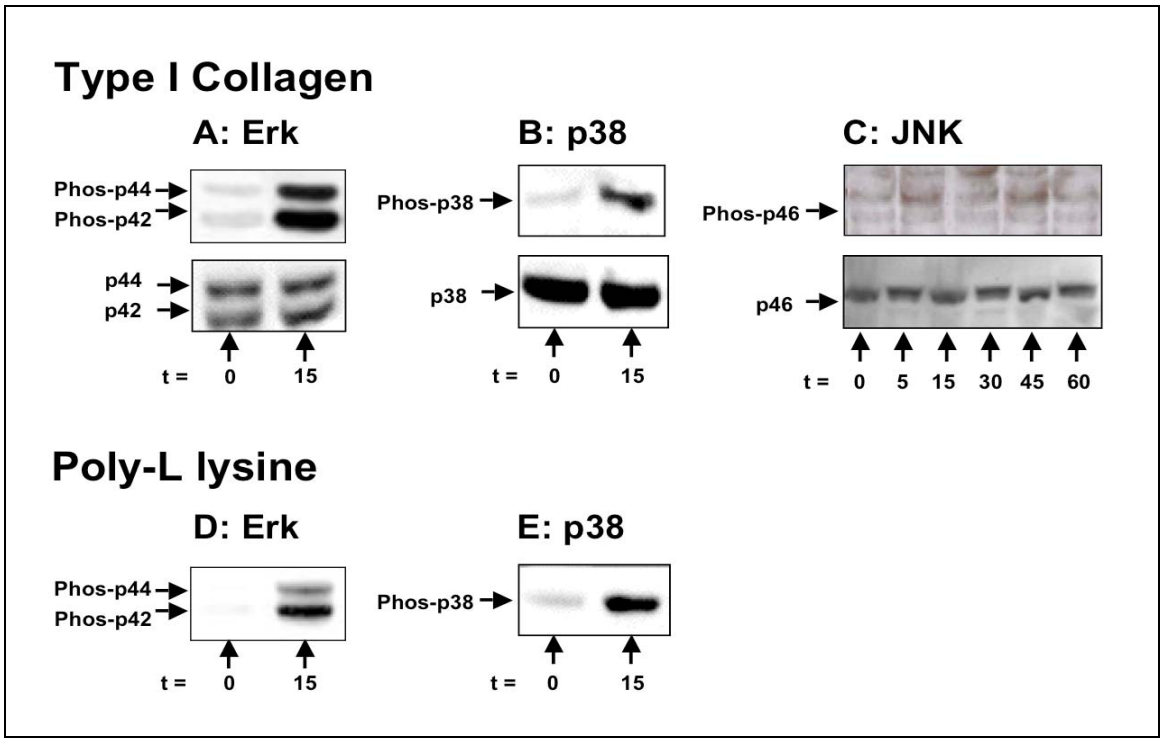


Figure 27: Western blot showing expression and activation of MAP kinase pathway gene products (Erk1/2, p38, and JNK) in keratinocytes on type I collagen (A, B and C) and poly-L lysine (D and E). A: Erk1/2 (Erk1=p44 and Erk2=p42) expression in keratinocytes on type I collagen prior to stimulation with growth factors (t=0) and following stimulation (t=15min). Lower images, at both times, show blots with antibody to un-phosphorylated forms, anti-Erk1/2. Upper images show phosphorylated forms visualized with anti-phospho-Erk1/2. B: p38 expression in keratinocytes, at the same times (t=0 and t=15min). The lower images show un-phosphorylated p38 (anti-p38) and upper images phosphorylated p38 (anti-phospho-p38). C: JNK expression. Lower images show un-phosphorylated JNK; upper images phosphorylated JNK. An extended time period for growth factor stimulation was used up to t=60 minutes. D: Erk1/2 expression in keratinocytes on poly-L lysine prior to stimulation with growth factors (t=0) and following (t=15min). Images of phosphorylated Erk1/2 only are shown (anti-phospho-Erk1/2). E: p38 expression the same times (t=0 and t=15min) images of phosphorylated p38 are shown (anti-phospho-p38).

For keratinocytes on type I collagen the Western blot for Erk1/2 (Figure 27: A) and p38 (Figure 27: B) show the presence of un-phosphorylated forms (Erk1/2: p44 and p42, p38: p38) that do not change with time (t=0 compared to t=15min, lower images). At t=0 low basal levels of the phosphorylated forms phospho-Erk1/2 (Phos-p44 and Phos-p42) and phospho-p38 are also seen (upper images t=0) and following growth factor stimulation for

15 minutes there are significant increases in these forms (upper images t=15min). There was a relative increase in intensity from t=0 of 6.7-fold for phospho Erk1/2 and a 4.5-fold increase for phospho-p38 (both comparisons were seen on repeated experiments and each showed similar findings). The low basal levels of phospho-Erk1/2 might reasonably be considered to represent activity in this pathway mediated by integrin-ECM stimulation (discussed in more detail later), while that seen for p38 probably reflects a response to physical stress (for example the stress of cell handling during the experiment and the tension generated by cellular of adhesion). The increase seen in phosphorylated forms following the addition of growth factors is due to stimulation in the MAP kinase pathways by those agents.

The Western blot for JNK (Figure 27: C) under the same conditions also showed the presence of un-phosphorylated JNK (p46) at a level that does not change with time (t=0 compared to t=60min, lower images). The corresponding phosphorylated form (Phos-p46) was undetectable at all times. The length of time for exposure to growth factors was increase to 60min to further explore the absence of detectable phosphorylated JNK. (The same results were demonstrated with keratinocytes in contact with poly-L lysine - data not shown).

For keratinocytes in contact with poly-L lysine, the expression of the phosphorylated forms of Erk1/2 and p38 were evaluated (Figure 27: D and E). Note: Detection of the un-phosphorylated form did not change with time (t=0 compared to t=15min) and this data is not shown. At t=0 there is no detectable phospho- Erk1/2 and low basal levels of the phospho-p38 (upper images t=0). Following growth factor stimulation for 15 minutes there was a significant increase in the level of the phosphorylated forms of both Erk1/2 and p38. A relative increase in intensity from t=0 of 6.0-fold for phospho-Erk1/2 and 4.6-fold for phospho-p38. This finding was confirmed in repeated experiments (three times).

Keratinocytes on poly-L lysine at t=0 showed negligible phosphorylated Erk1/2. The absence of phosphorylated Erk1/2 represents the absence of Erk MAP kinase pathway activity when there is no integrin-ECM stimulation, which is the case on poly-L lysine and when there is no stimulation by growth factors. At t=0 the level of phosphorylated p38 appeared to be the same as that seen on type I collagen and similarly might represent the same response in this pathway due to physical stress (for example the stress of cell handling

during the experiment and the tension generated by cellular of adhesion). The increase seen in phosphorylated forms of both Erk1/2 and p38 following the addition of growth factors are due to stimulation in the MAP kinase pathways by those agents.

5.5 Results: Confirmation that the JNK pathway was intact in keratinocytes and that the anti-phospho JNK antibody was functional

In view of the failure to detect phospho-JNK above, the following experiments were performed to demonstrate that the JNK pathway was intact and the anti-phospho JNK antibody functional. Western blot was performed on non-transfected keratinocytes prepared under the same conditions as for subsequent experimentation but following pre-exposure to Lipopolysaccharides (LPS) or TNFα. Of the three major MAP kinase cascades, TNFα has been reported to induce potent activation of the JNK pathway and to a much lesser extent the other SAPK pathway p38, while induced activation of the Erk pathway is minimal. The concentration of TNFα used was 50ng/ml and the exposure time 30 minutes. LPS are characteristic components of the cell wall of Gram-negative bacteria and are known to be potent stimulators of both of the SAPK pathways (JNK and p38) but not Erk MAP kinase pathway. The concentration of LPS used was 100ng/ml and the exposure time was 30 minutes. Cells were starved overnight in GF- media, trypsinized and prepared as if in preparation for a migration assay. They were re-suspended in GF- medium that had been supplemented with LPS or TNFα at the desired concentrations (see above). Cells were re-plated onto type I collagen at 40-50% confluence. After 30 minutes all cells were adherent and the medium was changed to GF+ again supplemented with TNFα or LPS at the same concentrations. After a further 15 minutes the medium was removed and the cells were lysed in preparation for Western blot. The lysates were normalized following spectrophotometric protein assay. 50μg total protein per lane was resolved on SDS-PAGE gel before blotting (Figure 28: A).

(Figure 28)

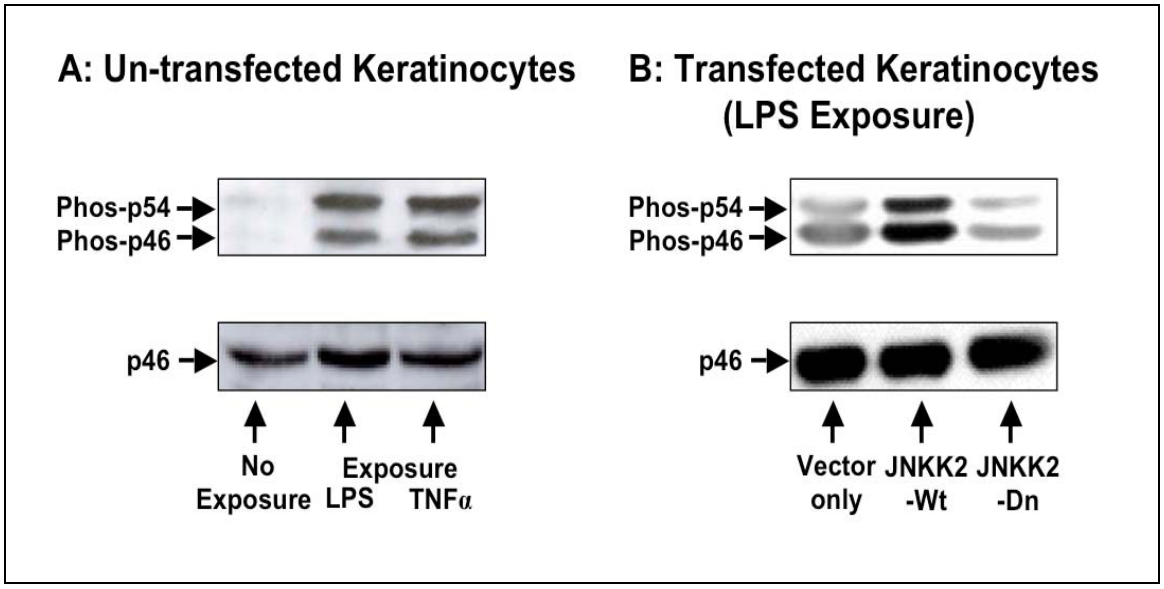


Figure 28: Western blot showing activity in the JNK MAP kinase pathway and JNKK2 dominant negative transgene function. A: Effects of LPS or TNFα exposure on expression of JNK [-p46 (-p54 not shown but displayed the same findings as -p46)] and phospho-JNK (-p46 and -p54) by un-transfected keratinocytes on type I collagen. Significant increase in levels of phosphorylated -p46 and -p54 were seen with exposure to both LPS and TNFα compared to the "No exposure" controls (≈ 7-fold increase). (Control blots show non-phosphorylated JNK (-p46) at t=15 minutes and demonstrate equal protein loading of samples). B: Effects of exposure to LPS on expression of non-phosphorylated JNK (-p46) gene product, and phosphorylated JNK (-p46 and -p54) gene products in keratinocytes transfected with Vector only, JNKK2-Wt or JNKK2-Dn on type I collagen. (Control blots show non-phosphorylated JNK (-p46) at t=15 minutes and demonstrate equal protein loading of samples). The gene products in A and B were visualized with anti-JNK and anti-phospho-JNK antibodies.

Following exposure of un-transfected keratinocytes to both LPS and TNFα, significant increases of the phosphorylated forms of JNK (phospho-p46 and phospho-p54) were seen compared to no-exposure controls (≈ 7-fold increase) demonstrating that the anti-phospho JNK antibody was functional and that the JNK pathway was intact in those cells. (Control blots show non-phosphorylated JNK (p46) at t=15 minutes and demonstrate equal protein loading of samples).

5.6 Results: Demonstration that the dominant negative (kinase inactive) JNKK2 was functioning as an inhibitor of downstream activity in the JNK MAP kinase pathway

LPS stimulation was used to demonstrate that the dominant negative (kinase inactive) JNKK2 was indeed functioning as an inhibitor of downstream activity in the JNK pathway. A Western blot was performed on cells transfected with vector only, JNKK2-Wt and JNKK2-Dn, following exposure to LPS, in each case under the same conditions as described in Section 5.5.

The JNKK2 dominant negative gene product had the appropriate inhibitory effect downstream in the JNK MAP kinase pathway. In keratinocytes transfected with the vector alone or the JNKK2-Wt transgene, responses of the JNK MAP kinase pathway to LPS stimulation were not impaired, as would be expected. In both cases (Vector only and JNKK2-Wt transfected cells) following LPS stimulation there was significant expression of phospho-JNK (-p46 and -p54) (Figure 28: B left and middle lanes). Although absolute (fold) comparisons are not possible this can be confirmed by comparison to un-transfected cells without LPS exposure (Figure 28: A left hand lane) and following LPS exposure (Figure 28: A middle lane). Keratinocytes transfected with JNKK2-Wt compared to those transfected with Vector only following LPS exposure showed a 3.6-fold higher level of phosphorylated JNK expression (demonstrating increased downstream activity in the JNK MAP kinase pathway beyond JNKK2). Keratinocytes expressing JNKK2-Dn (that is kinase inactive) did show expression of phospho-JNK (-p46 and -p54) (Figure 28: B right hand lane). However this was less (a 3.1-fold reduction) than that seen in those cells transfected with Vector only. This demonstrates a reduced production of the anticipated substrate of JNKK2 (that is phosphorylated JNK) in cells transfected with the dominant negative transgene JNKK2-Dn, as would be anticipated.

5.7 Inhibition of elements of the MAP kinase pathways (Erk, p38 and JNK) during human keratinocyte migration on type I collagen by transduction of dominant-negative genes and application of chemical inhibitors

During the migration of many cell types, the Erk pathway is consistently activated by soluble factors such as EGF, TGFα and HB-EGF mediated by binding to the EGF receptor (and by KGF binding to the FGF receptor 2) (Figure 25: B). Ligand binding activates Ras-Raf interactions that lead to MEK1/2 activation and ultimately to activation of the Erk MAP

kinase. Furthermore many of the effectors of this pathway are known mediators of migration (MLCK, FAK, paxillin, and calpain-2) and share many direct links to focal adhesions and the cytoskeleton. The functional role of the Erk pathway can be demonstrated by its inhibition with dominant negative genes or pharmacological agents.

The SAPK pathways JNK and p38, considered as mediators of the cellular response to stress, also share links to many of the same effectors. Most data relating to these pathways in migration demonstrate effects that are dependent upon the transcription of genes and the subsequent new protein synthesis that follows (e.g. keratinocyte migration pathways such as the Rho-ROCK1-MEKK1-JNK pathway and the MEKK1-p38 pathway). Accepting known links to migration, it may be anticipated that activation of the JNK and p38 MAP Kinase pathways will be detectable during migration of keratinocytes. Similarly to the Erk MAP kinase pathway, their functional importance can be determined by investigating whether inhibition of the pathways results in detectable changes in migration. However, assays used here both evaluate cellular migration over a much shorter timeframe that would be needed for the effects of gene transcription and protein synthesis to be seen. It has also been shown that soluble factors activating the Erk MAP kinase pathway can in some case activate both the JNK and p38 MAP kinase pathways downstream. In some cells this more immediate response (not dependent on transcription) might link activity in these pathways to migration. Downstream effects of such interactions include alterations in the formation of actin stress fibres (Huang et al., 2003). The JNK pathway activated in this manner has been linked to altered actin dynamics by activation of Spir, and this could promote keratinocyte migration (Otto et al., 2000). The p38 pathway activated in this way has been linked to the activation of MAPKAPK2/3 and subsequent phosphorylation of heat shock protein 27 (HSP27); this too might be an important and more immediate influence on migration. It is worth noting p38 and JNK are often activated in parallel, but independent activation of each has also been observed (Raingeaud et al., 1995). While soluble growth factors are not the primary means of activation of the two SAPK pathways nonetheless it is possible that the SAPK pathways may be active during keratinocyte migration, but their effects may or may not detectably alter the migration measured by the two assays used here.

In order to begin to understand the relationship between activities in these pathways during keratinocyte migration the simplest initial questions to be answered were which if any of the three well-characterized MAP kinase pathways are active during keratinocyte migration

and, if active, which pathways appear to be necessary. The presence or absence of activity in each pathway was investigated in keratinocytes migrating on pro-migratory type I collagen and non-migratory poly-L lysine, in the presence and absence of soluble growth factors. In addition under the same conditions the perturbing effects of the introduction of selected dominant negative genes and the application of chemical inhibitors (both chosen for inhibition of elements of the MAP kinase pathways) on keratinocyte migration and activity in the pathways was examined.

5.7.1 Results: Dominant negative inhibition of elements of the MAP kinase pathways during human keratinocyte migration

The dominant negative transgenes were delivered by means of a lentiviral gene transduction system detailed in Section 2.7.2. GFP gene transduction was performed as the procedural control and successful transduction, incorporation into the host genome, and subsequent expression of a transgene, was also confirmed by Western blot shown previously (Figure 26). The dominant negative genes used in this evaluation were p38α-Dn (derived from the p38α MAP kinase), MEK1-Dn (derived from the Erk MAP kinase kinase MEK1), and JNKK2-Dn (derived from the JNK MAP kinase kinase JNKK2) [details of the relevant source and activity of each transgene was provided in Section 2.7.2.1 and are summarized in (Table 3)]. Comparable "wild-type" genes p38α-Wt, MEK1-Wt and JNKK2-Wt (that are unaltered and with normal function) were also transduced for use as controls within the migration assays. Typically, four sets of cultured keratinocytes were prepared for each gene to be studied. Three sets were transfected respectively with, the lentivirus containing the vector pRRLsin alone (no transgene inserted), lentivirus containing a functional form of the gene ("wild-type"), and lentivirus containing the inactivated form of the same gene ("dominant negative"). The fourth set of keratinocytes was un-transduced. By studying both keratinocytes containing transduced genes and un-transduced keratinocytes it was hoped that some of the effects of the altered stoichiometry would be minimized. Potential cytotoxicity caused by gene over-expression was assessed by Trypan blue exclusion assay and cell proliferation studies. These assays were performed following completion of the gene transduction process in a subculture of the transfected keratinocytes that were prepared and handled identically to those undergoing the migration and wound assays. By either assessment, cytotoxicity did not occur following any of these gene transductions. In preparation for the assays, cells were starved overnight in GF- media, trypsinized and re-suspended in either GF- or GF+ media according to the protocol. They were then re-plated

(3000 viable cells/well) in each well of 12 well cell culture plates prepared with the two substrates; non-migratory poly-L lysine and pro-migratory type I collagen at concentrations shown in (Table 1). Migration was allowed to proceed for 16-hours.

The results of the colloidal gold migration assay are shown in (Figure 29). The results of identically prepared experiments repeated under the same conditions using the *in vitro* wound migration assay are shown in (Figure 30). (During the *in vitro* wound migration assay there was greater variability, but otherwise the results of the two experimental assays were in agreement). Note: In both Figures 29 and 30, un-transfected keratinocytes migrating on poly-L lysine were used as a negative control.

(Figure 29)

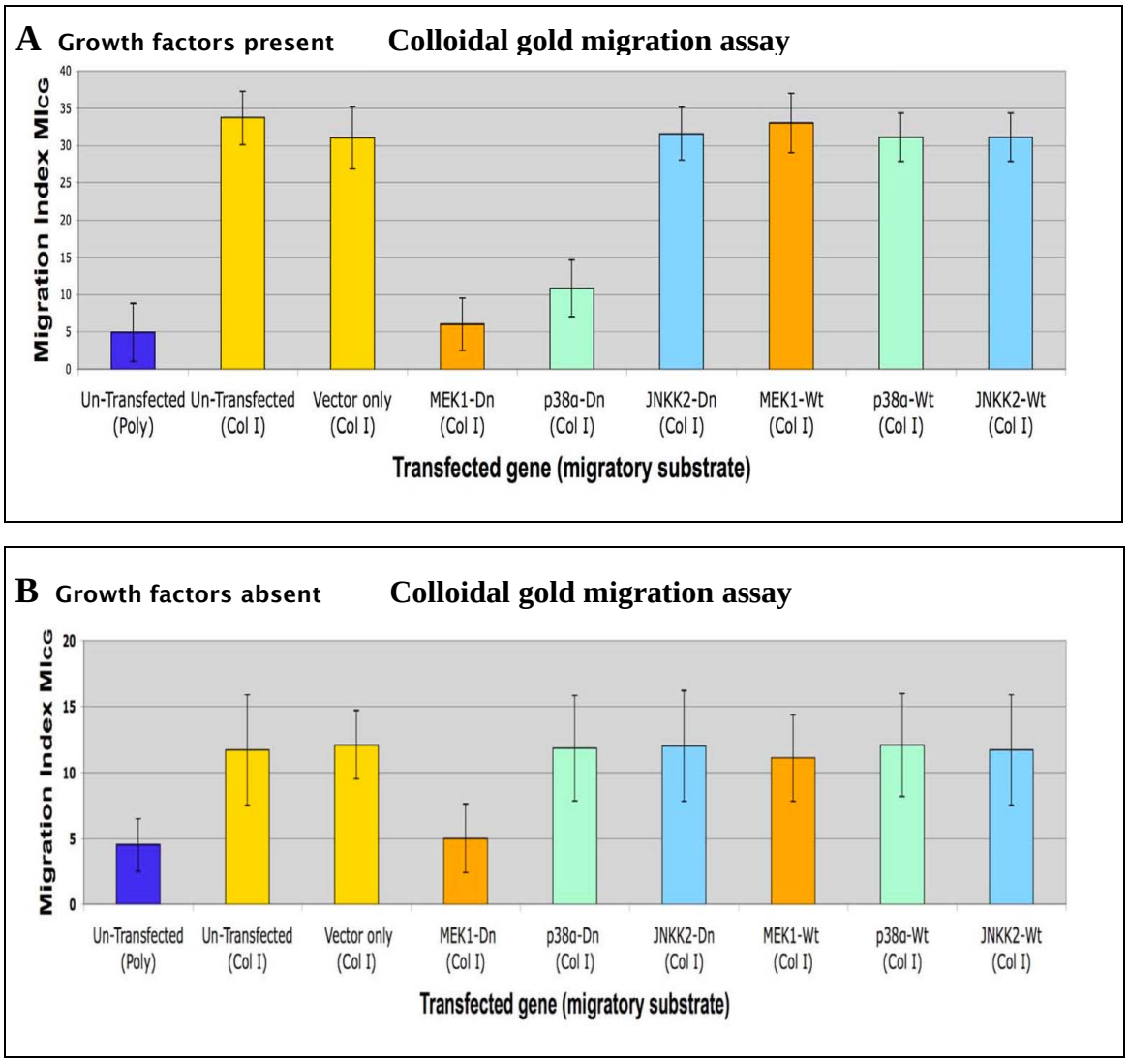


Figure 29: Colloidal gold migration assay of keratinocyte migration on type I collagen (Col I) and non-migratory poly-L lysine (Poly) following transfection with selected MAP kinase transgenes:

"wild-type" (-Wt) and dominant negative (-Dn) forms. A: Migration indices (MI_{CG}) for keratinocytes migrating in the presence of growth factors. B: Migration indices (MI_{CG}) for keratinocytes migrating in the absence of growth factors. In both cases keratinocyte migration indices are shown for cells with no transfected gene inserted (Un-transfected), following insertion of the pRRLsin vector (Vector only), as well as following transfection of the "wild-type" functional genes and non-functional dominant negative form of the genes. Transgenes p38α, MEK1, and JNKK2, from each of the three MAP kinase pathways p38α, Erk and JNK (respectively) were studied.

(Figure 30)

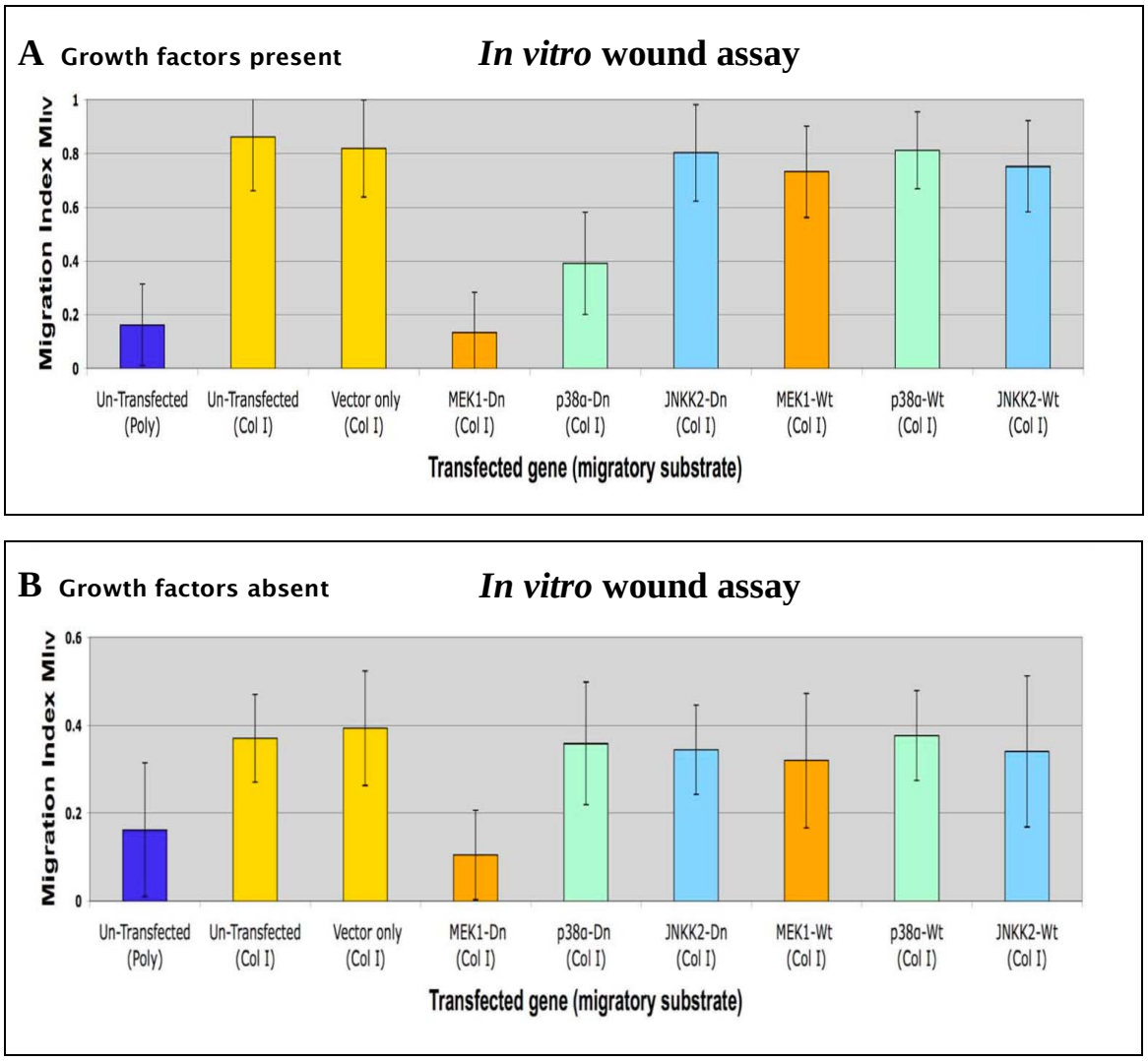


Figure 30: *In vitro* wound assay of keratinocyte migration on type I collagen (Col I) and non-migratory poly-L lysine (Poly) following transfection with selected MAP kinase transgenes, "wild-type" (-Wt) and dominant negative (-Dn) forms. A: Shows migration indices (MI_{IV}) for keratinocytes migrating in the presence of growth factors. B: Shows migration indices (MI_{IV}) for

keratinocytes migrating in the absence of growth factors. In both cases keratinocyte migration indices are shown for cells with no transfected gene inserted (Un-transfected), following insertion of the pRRLsin vector (Vector only), as well as following transfection of the "wild-type" functional genes and non-functional dominant negative form of the genes. Transgenes p38α, MEK1, and JNKK2, from each of the three MAP kinase pathways p38α, Erk and JNK (respectively) were studied.

In these experiments keratinocytes transduced with the vector pRRLsin (Vector only) and the un-transfected keratinocytes (Un-Transfected) migrated on type I collagen at the previously observed level of migration in both assays, and the MI's observed were not statistically significantly different from that seen previously. This was true in both the presence (Figure 29: A. MI_{CG}≈31-34 and Figure 30: A. MI_{IV}≈0.78-0.90) and absence (Figure 29: B. MI_{CG}≈10.5-13.0 and Figure 30: B. MI_{IV}≈0.34-0.39) of growth factors ($p<0.05$). Appreciable migration on poly-L lysine did not occur (MI_{CG}≈4-5 and MI_{IV}≈0.15-0.18 with or without growth factors), which was expected, and the level of migration observed was statistically different from that seen during the migration of keratinocytes transfected with both the vector alone and the un-transfected keratinocytes under otherwise similar conditions ($p<0.05$ for each comparison). Migration of all the "wild-type" gene-transduced cells (MEK1-Wt, p38-Wt, and JNKK2-Wt) was indistinguishable from that observed with keratinocytes transduced with the vector pRRLsin only and the un-transfected cells in both migration assays and presence and absence of growth factor (again $p>0.05$ for each comparison). However, migration of keratinocytes transduced with dominant negative genes (MEK1-Dn, p38-Dn, and JNKK2-Dn) was different in each case.

As might be expected from the literature, the inhibition of the Erk pathway by transduction with the MEK1 dominant negative (MEK1-Dn) had a significant effect on keratinocyte migration in the presence of growth factors. Surprisingly, this also occurred in their absence. In both cases with the Erk pathway inhibited (keratinocytes transfected with MEK1-Dn) keratinocyte migration was essentially blocked (MI_{CG}≈5-6 and MI_{IV}≈0.14-0.16) and these cells in the presence and absence of growth factors migrated no differently than those attached to the non-migratory poly-L lysine, ($p>0.05$ for each comparison).

We have already demonstrated (Chapter 4) that keratinocytes in the presence of a pro-migratory substrate such as type I collagen migrate in the presence and absence of growth factors in both the colloidal gold migration and *in vitro* wound migration assays. In the absence of growth factors migration was shown to occur at a low level, below that seen in the presence of growth factors but greater than that seen on a non-migratory substrate (poly-L lysine and laminin-1). The presence of growth factors significantly enhances keratinocyte migration on the pro-migratory substrate type I collagen in these same experimental systems. This result suggests that in the presence of Erk MAP kinase inhibition, keratinocytes contact with a pro-migratory substrate is no longer sufficient to produce migration with or without growth factor stimulation. It appears that in keratinocytes, functional Erk MAP kinase pathway activity (at least below the level of the MAP kinase kinase MEK) is necessary for migration. Also, it is interesting and seems reasonable to interpret these results as inhibition of the Erk MAP kinase pathway eliminates the contribution to migration from the interaction with the pro-migratory substrate (integrin-ECM stimulated) as well as from soluble growth factors (EGFR-GF stimulated) in these two migration assays.

Notably, and not previously reported during keratinocyte migration on type I collagen, inhibition of the p38 MAP kinase pathway also had a significant effect on migration. In this case however, the effect was only apparent in the presence of soluble growth factors, under these conditions migration was inhibited ($p<0.05$, compared to all controls, un-transfected, and cells transfected with vector only and with "wild-type" p38α-Wt). The level of migration observed in the keratinocytes transfected with dominant negative p38α was the same in the presence and absence of growth factors ($MI_{CG}\approx10.5\text{-}12$ and $MI_{IV}\approx0.38\text{-}0.4$) ($p>0.05$). In addition, in the presence and absence of growth factors, migration of the p38α-Dn keratinocytes was the same as that shown by unmodified keratinocytes and other controls (cells transfected with vector only and with "wild-type" p38α-Wt) migrating on type I collagen the absence of growth factors ($MI_{CG}\approx10.5\text{-}13.0$ and $MI_{IV}\approx0.34\text{-}0.39$) ($p>0.05$).

Possible downstream links between the p38 pathway and migration in a number of cells have been demonstrated by others and was summarized in section 5.2.3. This result suggests that this pathway may be involved (directly or indirectly) in keratinocyte migration. Here, in the presence of p38 MAP kinase inhibition, growth factors are no

longer able to augment keratinocyte migration on a pro migratory substrate, type I collagen. The elimination of this effect suggests an intact p38 MAP kinase pathway (at least below the level of the MAP kinase p38α) is necessary for growth factor-mediated augmentation of keratinocyte migration. Moreover, it is interesting that inhibition of the p38 MAP kinase pathway appears to eliminate only the contribution to migration from soluble growth factors (e.g. EGFR-GF stimulated), but has no effect on that from the interaction with the pro-migratory substrate (integrin-ECM stimulated) in these two migration assays.

Finally, and also not previously reported during keratinocyte migration, inhibition of the JNK/MAP kinase pathway had no significant effect on migration that could be detected by these assays. In both assays under all conditions the migration indices recorded for keratinocytes transfected with dominant negative JNKK2-Dn were the same as all pro-migratory controls (un-transfected, vector only and JNKK2-Wt cells). This result suggests that inhibition of the JNK MAP kinase pathway (at least at the level of the MAP kinase kinase JNKK2) does not influence keratinocyte migration on type I collagen (neither affecting the interaction with the pro-migratory substrate or downstream effects from soluble growth factors). Note: It was demonstrated above the JNKK2 "wild-type" and dominant negative transgenes were expressed and the JNK pathway was intact (and the anti-phospho JNK antibody functional).

5.7.1.1 Results: Demonstration that the dominant negative MEK1 transgene was functioning as an inhibitor of downstream activity in the Erk MAP kinase pathway

The downstream effects of over-expression of the dominant-negative gene product MEK1-Dn in the Erk/MAP Kinase pathway were visualized by Western blot. Cells [keratinocytes transfected with pRRLsin (Vector only), "wild-type" transgene (MEK1-Wt), and the dominant-negative transgene (MEK1-Dn)] were starved overnight in GF- medium, trypsinized and prepared otherwise as if for a migration assay. They were re-suspended in GF- medium and re-plated onto type I collagen at 40-50% confluence. After 30 minutes all cells were adherent and the GF- medium was changed to GF+ (except for one control group of cells transfected with Vector only that were maintained in GF- medium). After a further 15 minutes the medium was removed, the cells were lysed in preparation for Western blot and the lysates were normalized following spectrophotometric protein assay. 50μg total protein per lane was resolved on SDS-PAGE gel before blotting with anti Erk and anti-phospho-Erk (Figure 31).

(Figure 31)

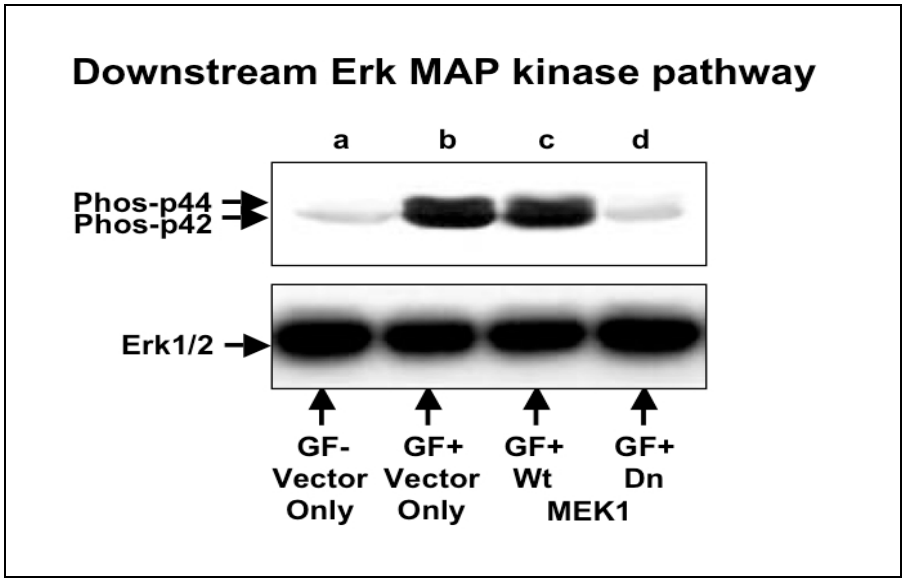


Figure 31: Western blot showing activity in the Erk MAP kinase pathway and expression of the MEK1 dominant negative transgene. Lower panel shows expression of non-phosphorylated Erk at t=15 minutes (and demonstrates equal protein loading of samples). Upper panel shows expression of phospho-Erk1/2 (Erk1-p44 and Erk2-p42). (Lysates visualized with anti-Erk1/2 and anti-phospho-Erk1/2 antibodies). All cells were plated on to type I collagen, and differences in experimental conditions are represented as; (a) Keratinocytes transfected with Vector only in GF- medium, (b) Keratinocytes transfected with Vector only in GF+ medium, (c) Keratinocytes transfected with MEK1 "wild-type" gene MEK1-Wt in GF+ medium (d) Keratinocytes transfected with MEK1 dominant negative gene MEK1-Dn in GF+ medium. The in absence of growth factor keratinocytes show minimal phosphorylated Erk1/2 (upper panel column a). In comparison keratinocytes transfected with Vector only (upper panel column b) and equally MEK1-Wt (upper panel column c) in the presence of growth factors show significantly increased phosphorylated Erk1/2 (both 4.1-fold increases). Keratinocytes transfected with MEK1-Dn (upper panel column d) show a low level of phosphorylated Erk1/2, a level not significantly different to that seen in keratinocytes in the absence of growth factor.

These results show that in the presence of over expression of the MEK1 dominant-negative gene, MEK1-Dn the downstream Erk MAP kinase is inhibited to a level of activity similar to that seem in the absence of growth factor. Over expression of the "wild-type" MEK1 (MEK1-Wt, the MAP kinase kinase) has no effect on the downstream activity in this pathway at the level of the MAP kinase Erk.

5.7.2 Results: Chemical inhibition of elements of the MAP kinase pathways during human keratinocyte migration

Inhibition of the MAP kinase pathways with dominant negative transfections above showed their involvement in human keratinocytes migrating on type I collagen, each pathway appearing to have a different effect. To confirm these findings and the relationships of such inhibition in each MAP kinase pathway, similar experiments were performed using an alternative technique, chemical inhibitors. This second approach was performed to provide complementary and corroborative data on the effects identified. Chemical inhibitors are cell-permeable compounds developed to study the actions of protein kinases such as those involved in the MAP kinase pathways. Inhibitors have several advantages for the study of cell signalling. They can be used simply and rapidly to assess the physiological roles of protein kinases, in unaltered cells as well as in transformed cell lines. These agents can inhibit the intended endogenous protein kinase without the need for over expression of transgenes (dominant-negatives), which can, by altered stoichiometry, cause the specificity of signalling to break down and lead to erroneous conclusions. However, most protein kinases have multiple targets and are activated by multiple means. Uniquely inhibiting the interaction of one kinase and one target is usually not possible with these agents. Even the most selective inhibitors affect at least one additional protein kinase in addition to that for which it was intended.

The choice of inhibitors was intended to match the dominant negative transgenes. U0126 is an inhibitor of MEK1/2 and SB202190 is an inhibitor of p38. However, no suitable inhibitor of JNKK2 (the MAP kinase kinase of the JNK/MAP kinase cascade) was commercially available. Therefore, SP600125, an inhibitor of JNK itself was chosen. Amongst the chemical inhibitors used, U0126 and SB202190 are known to be highly specific and the results of the two assays with these agents can be interpreted accordingly (Davies et al., 2000). However, SP600125 has been shown to be only a weak inhibitor of the JNK pathway and is also poorly specific, but was the best of the available choices at that time (Bain et al., 2003). It is accepted that interpretation of results from experiments with this inhibitor must consider these points. A summary of the properties of the inhibitors used is given here and provided in (Table 2).

The chemical inhibitor U0126 is reported to inhibit MEK1/2 (Favata et al., 1998), and has been widely used to investigate activation of the classical MAP kinase cascade in a number

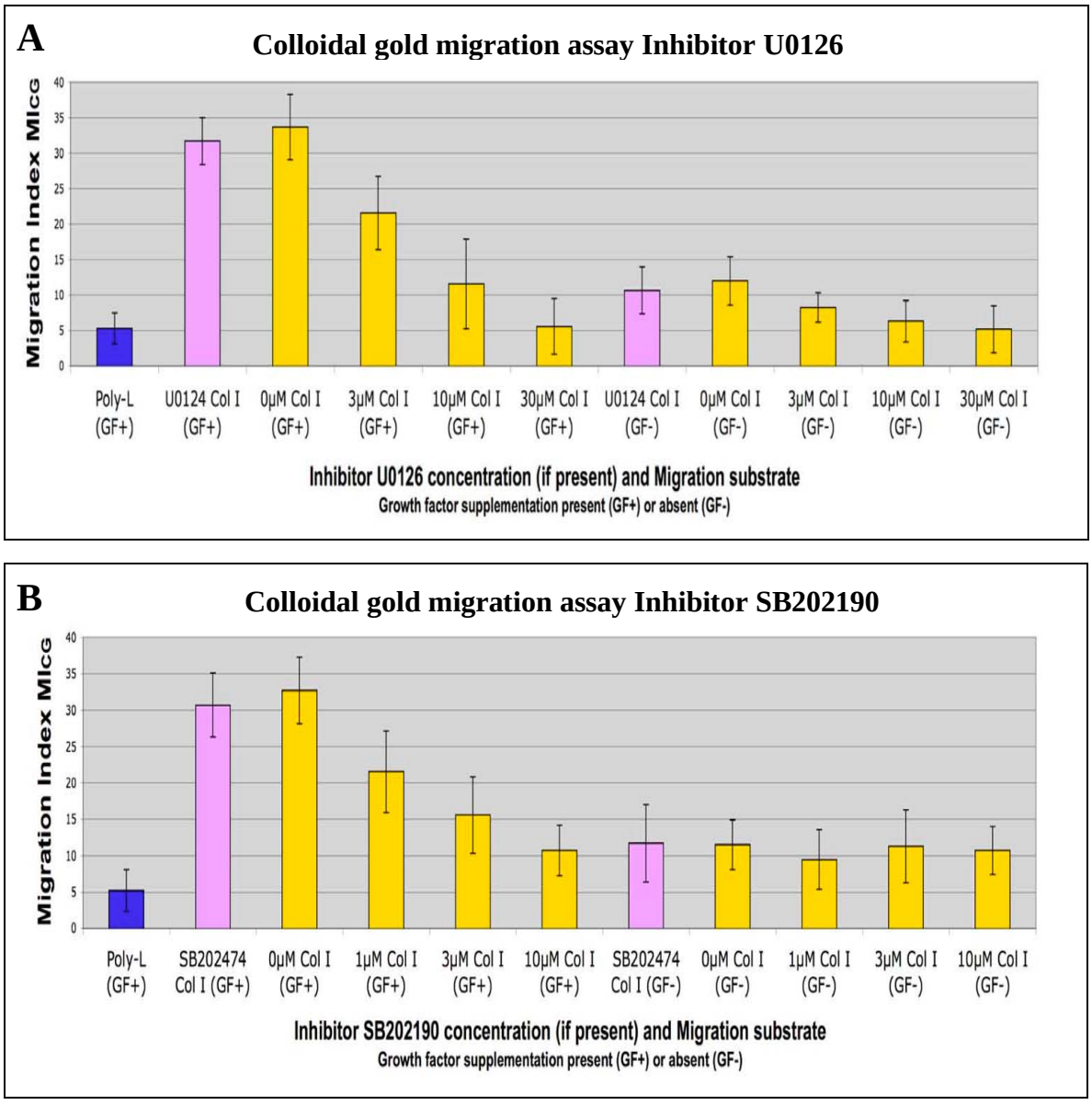
of cells (DeSilva et al., 1999). (The associated control was U0124). U0126 blocks the MAP kinase cascade by preventing the activation of the MAPK kinase MEK1/2, by Raf, (and not by inhibiting MEK1/2 activity directly). U0126 binds to MEK1/2 and not to Raf, so that other actions of Raf still occur e.g. U0126 does not inhibit the phosphorylation of myelin basic protein by Raf (DeSilva et al., 1999). U0126 was considered an excellent analogue to the dominant negative transgene MEK1-Dn. The chemical inhibitor SB202190 is reported to inhibit all p38 isoforms and has been widely used to suppress activation of the p38 MAP kinase cascade in a number of cell types (Eyers et al., 1998). (The associated control was SB202474). SB202190 prevents activation of p38 by directly binding to the kinase at the ATP-binding pocket of the enzyme, preventing its activity. The chemical inhibitor SP600125 was developed as an inhibitor of JNK and remains one of few such agents with specificity for kinases of this pathway. However, SP600125 is a relatively weak inhibitor of JNK isoforms (1/2) (Bennett et al., 2001). (An associated control was provided by the manufacture but is un-named). The isoforms of Erk and p38 MAP kinase and members of those cascades are inhibited minimally by SP600125. In addition, many other protein kinases are inhibited with similar or greater potency by SP600125 at the concentrations used here to inhibit JNK. SP600125 cannot be considered specific in its action (Bain et al., 2003). Potential cytotoxicity caused by any of the inhibitors was assessed by Trypan blue exclusion assays, and cell proliferation studies. These assays were performed under conditions reproducing those used in the colloidal gold migration assay and *in vitro* wound migration assay at the same concentrations subsequently investigated. Cytotoxicity by either assessment did not occur for any of the chemical inhibitors U0126, SB202190 or SP600125 at any of the concentrations used.

In preparation for the migration assays, keratinocytes were starved overnight in GF- media, trypsinized and re-suspended in either GF- or GF+ media with and without supplementation with each of the chemical inhibitors or their respective controls. Keratinocytes were then incubated for 30 minutes before being re-plated (3000 viable cells/well) into each well of 12 well cell culture plates. The plates were pre-coated with poly-L lysine (non-supportive of migration) and type I collagen (pro-migratory) at deposition concentrations listed in Table 1. Migration was continued for 16-hours.

The results of the colloidal gold migration assay with each chemical inhibitor (U0126, SB202190, and SP600125) and control are shown (Figure 32). Identical experiments were

repeated (three times) under the same conditions with the *in vitro* wound migration assay and the results of the two experimental assays were in complete agreement (results are not shown).

(Figure 32)



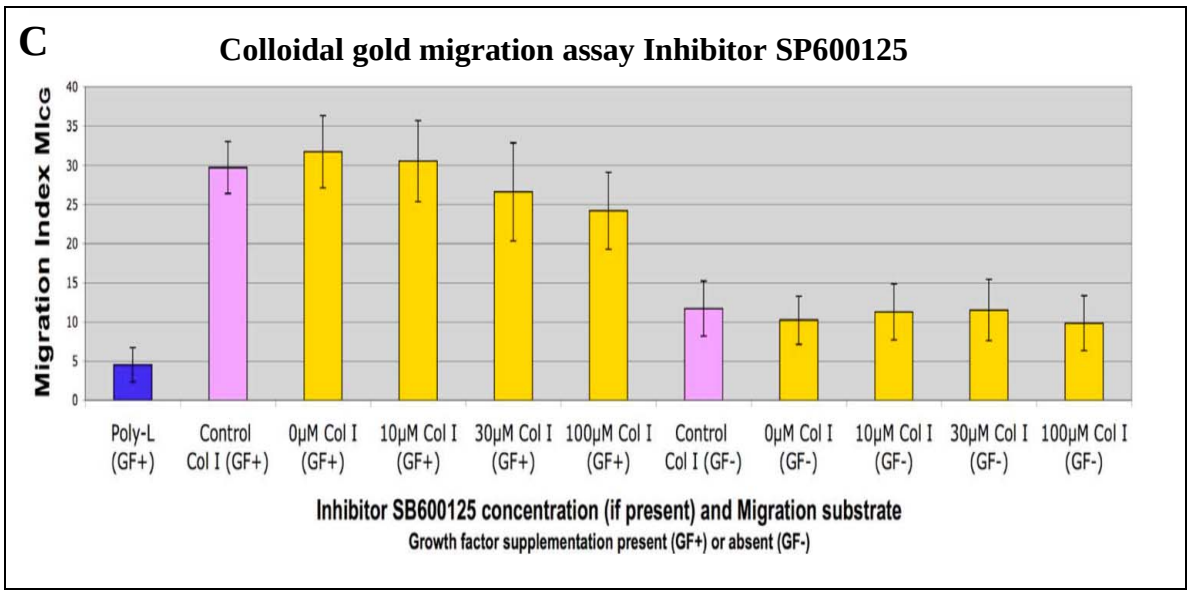


Figure 32: Chemical inhibition of key elements of the MAP kinase pathways during human keratinocyte migration on type I collagen (Col I) and non-migratory poly-L lysine (Poly-L) in the presence (GF+) and absence (GF-) of soluble growth factors. A: Results of the colloidal gold migration assay in the presence of chemical inhibitor U0126 (an inhibitor of MEK1/2) present during exposure at concentrations 0, 3, 10, and 30μM. (Results for exposure to the control agent U0124 are also shown, pink column). B: Results of the colloidal gold migration assay in the presence of chemical inhibitor SB202190 (an inhibitor of p38) present during exposure at concentrations 0, 1, 3, and 10μM. (Results for exposure to the control agent SB202474 are also shown, pink column). C: Results of the colloidal gold migration assay in the presence of chemical inhibitor SP600125 (an inhibitor of JNK) present during exposure at concentrations 0, 10, 30, and 100μM. (Results for exposure to the control agent un-named, are also shown, pink column). A further control, migration on poly-L lysine in the presence of growth factor and absence of exposure to the inhibitors is also shown. Note migration on poly-L lysine in the absence of growth factors (and absence of inhibitors) was performed and was not significantly different ($p>0.05$) from that obtained in the presence of growth factors (and absence of inhibitors), this data is not shown.

In the presence of growth factors, keratinocytes migrating on type I collagen in the presence of each control (U0124, SB202474, and un-named control of SP600125, respectively) showed MI_{CG} (and MI_{IV}), consistent with previously obtained vales (MI_{CG} ≈30-34 and MI_{IV}≈0.8-0.9). In all cases results were not significantly different from each other but were significantly different from migration seen on the non-migratory control substrate poly-L lysine (Poly-L) under the same conditions ($p<0.05$). In the absence of growth factors, keratinocyte migration on type I collagen in the presence of each control showed MI_{CG} (and

MI_{IV}) consistent with previously obtained vales (MI_{CG} ≈10-12.5 and MI_{IV}≈0.34-0.39) and in all cases results were not significantly different from each other, but were significantly different from migration seen on the non-migratory control substrate poly-L lysine under the same conditions (data is not shown for poly-L lysine migration in the absence of growth factors) ($p<0.05$).

Keratinocyte migration on type I collagen, in the presence of growth factor, showed a clear, dose-dependent inhibition by U0126 (MEK1/2) and SB202190 (p38). In the case of U0126 migration indices were reduced (at the highest concentration used 30μM) to the level seen on non-migratory poly-L lysine in the presence of growth factors. In the case SB202190 migration indices were reduced (at the highest concentration used 10μM) to that for keratinocytes migrating on type I collagen in the absence of growth factors. Note higher concentrations (>10μM) of SB202190 were evaluated and produced no further reduction in migration indices up to (100μM) data not shown. At high concentrations (>30μM) of SP600125 a reduction in migration index was also seen suggesting the same dose-dependent inhibition. However, as mentioned, SP600125 has been shown to be only a weak inhibitor of the JNK pathway and is also poorly specific (Bain et al., 2003). At such doses, other protein kinases in addition to JNK were also likely inhibited with this agent, making unequivocal interpretation difficult (discussed further below).

Keratinocyte migration on type I collagen in the absence of growth factors showed dose-dependent inhibition only by U0126 (MEK1/2). In this case migration indices were reduced (at the highest concentration used 30μM) to the level seen on non-migratory poly-L lysine in the absence of growth factors. In the absence of growth factors, SB202190 and SP600125 had no detectable effect on migration ($p>0.05$ for all comparison at all concentrations of inhibitor present). These results, with the provisos mentioned, reflect the same findings as those determined following dominant negative transfection.

5.7.2.1 Results: Demonstration of the causal relationship between effects of the chemical inhibitor U0126 (of MEK1/2 in the Erk MAP kinase pathway) and the decrease in keratinocyte migration

The Erk1/2 MAP kinases are the targets of the MEK1/2 MAP kinase kinases, and inhibition by U0126 (a MEK1/2 inhibitor) would be expected to result in dose-dependent reduction in activated phospho-Erk1/2. To demonstrate the possible causal relationship between the

downstream effects of U0126 and the dose-dependent decrease in keratinocyte migration on type I collagen, Western blot was performed to evaluate the levels of Erk1/2 and phosphor-Erk1/2 in keratinocytes on type I collagen at each concentration of inhibitor. Cells were starved overnight in GF- media, trypsinized and prepared as if for a migration assay. They were re-suspended in GF- media supplemented with the inhibitor U0126 at various concentrations (0μM, 3μM, 10μM, and 30μM). Cells were re-plated in these media onto type I collagen, at 40-50% confluence. By 30 minutes all cells were adherent and the medium was changed to GF+ (in the continued presence of the same concentrations of the inhibitor U0126; 0μM, 3μM, 10μM, and 30μM). A control assay was also prepared in which keratinocytes were plated onto type I collagen in the absence of growth factors (GF-) and with no inhibitor present (0μM). After a further 15 minutes the cells were lysed, and the lysates normalized after spectrophotometric protein assay, and 50μg total protein per lane was resolved on SDS-PAGE gel before blotting (Figure 33).

(Figure 33)

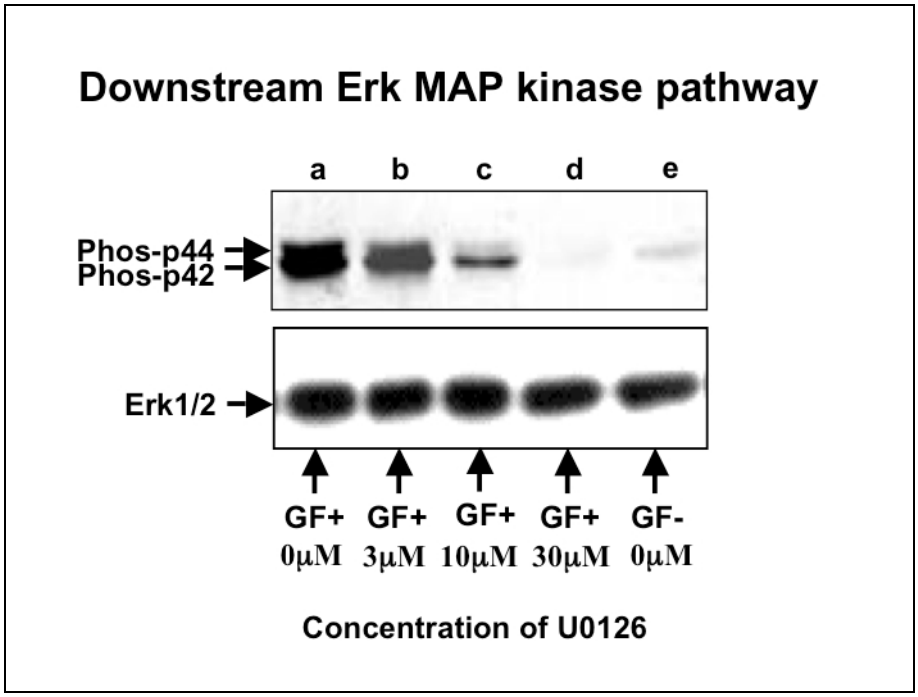


Figure 33: Western blot showing activity in the Erk MAP kinase pathway at the level of Erk1/2 in the presence of upstream inhibition of the Erk MAP kinase kinase MEK1/2 by the chemical inhibitor U0126 at various concentrations. Lower panel shows expression of non-phosphorylated Erk1/2 (at t=15 minutes and demonstrates equal protein loading of samples). Upper panel shows expression of phospho-Erk1/2 (Erk1 = -p44 and Erk2 = -p42). (Lysates visualized with anti-Erk1/2 and anti-phospho-Erk1/2 antibodies). All cells were plated on to type I collagen in the presence of soluble

growth factors (GF+) columns a-d and its absence (GF-) column e. Concentration of U0126 is shown (0μM=no exposure). Expression of phospho-Erk1/2 in the presence of growth factor (GF+) (column a) is greater than in the absence of growth factor (GF-) (column e). U0126 from 3 to 30μM, induced a dose-dependent inhibition of phospho-Erk1/2 expression (columns b-d). At high concentration of the inhibitor (d) the levels of phospho-Erk1/2 are undetectable.

The lower panel of Figure 33 shows the presence of Erk1/2 is unchanged at all concentrations of inhibitor and in the presence (GF+) and absence (GF-) of growth factors (and confirms equal loading of samples). The upper panel of (Figure 33) shows that in the presence of increasing concentrations of the MEK1/2 inhibitor U0126, and in the presence of growth factors (GF+) there is a dose-dependent reduction in levels of phospho-Erk1/2. Also apparent, and as was seen previously (Figure 27), keratinocytes on type I collagen in the absence of growth factors (GF-) show low basal levels of phosphorylated Erk1/2, column (e).

5.8 Discussion

The diverse and varying array of soluble factors (including growth factors) present during *in vivo* wound healing stimulates migration of local parenchymal cells adjacent to the wound and coordinates their continued activity. Sources of these factors include platelets (that act as a reservoir for many soluble mediators), serum (which is also a component of the milieu during the early phase of an acute wound), and the cells present in the wound, that are responsible for production and release of most of the more abundant soluble factors present. Cells both secrete and respond to soluble factors and at each phase, those factors and cells present, influence the events that are occurring and also guide progression towards the next phase and ultimately a completely healed wound. All of the effects of growth factors on cellular function are transduced into signals within the cell, typically via cell surface receptors. In addition, cells may respond by other means such as in response to physical stressors within the wound. Intracellularly, signals are propagated further by downstream networks of proteins and other chemicals that interact and lead ultimately to provision of the required cellular response to the initial signals.

In this chapter experiments were presented that explored the role of soluble growth factor signalling during keratinocyte migration on type I collagen. In particular, involvement of the MAP kinase pathways (Erk, p38 and JNK,) as downstream mediators of migration was considered. While the role of MAP kinases has been studied extensively in cellular functions including cellular migration, and in many different cell lines; more limited work has looked at their role during the migration of human keratinocytes.

Initially it was asked which if any of the three well-characterized MAP kinase pathways were active during keratinocyte migration. Basal levels of activity in the Erk pathway were shown to be present in keratinocytes plated on type I collagen in the absence of growth factors (above that seen on poly-L lysine under the same conditions). It seems reasonable to interpret this activity as a response to integrin-ECM interaction (Figure 27). The same finding were observed for the p38 pathway, although in this case a second interpretation, a cellular response to the assumed physical stress (of cell handling during the experiment and the tension generated by cellular of adhesion), was also possible and perhaps more likely, as a similar level of p38 pathway activity was also present in cells on poly-L lysine. In the presence of growth factors, not surprisingly, activity in both of these pathways was significantly increased compared to the level in their absence (Figure 27). Activity in the Erk pathway increased markedly above that seen in keratinocytes plated on poly-L lysine following the same exposure (comparing Figure 27: A and D at t=15min) while activity in the p38 pathway increased only in the same proportion to cells on poly-L lysine (comparing Figure 27: B and E at t=15min). Again, a reasonable interpretation of these findings is that the Erk MAP kinase pathway is stimulated by both integrin-ECM and soluble factors (perhaps synergistically) while the p38 pathway is stimulated by soluble factors and cellular stress (assumed) but not integrin-ECM interactions. Interestingly, activity in the JNK pathway was not demonstrated at any time or by any means of stimulation, physical (assumed), soluble or integrin-ECM (type I collagen) during the assays (functional activity in the pathway was demonstrated by exposure to LPS and TNFα) (Figure 28).

The presence or absence of activity in each pathway was further investigated in keratinocytes migrating on pro-migratory type I collagen and non-migratory poly-L lysine, in the presence and absence of soluble growth factors, by introducing the perturbing effects of selected dominant negative genes and the application of chemical inhibitors (both chosen for inhibition of elements of the MAP kinase pathways). Using both dominant negative

inhibitors and chemical inhibition provided complementary and corroborative data on the effects identified. Having reviewed the currently available information and results obtained so far in Chapter 4, it was reasonable to hypothesize that during migration of human keratinocytes, the Erk MAP kinase pathway would be active and have a direct effect on migration of keratinocytes on type I collagen. However, it was less clear whether the SAPK pathways, JNK and p38, would be involved under the same conditions.

When the Erk pathway was inhibited either with dominant negative transfection (MEK1-Dn) (Figure 29 and 30), or the chemical antagonist U0126 (a MEK1/2 inhibitor) (Figure 32: A) at a concentration of 30μM, keratinocytes did not migrate on type I collagen either in the presence or absence of growth factors. Under these conditions migration indices in both assays were no different than those from cells on non-migratory poly-L lysine. Furthermore the results shown in (Figures 31 and 33) suggest inhibition of this pathway at the level of MEK1/2 is directly responsible for the inhibition of migration observed. Considering these results and those shown in (Figure 27) relating to basal activity in this pathway, these findings are consistent with the idea that the Erk MAP kinase pathway is essential for keratinocyte migration. Inhibition of this pathway prevented both the migration following integrin-ECM stimulation alone and that seen with stimulation from both integrin-ECM interactions and soluble growth factors. In other words, when the Erk pathway is inhibited at the level of MEK1/2, integrin-ECM interactions can no longer initiate migration and soluble growth factors can no longer augment migration of keratinocytes on type I collagen.

When the p38 MAP kinase pathway was inhibited, either by dominant negative transfection (p38α-Dn) (Figure 29 and 30), or maximal chemical inhibition (SB202190) (a p38-α -β inhibitor) (Figure 32: B), keratinocyte migration on type I collagen in the presence of growth factors was inhibited, but remained unchanged in their absence. Furthermore, in the presence of growth factors, inhibition of the p38 pathway reduced the migration of keratinocytes on type I collagen to the same level as that seen in the absence of growth factors. Again, considering these results and those shown in (Figure 27), relating to basal activity in this pathway, it appears that inhibition of the p38 MAP kinase pathway blocks the contribution from soluble growth factors to keratinocyte migration on type I collagen, but has no effect on the response to interaction between pro-migratory type I collagen and integrins. The nature of the interaction between the p38 MAP kinase pathway and the downstream effectors of growth factor-stimulated migration in keratinocytes remains to be

determined. Data on this subject from keratinocytes and other cell lines is limited. Possibilities include activation by physical stress-inducing stimuli such as shearing force, hypoxia and heat shock, and several protein kinases, such as MAPK-activated protein kinase 2/3 (MAPKAP kinase 2/3) (McLaughlin et al., 1996), caldesmon (Goncharova et al., 2002) and paxillin (Hubner et al., 1996) as well as several transcription factors that are known downstream effector targets of the p38 MAP kinases and which are thought to be important in its functions during cellular migration. Activation of MAPKAPK2/3 by p38 and consequent phosphorylation of HSP27 is crucial for the migration of certain cells including endothelial cells and may be important for directionality of migration (Rousseau et al., 1997). p38 MAP kinase pathway activity leads to phosphorylation of paxillin at Ser-83 *in vitro* and in some tumour cell lines (Huang et al., 2004a). Additionally, the p38 MAP kinase pathway has also been implicated in regulating focal adhesion turnover, which may (or may not) relate to its phosphorylation of paxillin.

Finally, inhibition of the JNK MAP kinase pathway either by dominant negative transfection (JNKK2-Dn) (Figure 29 and 30), or maximal chemical inhibition (SP600125) (Figure 32: C) had no significant effect keratinocyte migration on type I collagen in either the presence or absence of growth factors. Migration indices appeared unaltered [apart from effects thought more likely to be due to broader inhibition produced by lack of specificity of the SP600125 inhibitor, which is known to be the case (Bain et al., 2003)]. Western blot demonstrated no activity in the JNK MAP kinase pathway at the level of JNK following stimulation by type I collagen interaction (alone) or that interaction in the presence of growth factors. However, functional activity in the JNK MAP kinase pathway and functionality of the dominant negative transgene JNKK2-Dn were demonstrable after exposure to LPS and TNFα (Figure 28). In some cells such as fibroblasts, activation of the JNK pathway by integrin-ECM stimulation has been observed. This occurs via a pathway independent of that for soluble factor activation via the EGF receptor (Miyamoto et al., 1995), in keratinocytes that does not appear to occur.

In summary, the experiments presented in Chapter 4 confirmed the hypothesis that initiation (and continued) migration of keratinocytes would depend on the presence of substrates such as type I collagen and others known to be present in the *in vivo* healing wound matrix, and also that in the presence of type I collagen and similar substrates (type IV collagen) that have been shown to be pro-migratory, migration of keratinocytes would occur with or

without the addition of soluble growth factors. In addition, it was shown that the presence of soluble factors would significantly augment keratinocyte migration. In this chapter the experiments further explored the role of soluble growth factor signalling during keratinocyte migration on type I collagen. In particular, involvement of the MAP kinase pathways (Erk, p38 and JNK) as downstream mediators of soluble factor signalling was considered. From these results it appears that a functional Erk pathway is necessary for migration and when this pathway is intact in the presence of a pro-migratory substrate such as type I collagen, keratinocyte migration is initiated and maintained for several hours without growth factor signalling, albeit at low level. It must be stressed that growth factor signalling alone at any level cannot induce keratinocyte migration in the absence of a pro-migratory substrate, as was shown in Chapter 4. The addition of growth factors and subsequent stimulation of their downstream signalling effectors significantly enhances keratinocyte migration on a number of substrates, notably type I collagen. This augmentation requires functionally intact and active Erk and p38 MAP kinase pathways, but apparently does not depend on the JNK MAP kinase pathway in keratinocytes.

Chapter 6 Downstream signalling of integrin-extracellular matrix Interactions and the role of FAK and Src in keratinocyte migration

6.1 Introduction

Thus far, this work has shown that a functional Erk MAP kinase pathway is necessary for migration, and when this pathway is intact in the presence of a pro-migratory substrate such as type I collagen, keratinocyte migration is initiated and maintained for several hours without growth factor signalling, albeit at a low level. (Growth factor signalling alone at any level cannot induce keratinocyte migration in the absence of a pro-migratory substrate). The addition of growth factors and subsequent stimulation of their downstream signalling effectors significantly enhances keratinocyte migration on a number of substrates, notably type I collagen. This augmentation requires functionally intact and active Erk and p38 MAP kinase pathways, but apparently does not depend on the JNK MAP kinase pathway in keratinocytes. In this chapter the effects of integrin-ECM stimulated signalling in keratinocyte migration mediated via FAK and Src is considered. In particular the relationship of FAK-Src activity to phosphorylation of paxillin (linked to focal adhesion turnover), and to the integrin-ECM activation of MAP kinase pathways (linked to a number of mediators of migration) are considered in addition to potential interactions between these events. A summary of important related interactions in these processes is provided first.

Integrins are involved in a number of *in vivo* and *in vitro* cellular processes including survival, proliferation and migration. The process of cellular migration as it occurs in wound healing involves integrins, and can be considered to be a complex and coordinated repeating series of changes in the actin cytoskeleton linked to concomitant changes in cellular adhesion (focal adhesions formation and dynamics). Integrins are involved directly in the attachment of cells to the extracellular matrix (adhesion), in the signalling pathways leading to the formation and maintenance of the polarized morphology, and to a number of other important changes in the actin cytoskeleton, in a process that enables the cell to move forwards.

Focal adhesion formation can be considered to begin with the expression of integrins on the cell surface that interact with the local environment, the extracellular matrix. Integrin binding to ECM leads to regional clustering of integrins. Like all cell signalling interactions, information gathered must be transmitted into the cell before its effects can be elaborated. In the case of integrins (which do not have enzymatic activity), subsequent intracellular effects are mediated by the profile of proteins that accumulate, their relative levels of tyrosine phosphorylation, and other activities such as their binding affinities. Downstream to many of the functions mediated by integrins and among the first proteins recruited to sites of integrin clustering following ligand binding is the focal adhesion kinase, FAK. FAK recruitment to sites of integrin clustering occurs via a region of the molecule termed FAT (focal adhesion targeting region). This region interacts with the intracellular portions of the β subunits of integrins in clusters (indirectly) by binding to structural proteins such as talin and paxillin (Figure 34). Thereafter, FAK is linked to other components of the cellular cytoskeleton (as was shown in Figure 3) and to the proteins that accumulate with the development of the focal adhesion (as was shown Figure 4).

(Figure 34)

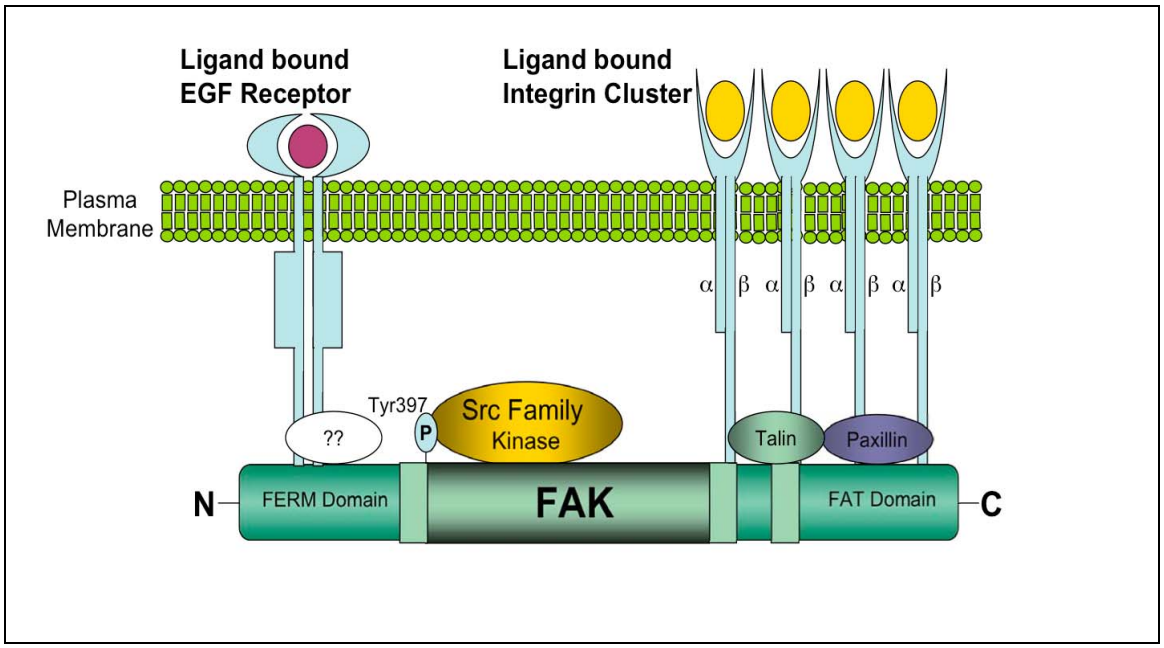


Figure 34: Schematic representation of the attachment between ligand bound integrin in clusters and FAK mediated via a region of FAK called the FAT (focal adhesion targeting) domain and the β chains of integrin in association with talin and paxillin. Also shown is the binding of ligand bound dimerized epidermal growth factor receptor (EGF Receptor) to a region of FAK referred to as the FERM (protein 4.1, ezrin, radixin and moesin homology) domain (the possibility of an intermediary

interaction in this process is represented by "??") and also the major FAK-Src family kinase interaction at Try-397 (the auto-phosphorylation site) on FAK.

Recruitment of FAK is concomitant with cytoskeletal changes that are manifested as a polarized cellular morphology. Physically there is the formation of actin stress fibres, lamellipodia and filopodia, the key features seen in cellular spreading and migration. Underlying the cytoskeletal and morphological changes observed are interlinked and interdependent interactions of FAK and the Rho family of small GTPases (Rac-1, Cdc42, and RhoA), phosphoinositide 3-kinases (PI3-kinases), microtubules, and other systems. FAK in focal adhesions has been shown to associate directly with activators and inhibitors of the Rho family, and also those influencing Ras (key interactions sites with FAK were shown in Figure 5). FAK has been directly linked to up-regulation of Rac-1 (leading to the formation of lamellipodia) via a paxillin (or $p130^{CAS}$) -Crk-DOCK180 (Dedicator of cytokinesis 180)-ELMO1 (Engulfment and cell motility 1) complex formation (Kiyokawa et al., 1998a; Kiyokawa et al., 1998b) and to inhibition of RhoA (contributing to the same effect) (Tsubouchi et al., 2002). At other sites FAK activity results in the down-regulation of actin stress fibre formation, an effect mediated through RhoA and the RhoA-GAP; GRAF (GTPase regulator associated with focal adhesion kinase). These and other similar interactions produce a polarized spatial distribution of these small GTPases that favours migration, with Rac1 activity greatest in protrusions at the advancing edge and a gradient of increasing RhoA activity posteriorly. Activators and regulators of the Erk MAP kinase pathway (including Ras) are also recruited to the site of focal adhesion formation (and are considered in more detail later). Briefly, within focal adhesions FAK is associated with the EGFR and along with Src activates Erk by mechanisms including interactions between Shc, Grb2 and SOS and the small GTPase Ras. Activated Erk has a number of downstream effectors related to migration such as calpain-2 that inactivate a number of key proteins and MLCK (myosin light chain kinase) that modulates actin-myosin contractility during migration (Fincham et al., 2000).

Simply, focal adhesions link the surroundings of the cell (the extracellular matrix) to its structural components (the cytoskeleton) via integrins. The processes occurring within and downstream of focal adhesions directly influence cytoskeletal changes and facilitate their own formation, translocation and dissolution. FAK is central to processes within focal adhesions during migration. It seems most likely from evidence derived from many cell

lines (though not specifically keratinocytes) that FAK (and FAK-Src) interactions within focal adhesions are particularly important in the regulation of adhesion turnover (and interrupt their continued maturation). This is achieved by FAK promoting focal adhesion disassembly at the proximal region of cellular protrusions. The rate of disassembly (turnover) is related to the level of tyrosine phosphorylation of FAK (and other proteins such as paxillin discussed further below); a significant proportion of which is due to the activity of Src family kinases. As described previously within focal adhesions, FAK binds to a number of proteins; most notably these include members of the Src family of phosphotyrosine non-receptor kinases (c-Src, Fyn, and Yes1). Integrin-ECM stimulation is associated with FAK recruitment, its auto-phosphorylation (at Tyr-397) and SH2 binding of Src (Schaller et al., 1994). Src bound to FAK undergoes a conformational change leading to an increase in its kinase activity. Src kinase activity is responsible for phosphorylation of a number of proteins including paxillin (and a number of sites on FAK) and with activation of a number of signalling pathways including the Erk MAP kinase pathway.

The Erk MAP kinase pathway is a well-known target of integrin signalling mediated by FAK and the FAK-Src complex. Erk has also been shown to localize to focal adhesions as they are formed at the leading edge of protrusions in migrating cells (Miyamoto et al., 1995) and the EGFR localizes to the same sites via interactions with FAK (shown in Figure 5). Shortly after FAK was identified, activation of the Erk MAP kinase cascade in response to fibronectin stimulation of fibroblasts was demonstrated (Schwartz et al., 1995) and the observation has subsequently been extended to a number of different cell types, although not to keratinocytes. While a number of debates are ongoing it seems most likely that integrin-ECM mediated influences on Erk MAP kinase pathway activity is cumulative from multiple sources. [These include the activation of tyrosine kinases, Rho family GTPases (Clark et al., 1998), serine-threonine kinases such as protein kinase C (Schlaepfer and Hunter, 1998) and lipid kinases such as PI-3 kinase (King et al., 1997)]. In contrast to the model of Erk MAP kinase pathway activity following stimulation by growth factors (shown in Figure 25), the molecular mechanisms of integrin-ECM signalling to the Erk MAP kinase pathway are best represented as a network of interactions across a number of different pathways that are acting in concert in a coordinated manner. Nevertheless, certain essential interactions are apparent such as those following integrin-ECM stimulation leading to activation of FAK and Src (as described above), and linked to Erk MAP kinase pathway activation.

Following integrin-ECM stimulated activation of FAK and Src, there is Src mediated tyrosine phosphorylation at a number of sites including Tyr-317 on the adaptor protein Shc and Tyr-925 on FAK itself. In both cases this creates an SH2 binding site to which the adaptor protein Grb2 can bind. Grb2 binding potentiates the translocation of the guanosine diphosphate-guanosine triphosphate (GDP-GTP) exchange protein SOS (Son of Sevenless) to the plasma membrane leading to an enhanced GTP exchange on Ras. The activation of the Erk-MAP kinase cascade is one target for the actions of GTP-bound Ras (as was shown in Figure 6). (Similarly and discussed previously, Grb2-SOS interactions (via Grb2 binding to the phosphorylated EGF receptor) are important mediators of activation of the Erk MAP kinase pathway from the growth factor receptor as was shown in Figure 25).

Src kinase kinase tyrosine phosphorylation of a number of other proteins including paxillin and other sites on FAK serves a number of functions relating to migration. Following integrin-ECM stimulation as described there is recruitment of FAK to focal adhesions where binding to paxillin stabilizes it in place. Subsequently, Src kinase activity phosphorylates paxillin at Tyr-118 this leads to a number of downstream effects including focal adhesion disassembly (Brown and Turner, 2004; Mitra et al., 2005). At different times paxillin phosphorylated at Tyr-118 can bind to a number of molecules such as the adapter protein Crk, p120RasGAP, Erk (and activated Erk), and Csk. Some of these interactions are related to the cytoskeletal changes seen in migration, such as binding of the adaptor protein Crk at Tyr-118 on paxillin that has been implicated in Rac1 activation and stimulation of cell motility (lamellipodia formation) (Kiyokawa et al., 1998a; Petit et al., 2000), or the binding of paxillin to the SH2 domain of p120RasGAP that displaces p190RhoGAP from that site, resulting in decreased RhoA activity and enhanced Rac1-RhoA polarization within the migrating cell (Iwasaki et al., 2002; Tsubouchi et al., 2002). Binding of Csk to Tyr-118 on paxillin negatively feeds back to inhibit Src activity by phosphorylation of Src at Try-527.

The key paxillin (Tyr-118) interaction relating to focal adhesion disassembly is the binding of activated Erk to paxillin at Tyr-118 that leads to phosphorylation of FAK at Ser-910. Phosphorylation of FAK at this site results in the dissociation of FAK from paxillin and away from the focal adhesion leading in turn to disassembly of the structure.

However, it is also known that activated Erk bound to paxillin can phosphorylate paxillin itself at a number of sites in a manner that favours FAK-paxillin association. This

phosphorylation promotes re-binding of FAK to paxillin that in turn favours formation of new focal adhesions in the migrating cell. Erk phosphorylation of paxillin in this manner is believed to occur at temporally and/or spatially different sites than those above. By these mechanisms, cyclical changes in FAK-Src activity, integrin-FAK-paxillin binding and Erk activity the focal adhesion turnover could be achieved (Liu et al., 2002).

Experiments presented here evaluate the roles of FAK and Src as central mediators of integrin-ECM stimulated keratinocyte migration. Firstly, experiments were performed to test the hypothesis that inhibition of a key binding and activation site of FAK (Tyr-397 the auto-phosphorylation site) and/or inhibition of FAK kinase activity would significantly decrease keratinocyte migration on type I collagen. This was investigated by use of two dominant negative inhibitors of FAK. It was anticipated that an intact FAK auto-phosphorylation site would prove to be essential to keratinocyte migration. Similarly, it was hypothesized that inhibition of the kinase activity of Src by use of dominant negative and chemical inhibitors would also have an effect on keratinocyte migration under the same conditions. In particular it was anticipated that such inhibition (of FAK and Src by these means) would affect migration on type I collagen both in the presence and absence of growth factors. Western blot was used to evaluate the effects of over-expression of FAK and Src "wild-type" transgenes and of dominant negative inhibitors FAK-F397, FAK-R454 and Src-KM on tyrosine phosphorylation of FAK and of significant known downstream mediators of migration such as paxillin. In the presence of these same transgenes and by the same means (Western blot), activity in the MAP kinase pathways (Erk, p38 and JNK) will be demonstrated. Under these conditions it was hypothesized that the changes in FAK phosphotyrosine levels and paxillin phosphorylation at Tyr-118 would be predictably altered in a manner directly linked to changes in keratinocyte migration. Similarly, it was anticipated that activity in the Erk MAP kinase pathway (and possibly in the SAPK pathways, JNK and p38) would prove to be linked to these changes in FAK and Src and to changes in migration. Again in both cases this was hypothesized to be likely to occur in the presence and absence of growth factors. Finally, in light of the findings that will be demonstrated (particularly in relation to the results observed following Src-Wt over-expression), an experiment was performed to show what if any effect on migration there might be from the inhibition of the p38 MAP kinase pathway using the chemical inhibitor SB202190 in keratinocytes transfected with FAK and Src "wild-type" transgenes and kinase deficient dominant negative transgenes (FAK-R454 and Src-KM) on type I collagen. The

hypothesis considered in this experiment was that the p38 MAP kinase pathway might prove to be more critical to migration under conditions that are associated with greater actin-myosin activity and greater turnover of focal adhesions (a circumstance thought to be true in the case of Src "wild-type" over expression).

To explore the keratinocyte migration under these circumstances and the involvement of intracellular pathways mediating these effects, selected cellular genes including FAK and c-Src were inhibited using chemical inhibitors (of Src) and transfection of dominant negative genes (of FAK and Src) delivered in lentiviral vector constructs. Generation of the FAK and Src lentiviral vectors for these experiments was constructed and experiments used in this regard are considered first.

6.2 Generation of FAK and Src lentiviral vectors

It was necessary to create lentiviral constructs containing the FAK and Src inserts (transgenes of "wild-type" and dominant negative forms). As described in Section 2.7.2 a lentiviral gene transduction system was used to deliver transgenes into keratinocytes for subsequent experiments. The system used was based on the human immunodeficiency virus (HIV) derived lentivirus vector pRRLsin (provided by Prof. Nori Kasahara, USC Institute of Genetic Medicine) and provided high gene transduction efficiency (over 90%) and sustained expression of the transduced gene in keratinocytes (Chen et al., 2003).

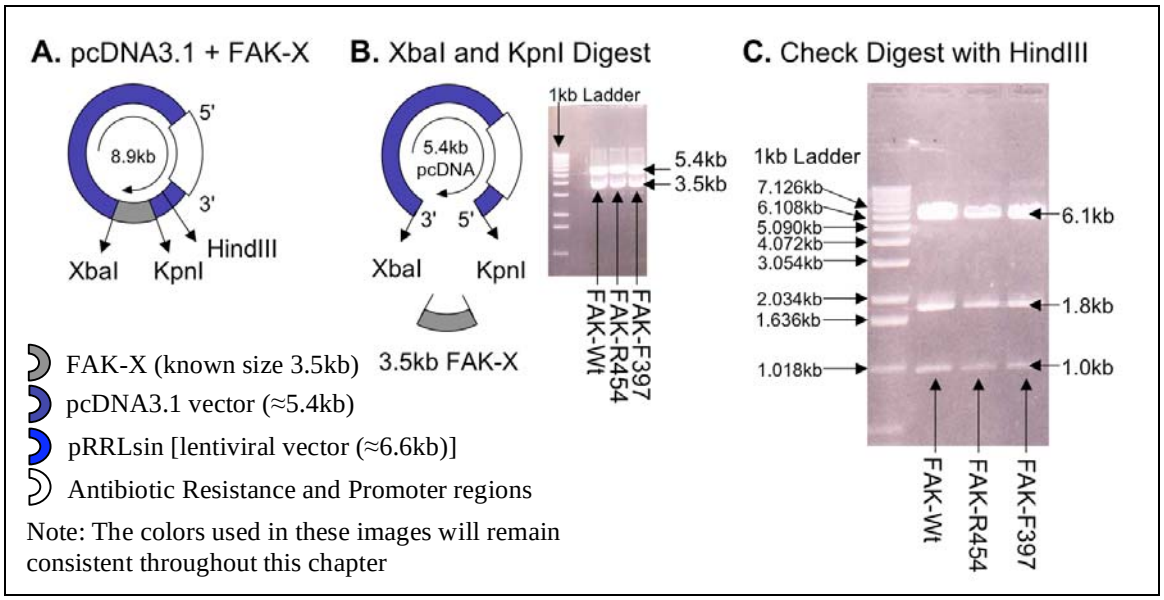
6.2.1 Results: Generation of the FAK lentiviral vectors and their transduction into keratinocytes

Three FAK transgenes were studied: "Wild-type" FAK (FAK-Wt) and two dominant negative mutants FAK-F397 and FAK-R454. The plasmid vectors (pcDNA3.1) containing the three FAK transgenes were provided as a gift from Professor D. Schlapfer, Department of Immunology, The Scripps Research Institute, CA, USA. The relevant source and activity of each transgene are provided in detail in Section 2.7.2.1 and are summarized in (Table 3). Briefly, the tyrosine residue at position 397 is the primary auto-phosphorylation site of FAK, and is the site for the interaction between FAK and the Src family of protein tyrosine kinases. The dominant negative gene FAK-F397 does not undergo auto-phosphorylation, which limits the subsequent interactions with Src (reduces Src kinase activity) and impairs a number of other important interactions. The activity of this dominant negative form FAK-

F397 has been widely studied in a number of cell types, notably fibroblasts (Schaller et al., 1994), but not in keratinocytes. The lysine residue at position 454 lies within the kinase domain of FAK. The dominant negative gene FAK-R454 has been documented to result in a significantly reduced FAK kinase activity and has been widely used and studied (Hildebrand et al., 1993). FAK-Wt is the unmodified transgene of FAK.

All three FAK transgenes were provided (individually) as inserts in the vector PcDNA3.1 at restriction sites, KpnI and XbaI. Confirmation of the presence of FAK-X ("-X" representing each of -Wt, -F397 and -R454) was performed using digests of the provided pcDNA3.1+FAK-X constructs with endonucleases KpnI and XbaI. The endonuclease digest confirmed the presence of appropriately sized FAK-X transgene fragments, 3.5kb in each case (Figure 35: A and B). An additional second confirmation digest of the constructs was performed using HindIII restriction endonuclease. pcDNA3.1 contains a single HindIII site and FAK-X contains an additional two HindIII sites. (Note: there are no HindIII sites in pRRLsin). It was anticipated that a digest of the pcDNA3.1+FAK-X constructs with HindIII would, in the presence of FAK-X, produce three fragments of length 1.0kb, 1.8kb and 6.1kb. This was confirmed to be the case (Figure 35: C).

(Figure 35)



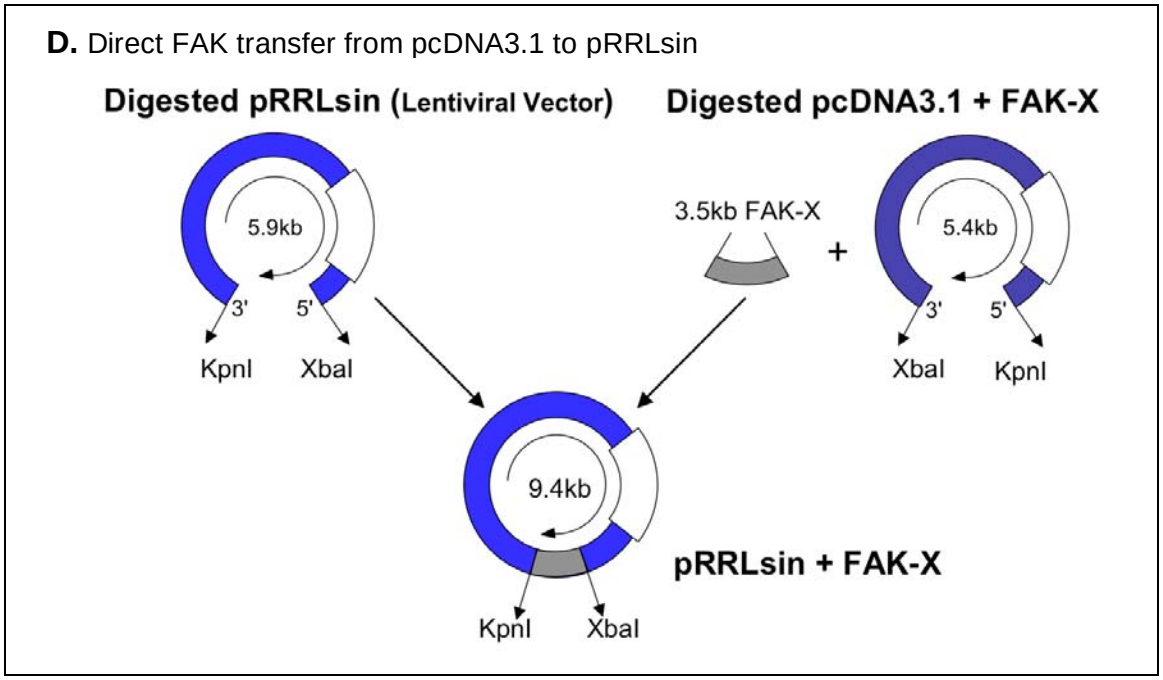


Figure 35: Endonuclease digest of pcDNA3.1+FAK-X constructs performed to confirm the presence of FAK-X ("-X" representing each of -Wt, -F397 and -F454) and a protocol for direct transfer of FAK-X from pcDNA3.1 to pRRLsin. A: Schematic showing FAK-X transgenes as provided incorporated into the pcDNA3.1 vector (and relevant restriction sites). B: Schematic and image showing fragments resulting from digestion (KpnI and XbaI) of the constructs pcDNA3.1+FAK-X. C: Image showing fragments resulting from HindIII digestion of pcDNA3.1+FAK-X. The images shown in B and C were taken following electrophoresis on agarose gel with DNA fragments visualized by UV illumination. Fragment size (position) was recorded by comparison to the standard 1kb ladder shown. D: Schematic outline of a protocol for transfer of FAK-X transgenes from PcDNA3.1 to pRRLsin. Note: This simple approach was not possible as the orientation of the FAK-X transgenes in the final pRRLsin-FAK-X vectors are in a reversed orientation.

The results in Figure 35: B and C were taken as confirmation of the presence of the FAK-X transgenes in the constructs provided. Consideration of restriction sites within the FAK-X genes and those present within the multiple cloning regions of the two vectors, pcDNA3.1 (Figure 35: A) and pRRLsin (Figure 11), showed that it was not possible, as it was initially hoped, to directly transfer the FAK genes from one vector to the other. Direct transfer at KpnI and XbaI (Figure 35: D) would have resulted in a final construct (pRRLsin-FAK-X) in which the FAK transgenes were inserted in a reversed orientation. Furthermore it was apparent that there were no alternative restriction sites present for a simple direct transfer of

the transgenes. Several alternative approaches were followed to create the correctly orientated final constructs (pRRLsin-FAK-X) to be used in further experiments, these will be described in detailed next.

6.2.1.1 Results: Use of an intermediate vector pSP73

As an alternative approach, the use of an intermediate vector, pSP73 was considered. This vector contained favourable restriction sites in its multiple cloning region that would allow the sequential transfer of the FAK-X transgenes from the vector pcDNA3.1 in which they were provided to pSP73 and then to the desired vector pRRLsin while maintaining the correct orientation. The steps that were performed using this approach are outlined in (Figure 36: A-G). Results for steps A to D are not shown, but the intermediate construct pSP73+FAK-X was successfully produced and the presence of FAK-X confirmed with SalI and EcoRV digestion (D). [The constructs pSP73+FAK-X were obtained following successful Midiprep of transformed competent cells DH5α. These cells were transformed (C) with ligated vector pSP73 and FAK-X inserts obtained from earlier steps (A and B)]. The this intermediate construct (pSP73-FAK-X) and the vector pRRLsin were digested with SalI and EcoRV (D and E respectively) and in each case bands of appropriate size were seen following electrophoresis. Later it became apparent that in the case of the vector only (pRRLsin) one of the two enzymes (SalI and EcoRV) in fact completed the digestion reaction and this was at least in part responsible for the failure of step (F) ligation of vector pRRLsin and the FAK-X inserts prepared as above.

(Figure 36)

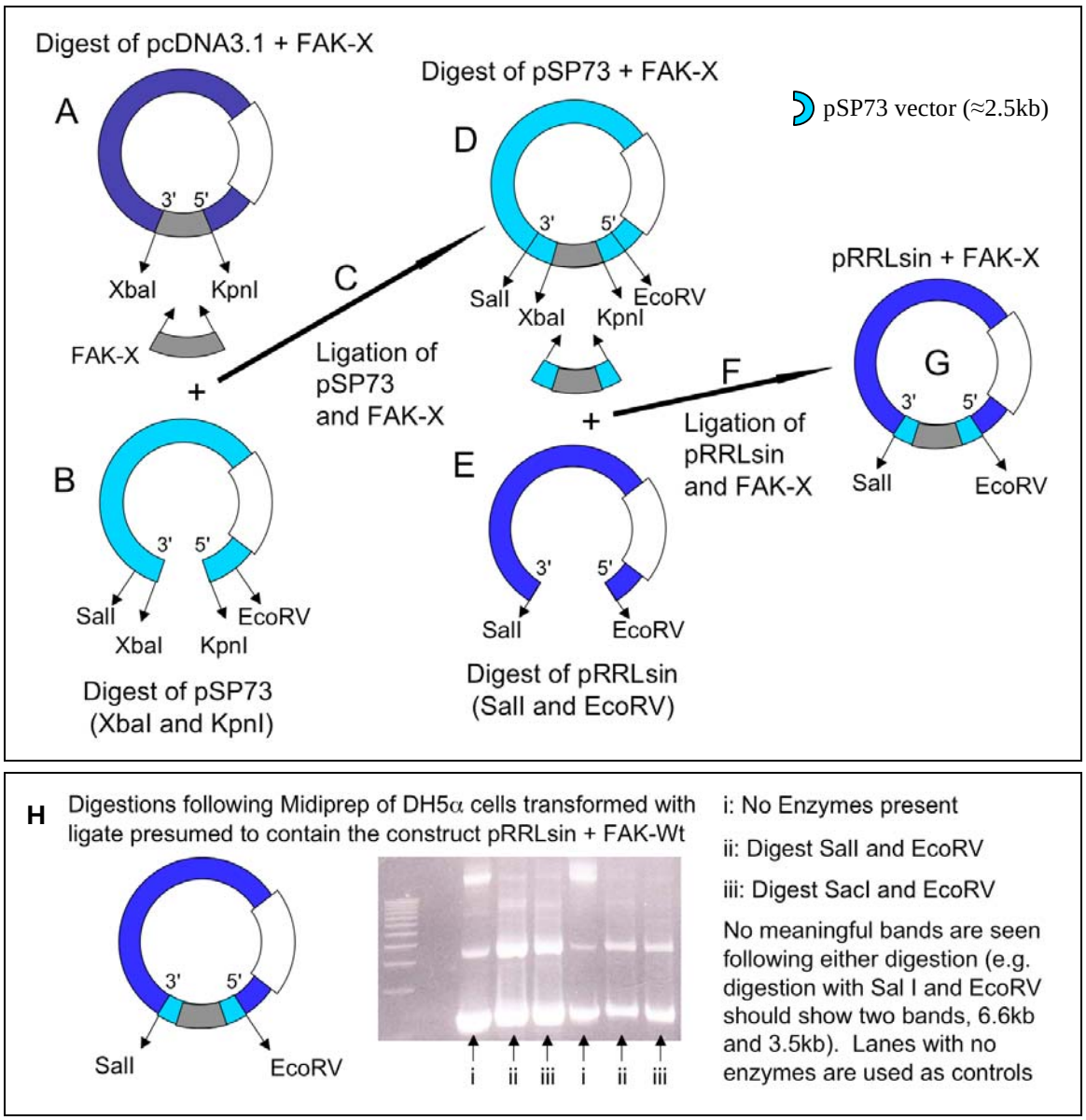


Figure 36: Schematic outline of the sequential transfer of FAK-X from pcDNA3.1 to an intermediary pSP73 and then to the desired vector pRRLsin. A: pcDNA3.1 vectors containing FAK-X inserts were digested with XbaI and KpnI, the FAK-X fragments were separated by electrophoresis and purified. B: The intermediate vector pSP73 was also digested with the same enzymes (XbaI and KpnI) and purified. C: The FAK-X fragments (each separately) and pSP73 vector were then ligated to produce new constructs pSP73+FAK-X. D: Following successful transformation of DH5α cells and Midiprep the intermediate pSP73+FAK-X was then digested with the SalI and EcoRV and again the FAK-X fragments were recovered by electrophoresis and purified. E: The vector pRRLsin was digested with the same enzymes (SalI and EcoRV) and purified. F: The FAK-X fragments and pRRLsin vector were then ligated to produce the final and desired construct pRLLsin+FAK-X. G: By this protocol pRRLsin containing the FAK-X transgenes constructs (with

FAK-X correctly orientated) could be obtained. H: Following transformation (of competent cells DH5α with the ligate (F) above) and Midiprep, digestions with two pairs of enzymes were performed. The specificities of these enzymes were chosen to match sites that should have been present following successful transformation (SalI and EcoRV, and SacI and EcoRV). The image shows a failure to digest (results for FAK-Wt are shown). This failure indicates the absence of the FAK-Wt insert in the DNA recovered from transformed colonies (identical results were obtained with the other FAK-X transgenes).

Despite repeated experiments varying a number of conditions the final construct was not formed pRRLsin+FAK-X, suggesting either it was not possible to achieve successful ligation (of FAK-X and pRRLsin) and/or transformation of the DH5α cells. Control transformations performed simultaneously were successful and experimentally transformed (transformed with the ligate obtained in step F) colonies did grow on agar indicating conferred antibiotic resistance in the transformed cells (Figure 36: F). However, analysis of these colonies following DNA recovery (typically 6-12 colonies were analyzed per experiment) with a number of pairs of enzymes (e.g. SalI and EcoRV (both sites within pRRLsin) and SacI (at a site within FAK-X) and EcoRV) did not show the presence of the desired construct pRRLsin+FAK-X (Figure 36: H). No meaningful bands of appropriate size were seen following digestion with either pair of enzymes and the migration patterns are similar to those in the absence of any enzymes.

Failure of this approach was considered most likely to be due to the close proximity of the two restriction sites SalI and EcoRV, in the vector pRRLsin. In pRRLsin, SalI (4665) and EcoRV (4644) are only 21 base pairs apart (Figure 11). The restriction enzyme SalI requires 31 base pairs to function efficiently and also EcoRV is known to be the more efficient of the two enzymes. Thus, it was considered possible that failure of ligation in step (F) was due to a preponderance of the situation in which EcoRV would cut pRRLsin first; leaving insufficient plasmid for the action of SalI that then could not act. (Additionally the action of EcoRV is to cleave plasmids with blunt ends, which creates a further inefficiency in any ligation that might have otherwise occurred successfully).

6.2.1.2 Results: Use of blunt-end ligation techniques

As an alternative approach conceived to overcome the potential difficulties identified as the likely cause of the failure described above, a "blunt-end" ligation technique was used. In this case EcoRV was used to digest the vector pRRLsin, producing blunt-ends, and the original constructs provided, pcDNA3.1+FAK-X were digested with XbaI and KpnI (non-blunt-end digestion). The FAK-X fragments obtained were separated by electrophoresis and purified. The pRRLsin (EcoRV digest) was then treated with CIP reagent that removes phosphate groups at blunt ends of DNA strands. This minimizes vector re-ligation, since the ligation reaction favours the phosphorylated insert over the unphosphorylated (CIP treated) vector. The purified FAK-X inserts were each treated with the Klenow reagent removing 3' overhangs and producing blunt ends. Following ligation, the construct was used to transform DH5α competent cells and also subsequently X-10 Gold ultra-competent cells. Again while the control transformations in both cases were invariably successful, few experimental colonies were obtained from this technique. (Note: sequencing (or selected restriction digests on) the ligated construct would have been necessary with this approach to identify the correctly orientated inserts). However, none of the preliminary digests following Midiprep (of the few colonies that did form) using the same pairs of enzymes described previously (SalI and EcoRV and SacI and EcoRV) revealed any bands indicating the presence of the desired vector, pRRLsin+FAK-X (data not shown). These experiments were repeated several times varying parameters of the protocol in attempts to favour successful ligation, but in each case the outcome was the same.

Failure of this approach was thought most likely to be due to the large size of the intended FAK-X inserts ≈3.5kb. To address this, ultra-competent cells (X-10 Gold) were used as mentioned, but this did not alter the outcome. The FAK-X inserts were the largest that had been attempted using the pRRLsin vector at that time.

6.2.1.3 Results: Use of the intermediate construct, pSP73+FAK-X with a partial digestion reaction and an existing construct, pRRLsin+MKK3 with favourable endonuclease sites

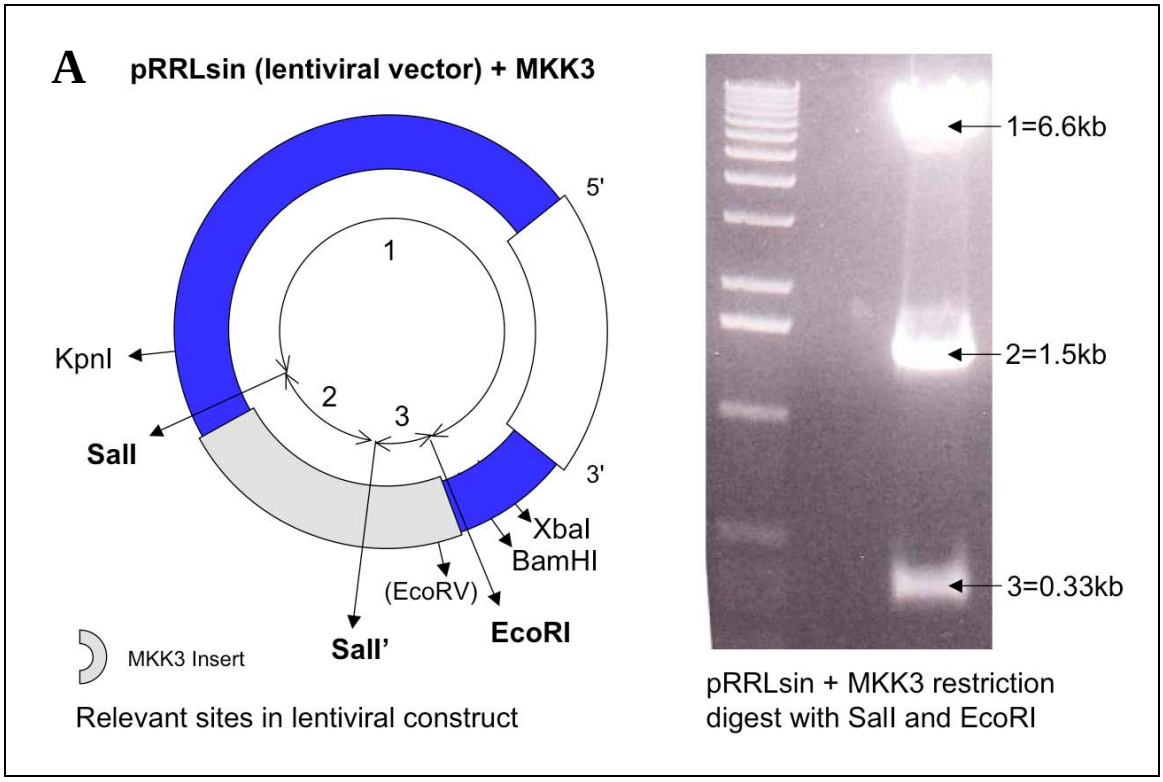
The next technique attempted for ligation and transformation of the transgenes FAK-X and vector pRRLsin, involved a partial digestion (an interrupted digestion) of the previously synthesized intermediate construct pSP73+FAK-X (Section 6.2.1.1). An existing construct pRRLsin-MKK3 (available in our lab) was also used as follows (Figure 37).

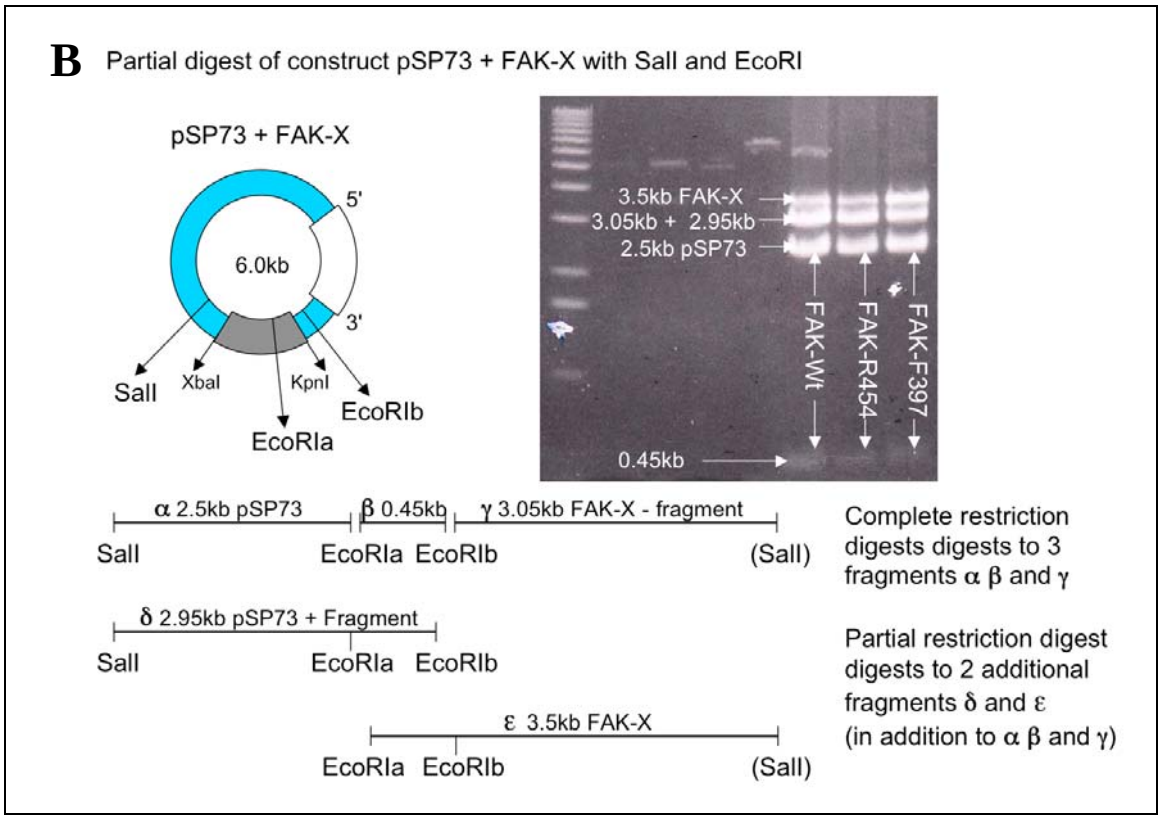
Comparing the restriction sites available in the multiple cloning region of pRRLsin (Figure 11) and those known to be present in our existing construct pRRLsin+MKK3 (Figure 37: A) it was apparent that many of the original pRRLsin multiple cloning region sites remained intact in the MKK3 construct; KpnI(4274), SalI(4664), EcoRI(4638), BamHI (4620), and XbaI(4608). Also known to be present was an additional SalI site within the MKK3 sequence, shown as SalI` (Figure 37: A).

The pRRLsin-MKK3 construct was chosen to avoid the difficulties experienced with the protocol using the intermediate vector pSP73 (Section 6.2.2.1) due to the close proximity of the SalI site to the EcoRV site in the pRRLsin vector. That is in the construct pRRLsin+MKK3, both the SalI` site within the MKK3 sequence and the Sal I site remaining in the multiple cloning region were separated by more than the 31 base pairs from any other potential sites that might be used, such as the EcoRI site; this separation would allow SalI to digest efficiently. Digestion of pRRLsin-MKK3 with SalI and EcoRI resulted in the 3 anticipated fragments (Figure 37: A). The 6.6kb fragment (the vector pRRLsin) was resolved by electrophoresis, isolated and then purified.

To prepare the inserts (FAK-X) with the same restriction specificities, the intermediate constructs pSP73+FAK-X made previously were partially digested (an interrupted digest reaction) with the same enzymes SalI and EcoRI. This partial reaction was expected to result in 5 fragments; 3 from complete digestion of the construct and 2 from its partial digest as follows: The desired FAK-X inserts were 3.5kb fragments (labelled ε in Figure 37: B) and resulted from the partially completed digestion. Other fragments included a 2.5kb fragment (labelled α in Figure 37: B) that was the pSP73 vector resulting from full digestion, a small 0.45kb fragment (labelled β in Figure 37: B) and a 3.05kb (labelled γ in Figure 37: B) both resulting from partial digestion, and a 2.95kb (labelled δ in Figure 37: B) the remaining fragment from complete digestion. Note: The latter two fragments 3.05kb (labelled γ) 2.95kb (labelled δ) overlap in the UV visualized agarose gel image shown (in Figure 37: B) but all other fragments were clearly visible. The 3.5kb FAK-X fragments were resolved by electrophoresis, isolated and then purified.

(Figure 37)


A
pRRLsin (lentiviral vector) + MKK3
5'
1
KpnI
2
3
SalI
3'
XbaI
BamHI
(EcoRV)
SalI'
EcoRI
MKK3 Insert
Relevant sites in lentiviral construct
1=6.6kb
2=1.5kb
3=0.33kb
pRRLsin + MKK3 restriction digest with SalI and EcoRI


B
Partial digest of construct pSP73 + FAK-X with SalI and EcoRI
pSP73 + FAK-X
5'
6.0kb
3'
SalI
XbaI
KpnI
EcoRIb
EcoRIa
3.5kb FAK-X
3.05kb + 2.95kb
2.5kb pSP73
FAK-Wt
FAK-R454
FAK-F397
0.45kb
α 2.5kb pSP73
β 0.45kb
γ 3.05kb FAK-X - fragment
SalI
EcoRIa EcoRIb
(SalI)
δ 2.95kb pSP73 + Fragment
SalI
EcoRIa EcoRIb
ε 3.5kb FAK-X
EcoRIa EcoRIb
(SalI)
Complete restriction digests digests to 3 fragments α β and γ
Partial restriction digest digests to 2 additional fragments δ and ε (in addition to α β and γ)

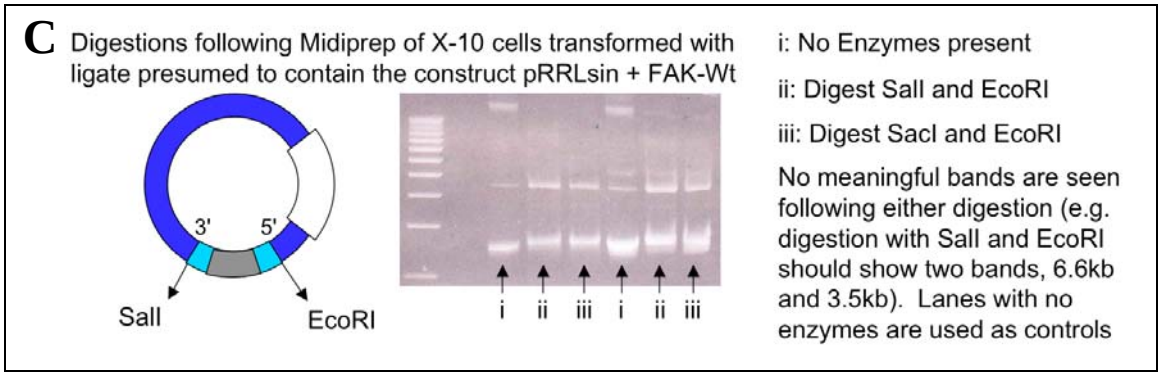


Figure 37: Use of a partial endonuclease digestion reaction (an interrupted digestion) of the intermediate construct pSP73+FAK-X and digestion of an existing construct pRRLsin+MKK3 (available in our lab) with favourable endonuclease sites (SalI and EcoRI) to produce the desired pRRLsin+FAK-X constructs. A: Schematic showing the relevant restriction sites present in the pRRLsin+MKK3 construct (MKK3 insert is shown in grey, it is a p38 MAP kinase pathway MAP kinase kinase). Also shown is an image of the aragose gel taken following digestion with SalI and EcoRI and electrophoresis, visualized under UV illumination. In this images 3 bands are indicated (1=6.6kb, 2=1.5kb and 3=0.33kb) corresponding to expected fragments obtained after digestion of the pRRLsin+MKK3 construct with these enzymes (the origin of these fragments is shown in the schematic as 1, 2, and 3). B: Schematic showing the previously created intermediate construct pSP73+FAK-X and its relevant restriction sites including sites of insertion of FAK-X (XbaI and KpnI) and sites of action of SalI and EcoRI (note: two EcoRI sites are shown and are labelled EcoRIa and EcoRIb). SalI and EcoRI digestion continued to completeness resulted in 3 fragments (labelled α=2.5kb, β=0.45kb and γ=3.05kb) indicated as lines showing the restriction sites, size of fragment and the protein contained in the fragment if any (e.g. α=2.5kb contains the vector pSP73). An interrupted digestion reaction with the same enzymes produced an additional (in addition to α, β, and γ) 2 fragments (labelled δ=2.95kb and ε=3.5kb) also indicated as lines showing the same information (note: ε=3.5kb contains the complete FAK-X insert). Also shown is an image of the aragose gel following partial endonuclease digestion of pSP73+FAK-X with SalI and EcoRI, and electrophoresis visualized under UV illumination. In this images 4 bands are indicated (3.5kb FAK-X, 3.05kb + 2.95kb, 2.5kb pSP73 0.45kb) corresponding to expected fragments obtained after interrupted digestion of the pSP73+FAK-X construct with these enzymes (only 4 bands are visible as the two fragments of size 3.05kb and 2.95kb lie too closely together in this image to be resolved). The FAK-X inserts obtained by partial digest and the vector pRRLsin digested with the same enzymes (SalI and EcoRI) were recovered purified and ligated, and the ligate transformed with X-10 Gold ultracompetent cells. C: Schematic of the intended (but not identified) construct pRRLsin+FAK-X. Also shown is an image following electrophoresis of DNA extracted from colonies produced after transformation and after endonuclease digestions with two pairs of enzymes (SalI and EcoRI and SacI and EcoRI). Samples prepared similarly but containing no endonucleases

were used as controls. The image demonstrates failure of either pair of enzymes to digest the recovered DNA. This failure indicates the absence of the FAK-WT insert (FAK-WT was used to indicate these findings, similar results were obtained with the other FAK-X transgenes).

Successful control transformations performed simultaneously were observed and multiple experimental colonies did grow following transformation of the ligate of FAK-X and pRRLsin, reflecting the presence of conferred antibiotic resistance in the transformed cells. However digestion with two of pairs of enzymes SalI and EcoRI, and SacI and EcoRI did not demonstrate the presence of the desired construct pRRLsin+FAK-X (Figure 37: C). Typically, 6-12 colonies following Midiprep were analyzed per experiment and repeated experiments were performed. Again, it was apparent that no meaningful digestion bands of appropriate size were seen following endonuclease digestion with either pair of enzymes and the migration patterns were similar to those in the absence of any enzymes.

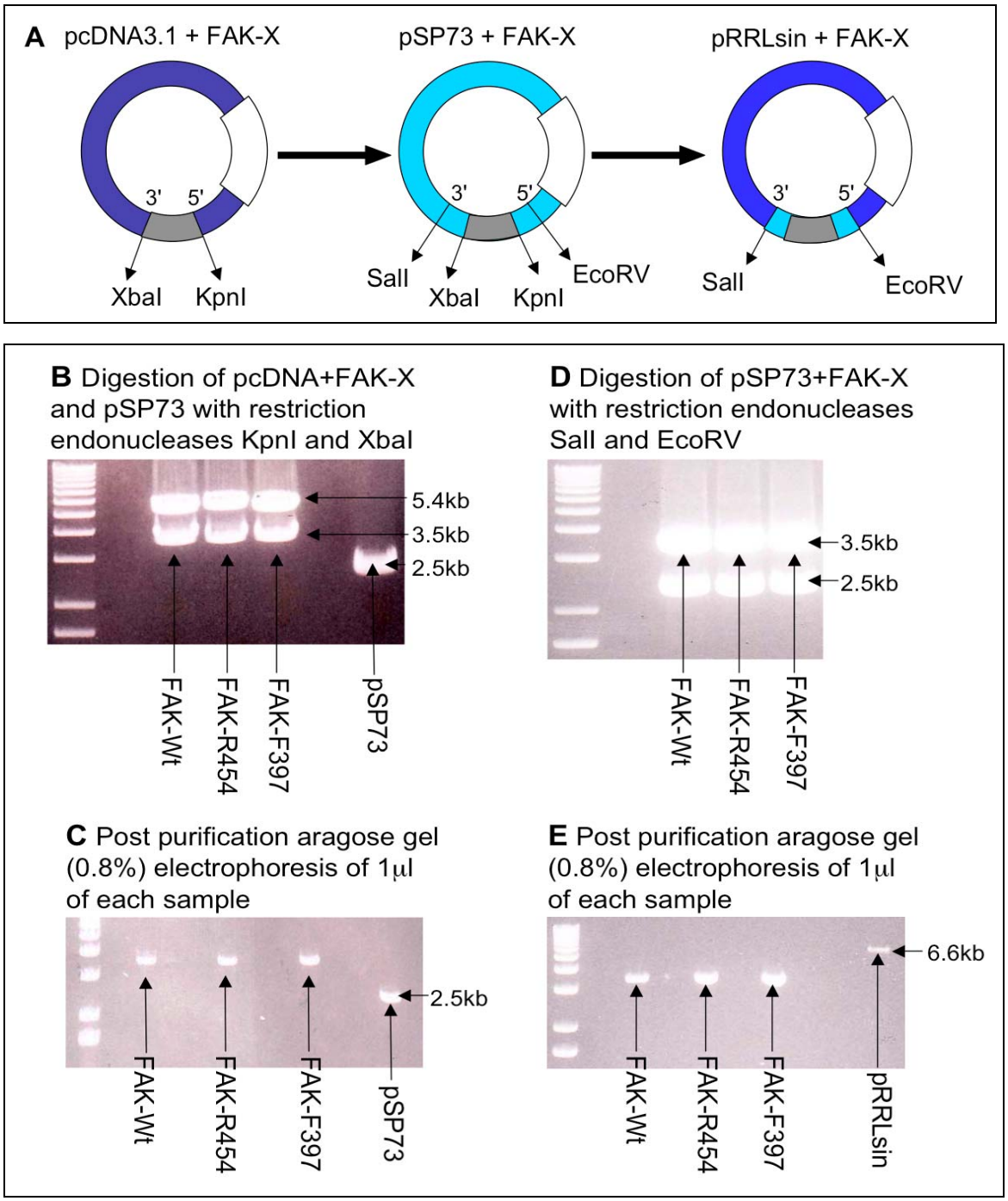
Failure of this method could not be easily explained but again the large size of the FAK-X inserts was thought to be a factor. In addition the relatively close proximity of the FAK-X inserts (3.5kb) to other fragments present from partial and complete digestion (3.05kb and 2.95kb) may also have contributed.

6.2.1.4 Results: Successful production of lentiviral constructs pRRLsin+FAK-X by use of the intermediate construct, pSP73+FAK-X and sequential digestion of pRRLsin with endonucleases SalI and EcoRV

Given the difficulties identified during the previous attempts to create the lentiviral constructs pRRLsin+FAK-X it was decided to attempt their production using a sequential digest of pRRLsin; first with SalI, followed, after electrophoresis, isolation and purification, by digestion with EcoRV. In this way it was hoped to overcome the close proximity of the SalI and EcoRV sites in the multiple cloning region of pRRLsin. Additionally the intermediate pSP73 vector was used to create the intermediate construct pSP73+FAK-X. This was then digested with SalI and EcoRV to produce FAK-X inserts with the required restriction specificities (as described in Section 6.2.1.1). In addition, transformation of the ligate of the FAK-X inserts and pRRLsin digest created as described above was performed with X-10 Gold ultra-competent cells. These cells were chosen to improve the probability of a successful transformation reaction. Finally the number of transformed colonies

analyzed was increased from approximately 12-24 in previous experiments to 40-60. This increase was based on the consideration that the probability of a successful ligation between these inserts and this vector at the restriction sites SalI and EcoRV was low - amongst a number of factors relating to this was the fact that EcoRV produces blunt ends. After a number of repeated experiments, successful production of the constructs was achieved. These results are shown in (Figure 38).

(Figure 38)



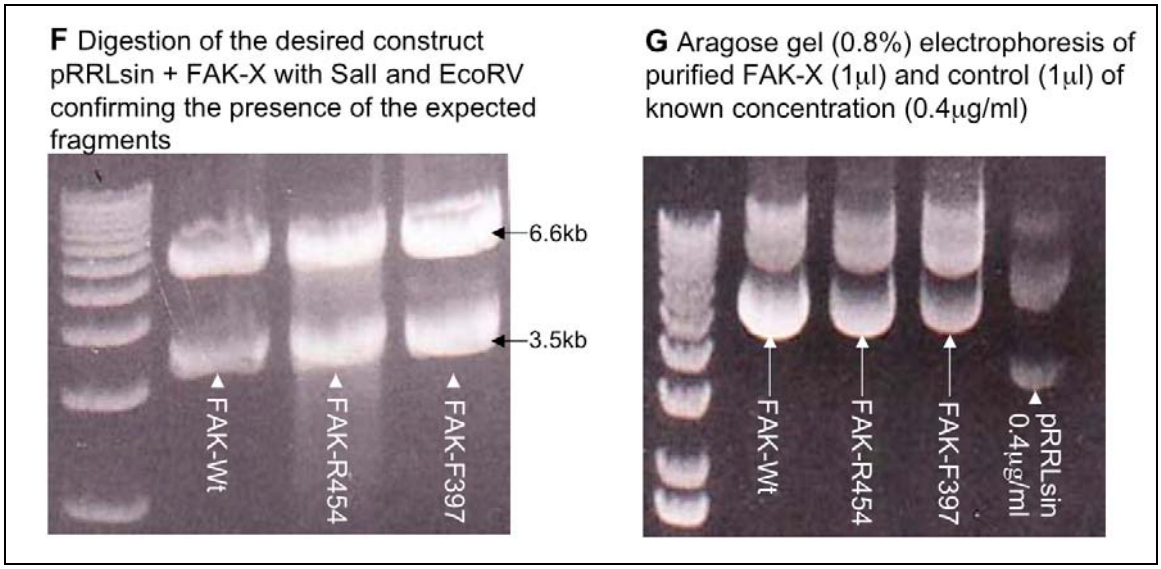


Figure 38: Use of the intermediate construct pSP73+FAK-X and sequential digest of pRRLsin both digested with SalI and EcoRV resulting in successful production of the lentiviral constructs pRRLsin+FAK-X. A: Schematic showing the intended transfer sequence and relevant restriction sites present in the initial constructs pcDND3.1+FAK-X, the intermediate constructs pSP73+FAK-X and the final constructs pRRLsin+FAK-X. The correct orientation of FAK-X transgenes is maintained throughout. B: An image showing KpnI and XbaI digest of the original constructs pcDNA3.1+FAK-Wt, -R454, and -F397 and the vector pSP73. The expected fragments are seen and indicated: pcDNA=5.4kb, FAK-X=3.5kb for each construct and pSP73=2.5kb. C: A post-purification image demonstrating the correct fragment sizes of FAK-X (3.5kb) and vector pSP73 (2.5kb) were present (and also the absence of any apparent contamination). Each insert FAK-X and vector pRRLsin were then separately ligated (as described in Section 6.2.1.1) to produce the intermediate constructs pSP73+FAK-X that were transformed into competent cells (DH5α). DNA from the transformed colonies was extracted and used in subsequent steps. D: An image showing SalI and EcoRV digest of all three intermediate constructs (pSP73+FAK-Wt, -R454 and -F397). The three FAK-X fragments indicated are at 3.5kb along with the vector pSP73 at 2.5kb. The vector pRRLsin was prepared separately by a sequential digestion with SalI, followed by EcoRV (data not shown). E: A post-purification image demonstrating the correct fragment sizes FAK-X (3.5kb) and vector pRRLsin (6.6kb) for each construct (and also the absence of any apparent contamination). Note the lower intensity signal of the vector at the same volume (1μl) is a reflection of the repeated digestion, purification, ligation, and transformation processes of the sequential digest. Each insert and vector were separately ligated to produce the constructs pRRLsin+FAK-X that were transformed into X-10 Gold ultra-competent cells. A large number of the colonies were seen after transformation and were analyzed (>50 for each construct) in each case only one colony with the desired construct pRRLsin+FAK-X was identified (See F). Midiprep of the successfully transformed colony was performed and the DNA recovered. F: This image shows a confirmatory

digest of the recovered DNA with SalI and EcoRV for each construct. It demonstrates that the correct fragment sizes were present FAK-X (3.5kb) and vector pRRLsin (6.6kb). G: An image showing 1μl of each successful construct (undigested) and 1μl of DNA of known concentration (in this case the solution was the vector pRRLsin at concentration 0.4μg/ml). Comparison of the intensities of the bands visualized allowed estimation of the concentration of the DNA present in each construct, which was 1μg/ml in each case. (For lentiviral transfection the concentration of the solution containing the plasmids was required).

The plasmid constructs pRRLsin+FAK-X were used for the lentiviral transfection of human keratinocytes (described in Section 2.7.2); these transduced keratinocytes were then used in the migration assay and other experiments presented here.

6.2.2 Results: Generation of the Src lentiviral vectors and their transduction into keratinocytes

Two Src (c-Src) transgenes were studied: "Wild-type" Src (Src-Wt) and a dominant negative mutant Src-K295M (referred to as Src-KM from now onwards). The plasmids containing the two c-Src genes were provided as a gift from Professor D. Schlapfer, Department of Immunology, The Scripps Research Institute, CA, USA. The relevant source and activity of each transgene are provided in detail in Section 2.7.2.1 and are summarized in (Table 3). Briefly, the lysine residue at position 295 lies within the kinase domain of Src. This site is modified in the dominant negative Src-KM, which has been well documented to be kinase inactive and has been widely used and studied (Mukhopadhyay et al., 1995). The plasmid constructs provided each contained one Src-X ("-X" representing each of -Wt, -KM) transgene that was inserted into a modified version of the vector pRK7 (referred to here as pRK7m for simplicity) at sites BamHI and EcoRI. Consideration of restriction sites within the Src genes and those within the two vectors, pRK7m and pRRLsin, showed that it was possible to directly transfer the Src genes from one vector to another at the same sites, BamHI and EcoRI while preserving the correct orientation of each gene. This simple approach is schematically shown in (Figure 39: A). As an initial stage of this protocol and to confirm the presence of the Src-X transgenes, digests of the pRK7m+Src-X constructs with BamHI and EcoRI were performed (Figure 39: B). A second confirmatory digest was performed with enzymes KpnI (acting at a site within pRK7m) and SphI (acting at a site within the Src-X genes). This data is not shown. Both digests demonstrated the presence of

appropriately sized fragments, in the case of the BamHI and EcoRI digest (Figure 39: B) this was Src-X=1.6kb in each case and the vector pRK7m 4.6kb. (The vector pRRLsin was also digested with BamHI and EcoRI and is shown as a band at 6.6kb on the left hand side of the image in Figure 39: B).

(Figure 39)

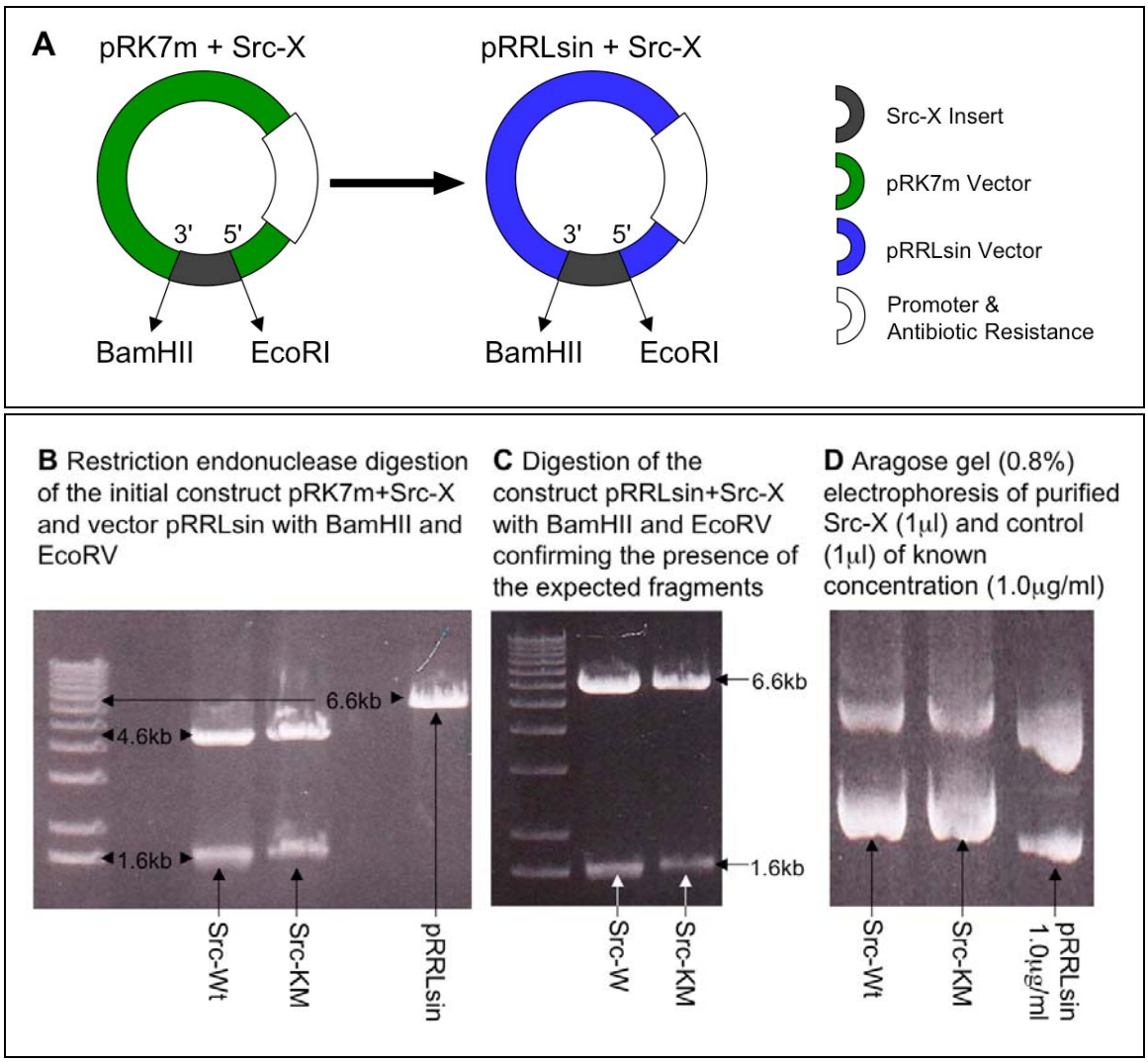


Figure 39: Direct transfer of the Src transgenes Src-X from the construct pRK7m+Src-X to the vector pRRLsin using restriction endonuclease digest with BamHI and EcoRI. A: Schematic showing transfer from the initial construct pRK7m+Src-X to the final pRRLsin+Src-X construct and relevant restriction sites BamHI and EcoRI. The correct orientation of Src-X transgenes is maintained. B: Image showing endonuclease digestion of the construct pRK7m+Src-X and vector pRRLsin with BamHI and EcoRI. The expected fragments, of the transgenes Src-Wt and Src-KM at 1.6kb, and of the vectors pRK7m and pRRLsin at 4.6kb and 6.6kb respectively are visible. C: Following purification and ligation of the transgenes Src-X and vector pRRLsin, Midiprep of transformed colonies was performed and the DNA (plasmids) recovered. Digestion with BamHI

and EcoRI of the plasmid DNA recovered (pRRLsin+Src-X) for each transgene resulted in the expected fragments. D: Image showing electrophoresis of 1μl of each construct pRRLsin+Src-X (undigested) and 1μl of a solution of plasmid DNA known concentration (this solution was the vector pRRLsin at concentration 1μg/ml). A comparison of the intensities of the bands allowed for an estimate of the concentration of the DNA present in each construct, which was 1μg/ml in each case.

These results confirm the presence of the Src-X transgenes in the constructs provided. In addition, the construct pRRLsin+Src-X was produced by ligation of purified insert Src-X and vector pRRLsin following BamHI and EcoRI digests. This ligate was transformed with DH5α competent cells and the construct pRRLsin-Src-X was recovered following Midiprep. Repeat digestion with BamHI and EcoRI confirmed the successful formation of the construct pRRLsin+Src-X (Figure 39: C). These constructs pRRLsin+Src-X were used in the lentiviral transfection of human keratinocytes (described in Section 2.7.2), and the transduced keratinocytes were then used in migration assay and other experiments presented here.

6.3 Results: Demonstration of transgene expression following lentiviral transfection of keratinocytes

The dominant negative transgenes described above were delivered to keratinocytes by means of lentiviral gene transduction system detailed in Section 2.7.2. In addition to the GFP control of the transfection process, Western blot was performed to demonstrate that all of the intended transgene products were subsequently expressed. The dominant negative genes used were FAK-F397 (auto-phosphorylation inactive) FAK-R454 (kinase inactive) and Src-KM (kinase inactive). Comparable "wild-type" genes FAK-Wt and Src-Wt (that are unaltered and with normal function) were used as controls. Cells were starved overnight in GF- media, trypsinized and prepared as if for a migration assay. They were then re-suspended in GF- media and re-plated onto type I collagen at 40-50% confluence. After 30 minutes all cells were adherent and the GF- media was changed to GF+. After a further 10 minutes the cells were lysed, the lysates normalized by spectrophotometric protein assay and 50μg total protein was resolved on SDS-PAGE gel before blotting (Figure 40).

(Figure 40)

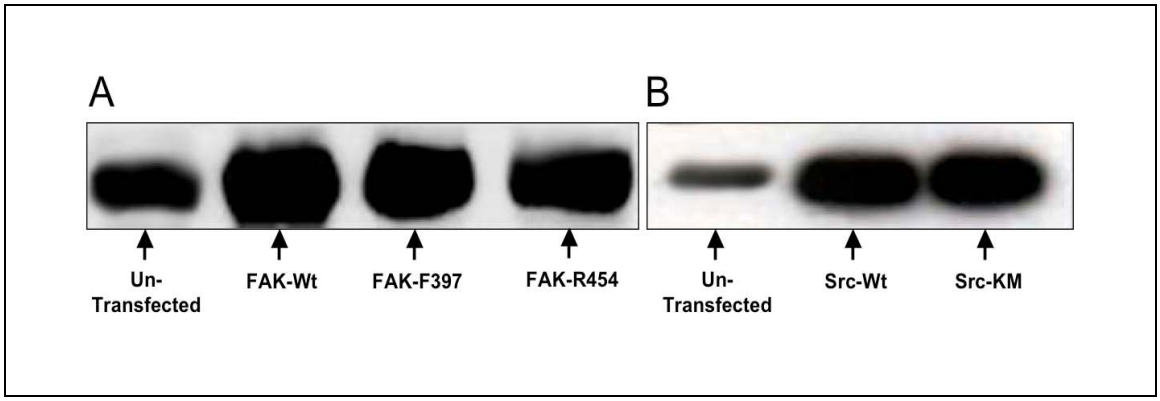


Figure 40: Western blots showing expression of the FAK and Src gene products in un-transfected and transfected keratinocytes. Using the lentiviral system of transgene delivery keratinocytes were transfected with "wild-type" (-Wt) and selected dominant negative transgenes (FAK-F397, FAK-F454 and Src-KM). A: FAK blots of whole cell lysates are visualized with anti-FAK and show a 4.2-fold increase in FAK-Wt, 3.8-fold increase in FAK-F397 and 3.5-fold increase in FAK-R454 expression. B: Src blots are visualized with anti-Src and show 5.3-fold increase in Src-Wt and 5.6-fold increase in Src-KM expression. For each gene product, expression in un-transfected keratinocytes was used as the reference to calculate increased expression.

These Western blots demonstrate over-expression of each of the transgene products (between 3.5-5.6 fold above levels in un-transfected keratinocytes). This finding was confirmed on three repeated experiments and the increases were each statistically significant (at $p<0.05$).

6.4 Results: Effects of FAK and Src "wild-type" and dominant negative mutants on the tyrosine phosphorylation of FAK

The level of tyrosine phosphorylation of FAK (largely due to activity of Src family kinases) has been shown in a number of cells (notably fibroblasts) to be linked to the rate of disassembly of focal adhesions. Repeated cycles of phosphorylation and de-phosphorylation of FAK (together with other proteins such as paxillin and $p130^{CAS}$) is thought to underlie the formation, translocation and disassembly of focal adhesions that, along with coordinated changes in the actin cytoskeleton and actin-myosin activity, enables cells to migrate.

The levels of tyrosine phosphorylation of FAK in cells transfected with FAK and Src transgenes (and in un-transfected cells) were evaluated by Western blot. Demonstration of the level of tyrosine phosphorylation present in each cell type provided relevant information and also a reference for interpretation of the subsequent migration assays evaluated under similar conditions, type I collagen and non-migratory poly-L lysine in the presence and absence of growth factors. Cells were starved overnight in GF- media, trypsinized and prepared as if for a migration assay. The cells were then re-suspended in GF- media and re-plated at 40-50% confluence onto either poly-L lysine (Poly) or type I collagen (Col I). Pairs of plates were prepared for each cell type to be used in the presence (GF+) and absence (GF-) of growth factor supplementation [4 pairs for un-transfected cells: Col I (GF+), Col I (GF-), Poly (GF+), Poly (GF-) and 2 pairs for all other cell types: Col I (GF+) and Col I (GF-)]. After 30 minutes, all cells were adherent and the GF- medium was changed to GF+. One plate from each pair was taken immediately for Western blot analysis and after a further 15 minutes the second plate of each pair was taken. In each case, the cells were lysed and the lysates normalized after spectrophotometric protein assay; 50μg total protein was resolved on SDS-PAGE gel before blotting (Figure 41).

(Figure 41)

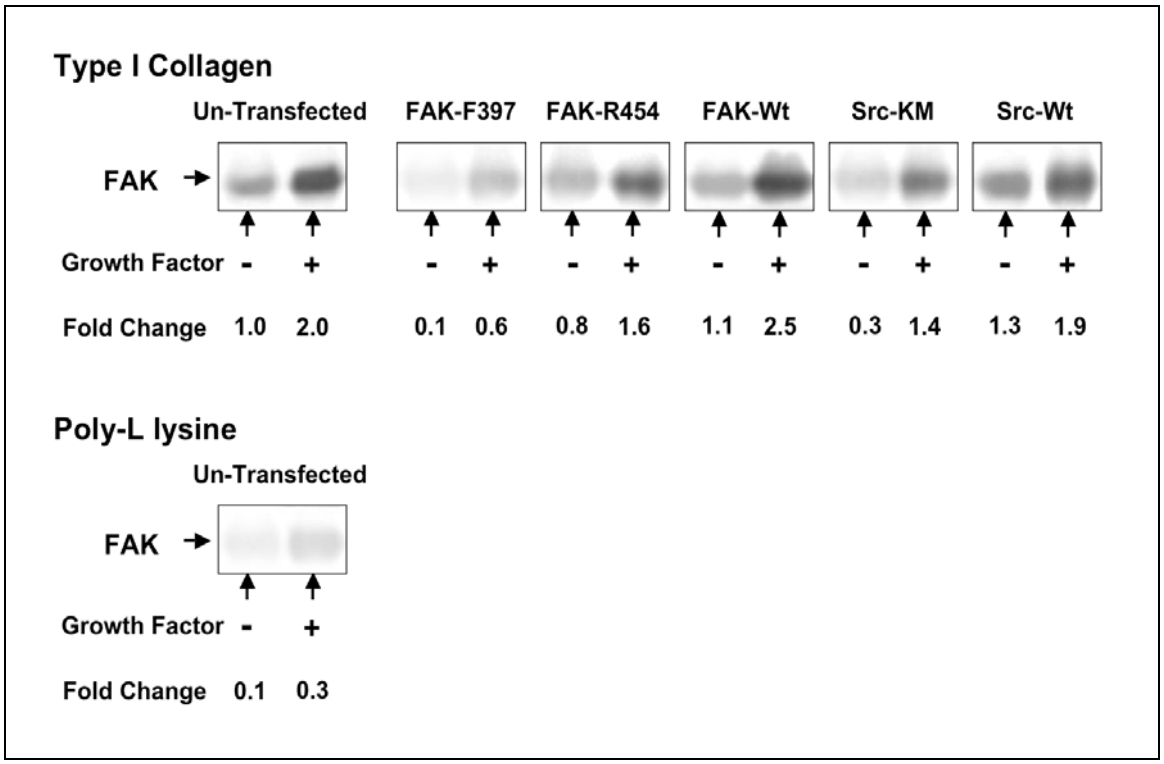


Figure 41: Western blot showing FAK tyrosine phosphorylation (p-Tyr) expression in keratinocytes transfected with FAK and Src "wild-type" and dominant negative genes (and in un-transfected keratinocytes) on type I collagen and poly-L lysine (un-transfected only) in the presence and absence of growth factors. For comparison of FAK p-Tyr expression between different cell types, the level of FAK p-Tyr expression in un-transfected keratinocytes on type I collagen in the absence of growth factors was used as a reference (1.0), top left image.

In all cases (all cell types and substrates) the presence of growth factors was associated with a significant ($p<0.05$) increase in FAK p-Tyr expression compared with its absence.

Un-transfected keratinocytes on poly-L lysine (lower half of Figure 41) showed minimal FAK p-Tyr expression: 0.1-fold in the absence of growth factors and 0.3-fold in their presence (relative to un-transfected cells on type I collagen in the absence of growth factor; upper row, first column, Figure 41). The addition of growth factors to un-transfected cells on type I collagen resulted in a 2.0-fold increase in FAK p-Tyr expression ($p<0.05$) (upper row, second column, Figure 41) and FAK p-Tyr levels in that case was significantly higher than the same cells in the presence of growth factor on poly-L lysine ($p<0.05$).

Transfected keratinocytes over-expressing FAK-Wt (upper row, fourth column, Figure 41) cultured on type I collagen, showed FAK p-Tyr expression of 1.1-fold in the absence and 2.5-fold in the presence of growth factors (relative to un-transfected cells on type I collagen in the absence of growth factors). The increase was significant only in the presence of growth factors ($p<0.05$). Note: FAK-Wt was over-expressed 4.2-fold in these cells in comparison to un-transfected cells (Figure 40). Over-expression of Src-Wt (upper row, sixth column, Figure 41) resulted in a level of FAK p-Tyr of 1.3-fold in the absence and 1.9-fold in the presence of growth factors (relative to un-transfected cells on type I collagen in the absence of growth factors). This increase was significant in the presence and notably in the absence of growth factors ($p<0.05$ in both cases). Note: In Src-Wt transfected cells, FAK was not over-expressed and levels of FAK were not significantly different from those in un-transfected cells, data not shown, Src-Wt was over expressed 5.3-fold (Figure 40).

For keratinocytes transfected with dominant negative genes, differing patterns of FAK p-Tyr were seen. Over expression of FAK-F397 (upper row, second column, Figure 41) in keratinocytes plated on type I collagen resulted in minimal FAK p-Tyr expression: 0.1-fold in the absence, and 0.6-fold in the presence of growth factors. This level of FAK p-Tyr expression was similar to that shown by keratinocytes on poly-L lysine, and in both the presence and absence of growth factors, and was significantly lower ($p<0.05$) than that shown by un-transfected keratinocytes under the same growth factor exposure on type I collagen. Over-expression of FAK-R454 (upper row, third column, Figure 41) in keratinocytes on type I collagen resulted in 0.8-fold increase expression of FAK p-Tyr in the absence of growth factors and 1.6-fold in their presence. This level of FAK p-Tyr expression was significantly lower ($p<0.05$) in both cases than that shown by un-transfected cells under the same growth factor exposure on type I collagen, but was significantly ($p<0.05$) greater than that seen with over-expression of FAK-R397. Keratinocytes on type I collagen, over-expressing Src-KM (upper row, fifth column, Figure 41) showed FAK p-Tyr at 0.3-fold in the absence of growth factors and 1.4-fold increase in their presence. This level was intermediated (in both cases) between that seen with FAK-F397 (lower expression) and FAK-R454 (higher expression). It was significantly ($p<0.05$) lower in both the presence and absence of growth factors than the FAK p-Tyr expression shown by un-transfected cells under equivalent growth factor exposure on type I collagen and significantly higher ($p<0.05$) than on expression on poly-L lysine.

6.5 Inhibition of FAK and Src during human keratinocyte migration on type I collagen following transduction of "wild-type" and dominant-negative genes and exposure to chemical inhibitors

Briefly, integrins are involved directly in the attachment of cells to extracellular matrix (adhesion), in signalling leading to the formation and maintenance of the migratory phenotype, and in important changes occurring in the actin cytoskeleton linked to migration. Integrin binding to extracellular matrix leads to clustering and intracellular accumulation of large numbers of proteins of which FAK is among the first to be recruited. FAK has been linked to the formation, maturation and particularly the turnover (disassembly) of focal adhesions. The rate of turnover regulates the strength of cellular adhesion. FAK activation following integrin-ECM stimulation begins with its auto-phosphorylation at Tyr-397 and SH2-mediated binding of Src (Schaller et al., 1994). Src activation begins with its translocation to sites of developing focal adhesions by a process that is actin-dependent, but independent of FAK (Fincham and Frame, 1998). Once activated, Src phosphorylates a number of proteins including FAK - maximal integrin-ECM stimulated FAK activity is correlated with the binding and kinase activity of Src family members. FAK and Src have many activities that are related to interdependent functioning (and some functions that are independent of each other).

To explore the involvement of FAK and Src (c-Src) in mediating these effects during keratinocyte migration, functions of FAK and separately Src, were inhibited by transfection of dominant negative genes delivered in lentiviral vector constructs (as derived in Section 6.2). Experiments were performed to test the hypothesis that dominant negative inhibition of the key binding and activation site of FAK, Tyr-397 and or dominant negative inhibition of FAK kinase activity would significantly decrease keratinocyte migration on the pro-migratory substrate type I collagen. Similarly, dominant negative inhibition of the kinase activity of Src (c-Src) that is required for maximal activation of FAK would also have an effect on keratinocyte migration under the same conditions. To corroborate these results, inhibition of Src, using PP2 a specific chemical inhibitor of Src, was also evaluated under the same conditions (a similar inhibitor of FAK was not available). The effects of such inhibition were evaluated on keratinocyte migration on type I collagen in the presence and absence of growth factors. It was anticipated that such inhibition by either method would reduce migration. In addition, the effects of inhibition of FAK and Src on tyrosine

phosphorylation of downstream targets such as paxillin and elements of the MAP kinase pathways (Erk, p38 and JNK) were evaluated by Western blot.

6.5.1 Results: Dominant negative inhibition of FAK and Src during human keratinocyte migration on type I collagen

The dominant negative transgenes delivered via the lentiviral gene transduction system (detailed in Section 2.7.2, with GFP gene transduction performed as the procedural control) were FAK-F397 (auto-phosphorylation inactive), FAK-R454 (kinase inactive) and Src-KM (kinase inactive). "Wild-type" genes (unaltered and with normal function) FAK-Wt and Src-Wt were transduced for use as controls within the migration assays assay. Typically, four types of cultured keratinocytes were prepared for each gene to be studied. Three of these were keratinocytes transfected respectively with; lentivirus containing the vector pRRLsin alone (no transgene inserted), lentivirus containing "wild-type" gene, and lentivirus(es) containing the inactivated (dominant negative) form(s) of the same gene. The fourth type of keratinocytes was un-transduced. By studying both keratinocytes containing transduced genes and un-transduced keratinocytes it was hoped that some of the effects of the altered stoichiometry would be minimized. Potential cytotoxicity caused by gene over-expression was assessed by Trypan blue exclusion assay, and cell proliferation studies. These assays were performed following completion of the gene transduction process, in a subculture of each transfected keratinocyte type, prepared and handled identically to those undergoing the migration and wound assays. By either assessment, cytotoxicity did not occur following any of these gene transductions. In preparation for the assays cells were starved overnight in GF- media, trypsinized and re-suspended in either GF- or GF+ medium. They were then re-plated (3000 viable cells/well) in each well of 12 well cell culture plates prepared with the two substrates; non-migratory poly-L lysine and pro-migratory type I collagen. Migration was allowed to proceed for 16-hours. The results of the colloidal gold migration assay are shown in (Figure 42).

(Figure 42)

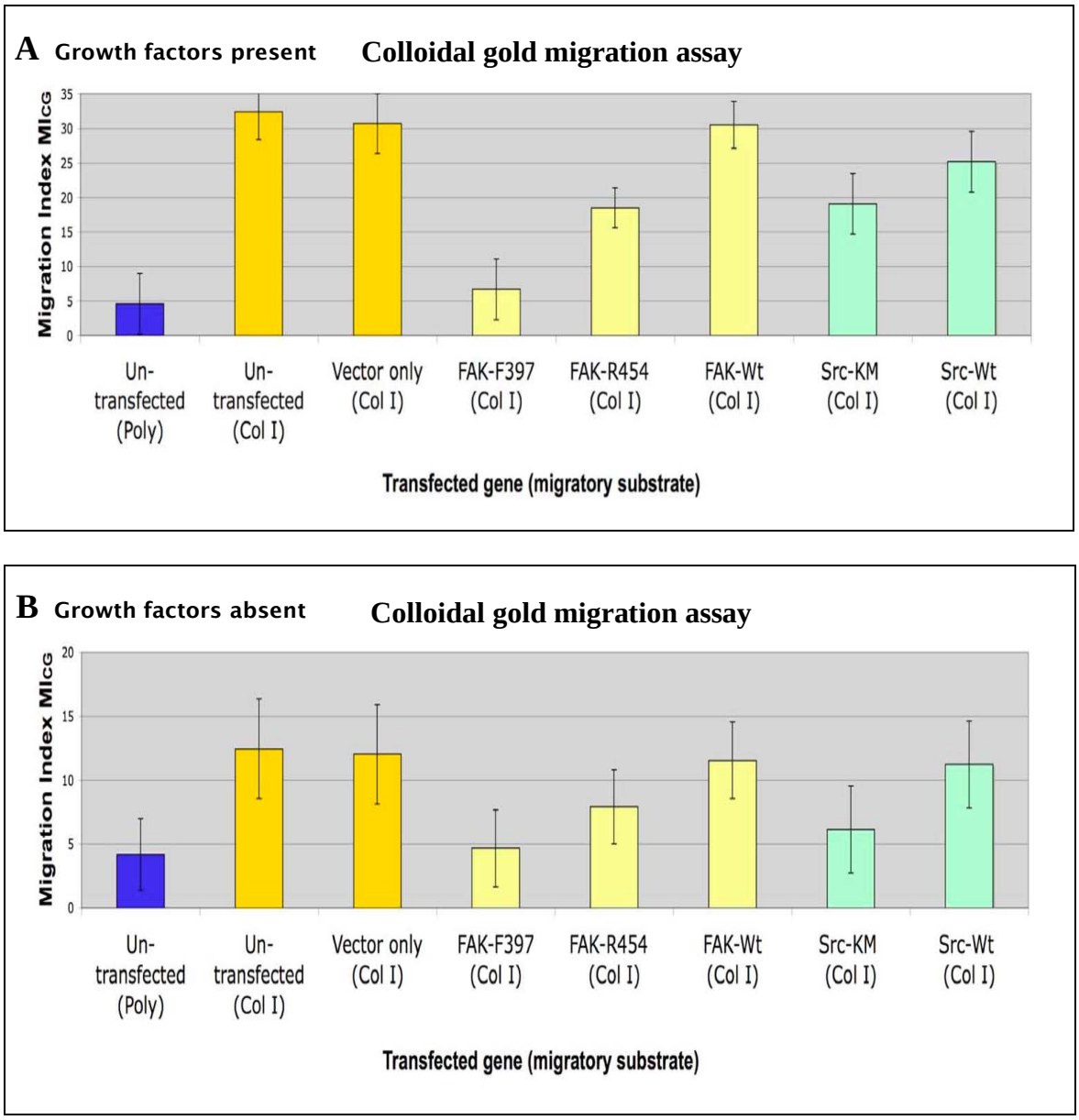


Figure 42: Colloidal gold migration assay of keratinocyte migration on type I collagen (Col I) and non-migratory poly-L lysine (Poly) following transfection with FAK and Src non-receptor tyrosine kinase transgenes "wild-type" and dominant negative forms. A: Shows migration indices (MI_{CG}) for keratinocytes migrating in the presence of growth factors. B: Shows migration indices (MI_{CG}) for keratinocytes migrating in the absence of growth factors. In both cases keratinocyte migration indices are shown for cells with no transfected gene inserted (Un-transfected), following insertion of the pRRLsin vector (Vector only), as well as following transfection of the "wild-type" (-Wt) functional genes and non-functional dominant negative form of the genes. Dominant negative transgenes studied for FAK include; FAK-F397 (auto-phosphorylation inactive) and FAK-R454 (kinase inactive). The dominant negative transgene for studied for Src was Src-KM (kinase

inactive). (Un-transfected keratinocytes migrating on poly-L lysine were used as a negative control).

In these experiments un-transfected keratinocytes and keratinocytes transduced with the vector pRRLsin only (Vector only) migrated on type I collagen at the previously observed level in both assays, and the MI's observed were not statistically significantly different from those seen previously ($p>0.05$ for each comparison). This was true in both the presence (Figure 42: A. $MI_{CG}\approx31$-33) and absence (Figure 42: B. $MI_{CG}\approx11.5$-12.4) of growth factors. As expected, appreciable migration on poly-L lysine did not occur ($MI_{CG}\approx4$-5 with or without growth factors), and the level of migration observed was statistically different from that seen during the migration of keratinocytes transfected with both the vector alone and the un-transfected cells under otherwise similar conditions ($p<0.05$ for each comparison).

Following transduction and over-expression of FAK-Wt, migration of keratinocytes in both migration assays and in the presence and absence of growth factors was indistinguishable from that observed for keratinocytes transduced with the vector pRRLsin only and the un-transfected cells ($p>0.05$ for each comparison). Notably, keratinocytes over-expressing Src-Wt showed significantly reduced migration ($MI_{CG}\approx25$) ($p<0.05$) in the presence of growth factors, although it was unchanged ($MI_{CG}\approx11.2$) ($p>0.05$) in the absence of growth factors, compared to un-transfected controls (that showed $MI_{CG}\approx33$ and $MI_{CG}\approx12.4$ respectively).

Over-expression of dominant negative genes (FAK-F397, FAK-R454 and Src-KM) produced different effects compared to un-transfected cells and to each other. Over-expression of all dominant negative genes significantly reduced keratinocyte migration on type I collagen in both the presence and absence of growth factors ($p<0.05$ for all comparisons). As expected from the literature and as predicted, over-expression of the dominant negative mutant FAK-F397 (that prevents auto-phosphorylation of FAK a central event in focal adhesion formation and turnover) had the most profound effect on keratinocyte migration that was reduced to a level no different ($p>0.05$ in each case) from that of keratinocytes attached to non-migratory poly-L lysine in both the presence ($MI_{CG}\approx6.6$) and absence of growth factors ($MI_{CG}\approx4.6$). This level of migration was also significantly lower than that seen following either FAK-R454 or Src-KM transfection in the presence of growth factors ($p<0.05$ for all comparisons). In the absence of growth factors

Src-KM produced equally low migration that was not statistically different from FAK-F397 ($p>0.05$), while FAK-R454 showed reduced migration significantly less than un-transfected cells but significantly greater than FAK-F397 (and un-transfected cells on poly-L lysine) ($p<0.05$).

Transduction and over-expression of the dominant negative mutants FAK-R454 and Src-KM, which lack kinase activity, produced similar effects on the migration of keratinocytes in the presence and absence of growth factors. Both reduced migration compared to un-transfected cells in the presence and absence of growth factors and their migration was not significantly different from each other in each case ($p>0.05$). As mentioned their migration was greater than that of FAK-F397 transfected cells in the presence of growth factors. Both FAK-R454 and Src-KM resulted in reduced migration in the absence of growth factors (below that seen by un-transfected cells on type I collagen), but only Src-KM reduced migration to a level no different from cells on poly-L lysine or FAK-F397 transfected cells.

The data presented in these experiments confirmed the hypothesis that dominant negative inhibition of the FAK auto-phosphorylation site and FAK and Src kinase activity would significantly decrease keratinocyte migration on the pro-migratory substrate type I collagen both in the presence and absence of growth factors. It has already been demonstrated (in Chapter 4 and Chapter 5) that keratinocytes in the presence of a pro-migratory substrate such as type I collagen migrate less ($MI_{CG}\approx12$) in the absence of growth factors than in the presence of growth factors ($MI_{CG}\approx32$), but more than that seen on a non-migratory substrate poly-L lysine ($MI_{CG}\approx5$). The presence of growth factors significantly enhances keratinocyte migration on the pro-migratory substrate type I collagen in these same experimental systems. Additionally it was shown here that inhibition of FAK auto-phosphorylation (by over-expression of FAK-F397) produced a profound decrease in migration, both in the presence and absence of growth factors ($MI_{CG}\approx4.6$-6.6). A reasonable interpretation of these findings is that inhibition of FAK auto-phosphorylation negates the stimulation of migration from the interaction with the pro-migratory substrate (integrin-ECM stimulated) as well the augmentation of migration that typically follows exposure to soluble growth factors (GF -stimulated EGFR). Inhibition of either FAK or Src kinase in keratinocytes migrating on type I collagen also reduced the migration previously observed and proposed to be induced by integrin-ECM stimulation alone (that is in the absence of growth factors). Under these conditions (absence of growth factors) Src kinase inhibition produced greater

inhibition (like FAK-F397) to a level no different to that seen on poly-L lysine, while inhibition of FAK kinase reduced migration significantly below that normally seen on type I collagen but not to the same low level. In both cases as was hypothesized, inhibition of either FAK- or Src-kinases would limit the enhancement of migration typically seen following growth factor stimulation - while this augmentation did occur it was significantly less than is usually seen for both transgenes FAK-R454 and Src-KM. Inhibition of these kinases might reasonably be interpreted as reducing (but not abolishing) the effects of both integrin-ECM and growth factor stimulated migration.

Interestingly over-expression of FAK-Wt had no detectable effect on migration following stimulation by either integrin-ECM or growth factors. However, over-expression of Src-Wt, prevented the full augmentation of migration induced by growth factor stimulation but had no detectable effect on integrin-ECM stimulated alone (in the absence of growth factors). This unexpected and unreported finding remains unexplained, although a proposed explanation for this finding is discussed and evaluated further in Section 6.5.4 below.

6.5.2 Results: Chemical inhibition of Src during human keratinocyte migration on type I collagen

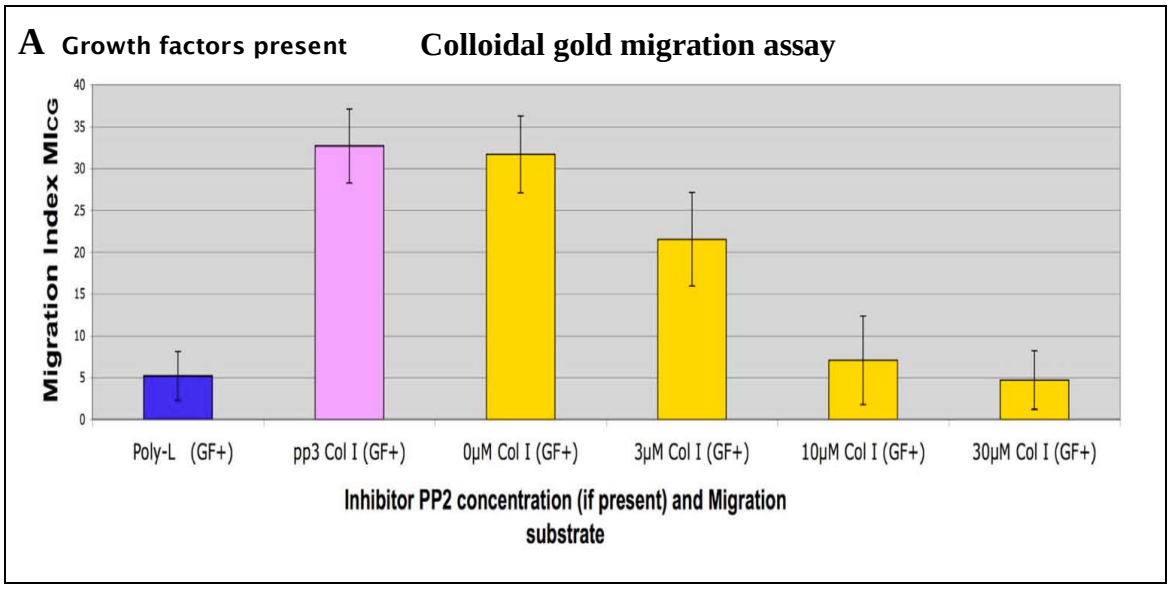
Inhibition of FAK (auto-phosphorylation and kinase activity) and Src (kinase activity) by over-expression of dominant negative genes demonstrated involvement of these proteins in the migration of human keratinocytes on type I collagen. Similar experiments using chemical inhibition were performed to further evaluate the effect of Src inhibition on migration (no suitable FAK inhibitors were available). This alternative approach provided complementary data to corroborate the dominant negative transgene results and gave additional findings. As described previously, chemical inhibitors are cell-permeable compounds that can be used to assess the physiological roles of protein kinases in unaltered and transformed cell lines. These agents inhibit an endogenous protein kinase without the need for over expression of transgenes (dominant-negatives), which can (by altered stoichiometry) cause the specificity of signalling to break down and lead to erroneous conclusions. However, uniquely inhibiting the action of only one kinase is usually not possible even with the most selective inhibitors.

A selective inhibitor of Src kinase activity was chosen to complement the dominant negative transgene Src-KM (kinase inactive) in these experiments. The Src inhibitor PP2, a

pyrazolopyrimidine, (the corresponding negative control was PP3) was used at concentrations previously described 1, 3, 10, 30μM (Hanke et al., 1996). At these concentrations the PP2 inhibitor has been shown to competitively inhibit the kinase activity of several members of the Src family; c-Src, Fyn, and others (p56lck etc.). Inhibition of other kinases such as JAK and of EGFR has been shown to occur but only at much higher concentrations than these. (FAK kinase activity is not inhibited directly by PP2, but inhibition of Src family members by PP2 is associated as might be expected with reduced phosphorylation at Try-577 and Tyr-576 on FAK). A summary of its properties is provided in (Table 2).

Potential cytotoxicity was assessed by Trypan blue exclusion assays, and cell proliferation studies. Cytotoxicity assays were performed under identical conditions and at the same concentrations used in subsequent migration assays and by either assessment did not occur. In preparation for the migration assays, keratinocytes were starved overnight in GF- media, trypsinized and re-suspended in either GF- or GF+ media with and without supplementation with PP2 or the control PP3. Keratinocytes were then incubated for 30 minutes before being re-plated onto poly-L lysine and type I collagen (at deposition concentrations in Table 1). Migration was continued for 16-hours. The results of the colloidal gold migration assay in the presence of inhibition of Src family kinases with PP2 and control PP3 are shown (Figure 43). Identical experiments were repeated (three times) under the same conditions.

(Figure 43)



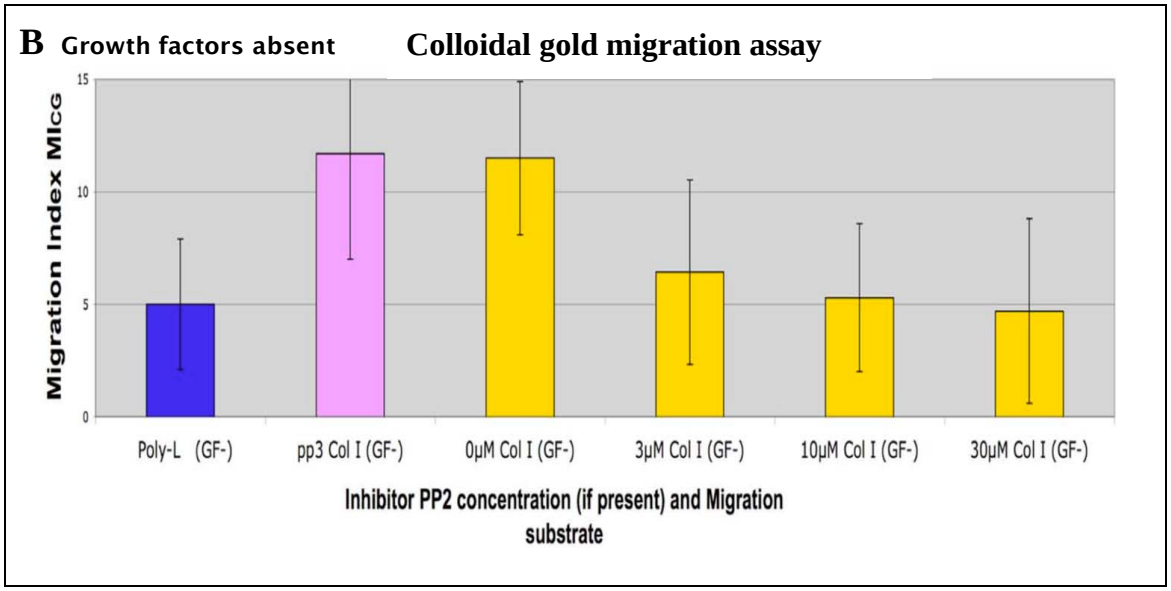


Figure 43: Chemical inhibition of Src family kinases by PP2 during human keratinocyte migration on type I collagen (Col I) and non-migratory poly-L lysine (Poly-L) in the presence (GF+) and absence (GF-) of growth factors. A: Migration indices (MI_{CG}) for keratinocytes migrating in the colloidal gold assay in the presence of growth factors. B: Migration indices (MI_{CG}) for keratinocytes migrating in the absence of growth factors. (Results for exposure to the control agent PP3 are also shown, pink column). A further control, migration on poly-L lysine in the presence and absence of growth factors, and absence of exposure to the inhibitor is also shown.

In the presence and absence of growth factors, keratinocytes migrating on type I collagen exposed to the control PP3 (pink column) showed MI_{CG} not significantly different ($p>0.05$) from keratinocytes migrating in the absence of PP2 (concentration 0μM). The results were consistent with previously obtained vales ($MI_{CG} \approx 32$ in the presence of growth factors and $MI_{CG} \approx 12$ in their absence). In all cases (the presence of PP3 and absence of PP2), the MI_{CG} observed on type I collagen were significantly different ($p<0.05$) from that seen on the non-migratory control substrate poly-L lysine (Poly-L) under the same conditions (MI_{CG} for poly-L lysine is ≈ 5 in the presence and absence of growth factors). Additionally, keratinocyte migration on type I collagen, in the presence and absence of growth factors, showed a clear, dose-dependent inhibition by PP2. Migration indices were reduced (at concentrations of 10μM and above) to the level seen on non-migratory poly-L lysine under the same conditions.

More dramatic inhibition of keratinocyte migration was seen with the Src-family kinase inhibitor PP2 in comparison to that seen with over-expression of Src-KM. In the case of PP2, migration indices MI_{CG}, were reduced (at concentrations of 10 or more μM) to those seen on poly-L lysine ($MI_{CG} \approx 5$) in both the presence and absence of growth factors. Src-KM represents c-Src kinase inhibition only. Keratinocytes over-expressing Src-KM showed the same inhibition of migration in the absence of growth factors as was seen with PP2 (at concentrations of 10 or more μM). But in the presence of growth factor Src-KM inhibition resulted in only moderately reduced migration $MI_{CG} \approx 19$ (that was significantly ($p<0.05$) greater than that seen on poly-L lysine with growth factor exposure and also greater than that seen by un-transfected cells on type I collagen in the absence of growth factors).

The effects on keratinocyte migration demonstrated with PP2 exposure seem likely to be due to the inhibition of kinase activity in all Src-family members rather than just in c-Src as occurred with Src-KM over-expression. Inhibition of all Src family kinases (PP2 inhibition) is similar in its effect on migration to that demonstrated with inhibition of the auto-phosphorylation site of FAK by the dominant negative FAK-F397.

These findings lead reasonably to the conclusions that the auto-phosphorylation of FAK and kinase activity distributed amongst the Src-family kinases are required for keratinocyte migration on type I collagen. Their inhibition negates contributions to migration from integrin-ECM stimulation as well the augmentation of migration that follows exposure to soluble growth factors (EGFR-GF stimulated) in these two migration assays.

6.5.3 Results: Effects of inhibition with dominant negative FAK and Src on activity in the MAP kinase pathways (Erk, p38, and JNK) and on phosphorylation on Tyr-118 a key a site on paxillin

Western blot was used to evaluate the effects of over-expression of FAK and Src transgenes ("wild-type" and dominant negative inhibitors) on tyrosine phosphorylation of the downstream mediator of migration paxillin. In the presence of these same transgenes and by the same means (Western blot) activity in the MAP kinase pathways (Erk, p38 and JNK) was evaluated. Under these conditions it was hypothesized that the changes in paxillin phosphorylation (at Tyr-118) would be predictably altered and linked to observed changes in keratinocyte migration in the two assays. Similarly, it was anticipated that activity in the Erk MAP kinase pathway (and possibly in the SAPK pathways; JNK and p38) would prove

to be linked to changes in FAK and Src activity and again to changes in migration. In all cases this is hypothesized to be likely to occur in the presence and absence of growth factors and if true will demonstrate a critical link between integrin-ECM and growth factor stimulated migration. A similar analysis was performed on un-transfected keratinocytes plated on poly-L lysine as a non-migratory control.

Activity in the JNK MAP kinase pathway in the presence of FAK and Src transgenes was absent, showing the same response to anti-JNK and anti-phospho-JNK as demonstrated in Section 5.5 (Figure 27); further results are not shown. Similarly to Section 5.6 (Figure 28) confirmation was made that showed the JNK pathway was intact in FAK and Src transfected keratinocytes by exposure to LPS and TNFα; results are not shown.

In preparation for Western blot, all cells were starved overnight in GF- medium, trypsinized and prepared otherwise as if for a migration assay. They were re-suspended in GF- medium and re-plated onto type I collagen at 40-50% confluence. After 30 minutes all cells were adherent and the GF- medium was changed to GF+ (except for one control group of cells transfected with Vector only that were maintained in GF- medium). After a further 15 minutes the medium was removed, the cells were lysed in preparation for Western blot analysis and the lysates were normalized following spectrophotometric protein assay. 50μg total protein per lane was resolved on SDS-PAGE gel before blotting with specific antibody to phospho-Erk, phospho-p38 and phospho-paxillin (Tyr-118).

The results of these Western blots are shown in (Figure 44). Note: Un-transfected keratinocytes and keratinocytes transfected with the vector pRRLsin, were also subjected to Western blot under the same conditions as the “wild-type” and dominant negative transfected cells, that is migration on type I collagen in the presence and absence of growth factors. The data is not shown here for simplicity, but was previously demonstrated and shown in (Figure 27) for un-transfected cells. Vector only transfected cells produced the same results as un-transfected keratinocytes.

(Figure 44)

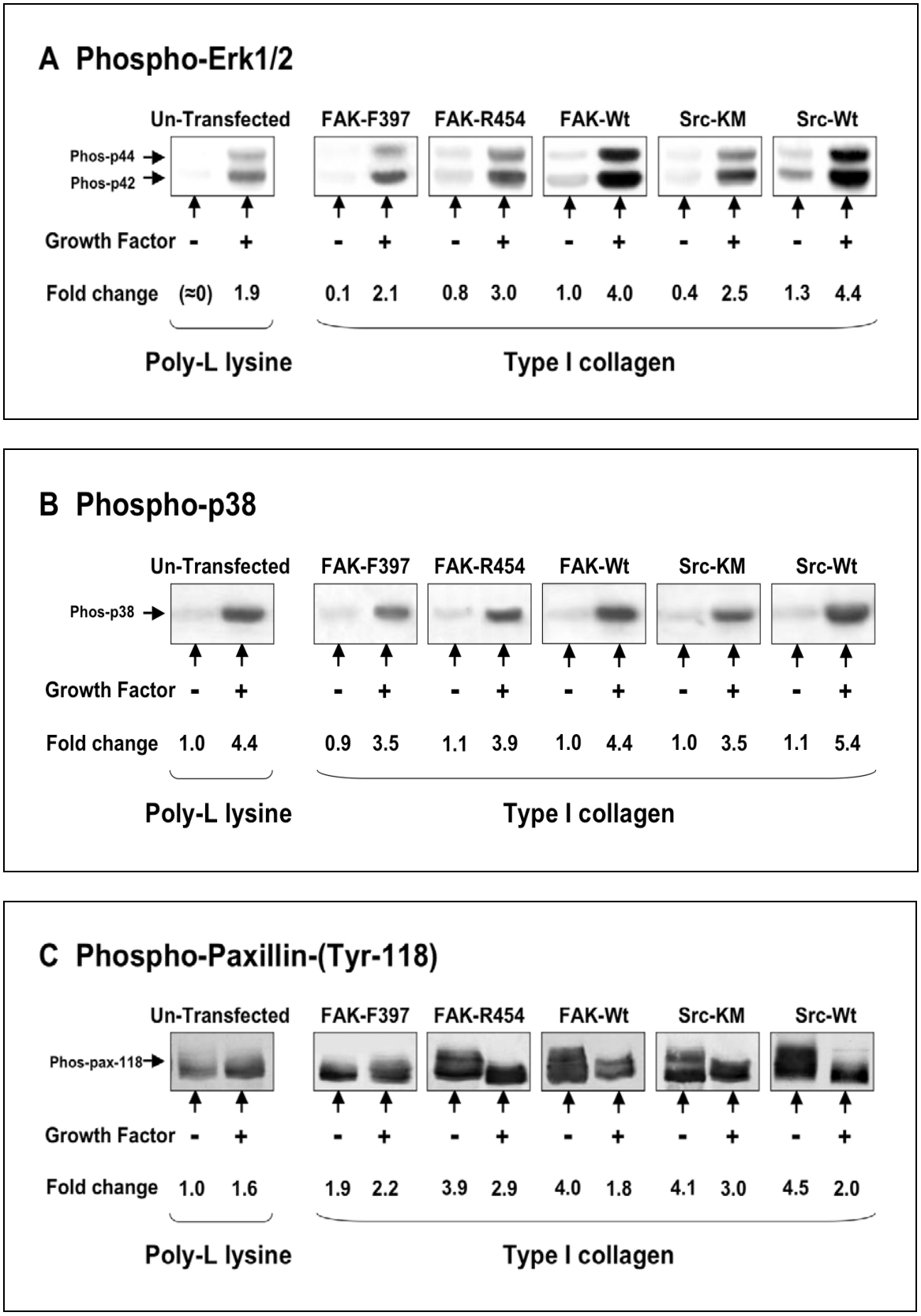


Figure 44: Western blot of keratinocytes migrating on type I collagen (and poly-L lysine) showing expression of phosphorylated Erk1/2, p38, and paxillin (Tyr-118) in the presence and absence of

growth factors. Results for "wild-type" FAK-Wt and Src-Wt and dominant negative inhibitors FAK-F397, FAK-R454, and Src-KM are shown in addition to those for un-transfected keratinocytes migrating on poly-L lysine. A: Phosphorylated Erk1/2 expression (Phos-p44/Phos-p42) visualized with anti-phospho-Erk1/2 in each cell type in the presence (+) and absence (-) of growth factors. Fold change is also shown for each cell type and condition relative to the level of expression shown in FAK-Wt cells in the absence of growth factors (defined as 1.0). B: Phosphorylated p38 expression (Phos-p38) visualized with anti-phospho-p38 in each cell type in the presence (+) and absence (-) of growth factors. Fold change is also shown for each cell type and condition relative to the level of expression shown in FAK-Wt cells in the absence of growth factors (defined as 1.0). C: Phosphorylated paxillin (Tyr-118) expression (Phos-pax-118) visualized with anti-phospho-paxillin-(Tyr-118) in each cell type in the presence (+) and absence (-) of growth factors. Fold change is also shown for each cell type and condition relative to the level of expression shown in un-transfected cells on poly-L lysine in the absence of growth factors (defined as 1.0).

Expression of phospho-Erk1/2 (phos-p44/phos-p42) is shown in (Figure 44: A), phospho-p38 (phos-p38) in (Figure 44: B) and phospho-paxillin-(Tyr-118) (phos-pax-118) in (Figure 44: C). Keratinocytes transduced with FAK-Wt (fourth column in panels A and B) showed an identical pattern of phospho-Erk1/2 and phospho-p38 activity as un-transfected cells (data not shown here) on type I collagen, in both the presence (+) (4.0-fold and 4.4-fold increases respectively), and absence (-) of growth factors (1.0-fold in each case). Data from FAK-Wt cells was used as positive migratory controls (and the level of expression of phospho-Erk1/2 and phospho-p38 in the absence (-) of growth factors was taken as the reference/control for all other changes and defined as 1.0 in each case).

Un-transfected keratinocytes on poly-L lysine substrate were used as a non-migratory control (left hand column in each panel). In the absence of growth factors (-) no significant phospho-Erk1/2 (≈0) was seen and levels of phospho-p38 expression (1.0-fold) were unchanged compared to the positive migratory control. In fact in the absence of growth factors (-) levels of phospho-p38 were not significantly different for all cell types (both transfected and un-transfected) on poly-L lysine and on type I collagen (0.9-1.1 fold) ($p>0.05$ in all cases). Un-transfected keratinocytes on poly-L lysine exposed to growth factor stimulation (+) showed elevated levels of phospho-Erk1/2 (1.9-fold) and of phospho-p38 (4.4-fold) compared to those in its absence (-). This was also shown previously in (Figure 27). In the absence of growth factors (-) un-transfected keratinocytes on poly-L

lysine were expressing phos-Tyr-118. This level of expression was used as the reference/control level for all other changes in phospho-paxillin-Tyr-118 and defined as 1.0. Stimulation of these cells with growth factor (+) resulted in a significant increase ($p<0.05$) in expression (1.6-fold increase).

In keratinocytes over-expressing FAK-F397 (auto-phosphorylation inactive), on type I collagen (second column in each panel) the presence of growth factor stimulation (+) compared to its absence (-) resulted in significant increases in expression of both phospho-Erk1/2 and phospho-p38 ($p<0.05$ in both cases). This finding was true for all cell types transfected and un-transfected. In the absence of growth factor stimulation (-) FAK-F397 cells showed low phospho-Erk1/2 expression (0.1-fold) that was unchanged ($p>0.05$) compared to un-transfected cells on poly-L lysine (non-migratory) controls, but represented significantly ($p<0.05$) reduced expression when compared to FAK-Wt on type I collagen (migratory) controls. The level of phospho-p38 expression (-) observed was not significantly different from either non-migratory and FAK-Wt (migratory) controls, which as described above, was typically the case for all cells in the absence (-) of growth factor. In the presence of growth factor stimulation (+) phospho-Erk1/2 expression (2.1-fold) was significantly greater than that seen in non-migratory controls and significantly reduced compared to migratory controls ($p<0.05$ in both cases). Under the same conditions (+) levels of phospho-p38 expression (3.5-fold) were significantly reduced compared to both non-migratory and migratory controls ($p<0.05$ in both cases). Phospho-paxillin-Tyr-118 expression in FAK-F397 cells was increased significantly compared to non-migratory controls in the presence (+) and absence (-) of growth factors ($p<0.05$ in both cases). In FAK-F397 cells expression of phospho-paxillin-(Tyr-118) in the absence (-) of growth factors (1.9-fold) were significantly less than in their presence (+) (2.2-fold) ($p<0.05$). This pattern was also observed in un-transfected keratinocytes on poly-l lysine (1.0-fold compared to 1.6-fold).

Keratinocytes over-expressing FAK-R454 (kinase inactive), on type I collagen (third column in each panel) in the absence of growth factor stimulation (-) showed levels of phospho-Erk1/2 expression (0.8-fold) that were significantly increased compared to non-migratory controls, but decreased compared to FAK-Wt ($p<0.05$ in both cases). In the presence of growth factors (+) phospho-Erk1/2 expression (3.0-fold) showed the same pattern, while phospho-p38 expression (3.9-fold) in this case (+) was significantly less

compared to both non-migratory and migratory controls ($p<0.05$ in all cases). FAK-R454 cells in the absence of growth factors (-) showed higher levels of phospho-paxillin-(Tyr-118) (3.9-fold) than in its presence (+) (2.9-fold) ($p<0.05$). This pattern was also observed in other transfected cells; FAK-Wt, Src-KM and Src-Wt, but was reversed in un-transfected keratinocytes on poly-l lysine and cells transfected with FAK-F397 on type I collagen.

Keratinocytes over-expressing Src-KM (kinase inactive), on type I collagen (fifth column in each panel) showed phospho-Erk1/2 expression in both the absence (-) and presence (+) of growth factor stimulation (0.4 and 2.5 fold respectively). These cells also expressed phospho-p38 in the absence (-) and presence (+) of growth factors (1.0 and 3.5 fold respectively). The pattern of significance was identical to that of FAK-R454 cells. However, in each case the expression of phospho-Erk1/2 and phospho-p38 in Src-KM cells was significantly less compared to that in FAK-R454 cells ($p<0.05$ in all cases); excluding the expression of phospho-p38 in the absence of growth factors, which as mentioned before remained unchanged in all cell types. In Src-KM cells the expression of phospho-paxillin-(Tyr-118) in the absence (-) of growth factors (4.1-fold) was significantly greater than in its presence (+) (3.0-fold) ($p<0.05$). As with FAK-R454 this pattern was also observed in other transfected cells; FAK-Wt, and Src-Wt, but was reversed in un-transfected keratinocytes on poly-l lysine and cells transfected with FAK-F397 on type I collagen.

Keratinocytes over-expressing Src-Wt ("wild-type"), on type I collagen (right hand column in each panel) in both presence (+) and absence (-) of growth factor stimulation showed the greatest expression of phospho-Erk1/2 (4.4-fold (+) and 1.3-fold (-) respectively). Both levels were significantly increased compared to un-transfected cells on poly-L lysine (non-migratory control cells) and FAK-Wt cells (migratory control cells) on type I collagen ($p<0.05$ in all cases). Expression of phospho-p38 was similarly increased to the highest levels observed in the presence (+) of growth factors (5.4-fold), and again this was shown to be a significant increase above expression seen in non-migratory and migratory controls. Phospho-paxillin-(Tyr-118) expression in Src-Wt cells was 4.5-fold in the presence (+) of growth factors and 2.0-fold in their absence (-). This was the highest level of expression seen in the absence (-) of growth factors. Notably, in the presence (+) of growth factors the expression was significantly lower than in FAK-F397, FAK-R454 and Src-KM ($p<0.05$ in each case). The level of phospho-paxillin-(Tyr-118) seen in FAK-Wt cells (4.0-fold) was significantly ($p<0.05$) lower than that in Src-Wt (4.5-fold). However, Src-Wt transfected

cells showed the greatest change in paxillin phosphorylation levels with the addition of growth factors (amongst all cells considered). (This observation was also true for the changes in phospho-Erk1/2 and phospho-p38 following growth factor stimulation compared to its absence).

6.5.4 Results: Effects on migration due to chemical inhibition of the p38 MAP kinase (with SB202190) in "wild-type" and dominant negative (kinase deficient) transfected keratinocytes

The hypothesis considered in this experiment (which was considered preliminary to further investigation) was that the p38 MAP kinase might prove to be more critical to keratinocyte migration under conditions that are associated with greater actin-myosin activity and with greater focal adhesion turnover. Increasing focal adhesion turnover and greater cytoskeletal tension are both associated with a reduction in phospho-paxillin-(Tyr-118) and in most instances of cells showing greater migration (such as frequently followed growth factor stimulation) (Wichert et al., 2003; Zaidel-Bar et al., 2005).

In the formulation of this hypothesis particular consideration was given to the results obtained with FAK and Src "wild-type" over-expression. Src-Wt over-expression showed a surprising significant reduction migration ($MI_{CG}\approx25$) in the presence of growth factor compared to un-transfected keratinocytes (Figure 42: A). In addition, Src-Wt migration was shown to be associated with the following: the largest change in phospho-paxillin-(Try-118) expression between conditions of absence to growth factor and their presence; the highest levels phospho-p38 expression in the presence of growth factor (Figure 44: B); and the highest levels of tyrosine phosphorylation of FAK (Figure 41) and phospho-paxillin-(Try-118) (Figure 44: B) in the absence of growth factors. FAK-Wt over-expression was associated with high levels of migration ($MI_{CG}\approx12$ in the absence of growth factors, and $MI_{CG}\approx32$ in their presence) that was not significantly different from migration in un-transfected cells. Stable over-expression of FAK in some other cell lines [e.g. in Chinese hamster ovary cells (Cary et al., 1996)] has been shown to cause enhanced cell migration, but this was not overtly demonstrated here.

Since p38 MAP kinase mediates pathways related to physical stressors it was hypothesized that its inhibition in the presence of over-expression of Src-Wt and FAK-Wt might best, but indirectly demonstrate such a link between this pathway and migration. It was hoped that

this would be most apparent in situations associated with greater actin-myosin activity (a circumstance likely to be linked to greater physical tension within the cell) such as those described for Src-Wt and also cases of more rapid migration such as with FAK-Wt. FAK-R454 and Src-Km dominant negative mutants were also evaluated.

The chemical inhibitor SB202190 was described previously and its properties are summarized in (Table 2)(Eyers et al., 1998). The associated control was SB202474. SB202190 was used at a concentration of 10μM shown previously to produce inhibition of keratinocyte migration on type I collagen in the presence of growth factors. In preparation for the migration assays, keratinocytes were starved overnight in GF- media, trypsinized and re-suspended in either GF- or GF+ media with and without supplementation with the chemical inhibitor or its control. Keratinocytes were then incubated for 30 minutes before being re-plated (3000 viable cells/well) into each well of 12 well cell culture plates. The plates were pre-coated with poly-L lysine (non-supportive of migration) and type I collagen (pro-migratory) at deposition concentrations listed in (Table 1). Migration was continued for 16-hours. Results of the colloidal gold migration assay in the presence of growth factors are shown in (Figure 45).

(Figure 45)

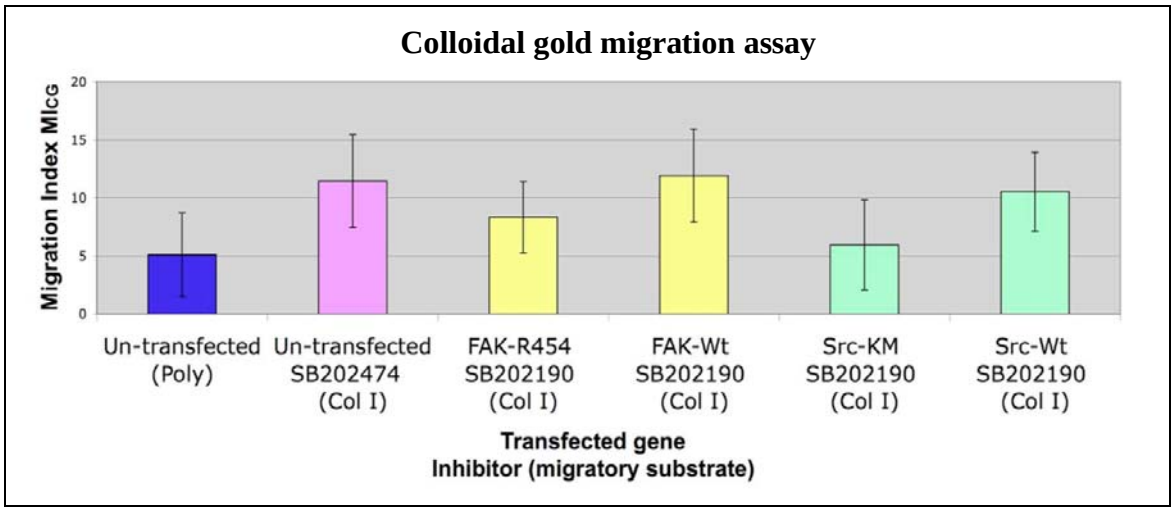


Figure 45: Chemical inhibition of p38 kinase with SB202190 during the migration of "wild-type" FAK-Wt and Src-Wt and dominant negative inhibitors FAK-R454 and Src-KM on type I collagen in the presence of growth factors. Results of the colloidal gold migration assay are shown. SB202190 was used at a concentration of 10μM in each case where indicated and migration of un-transfected keratinocytes in the presence of the control SB202474 on type I collagen is shown (pink column). A

further control, migration of un-transfected keratinocytes on poly-L lysine in the presence growth factors, and absence of exposure to the inhibitor is also shown.

The results of this assay in the presence of growth factors showed no significant difference in migration indices of MI_{CG}≈10.5-12.0 for the migratory control, un-transfected keratinocytes in the presence of SB202474 (pink column) and for cells over-expressing FAK- and Src-Wt in the presence of the inhibitor SB202190. The same observation is also true for un-transfected cells on poly-L lysine and for cells over- expressing kinase deficient FAK-R454 and Src-KM, MI_{CG}≈4.8-6.5 ($p>0.05$ for all comparisons).

A comparison of the results in Figure 42: B (in the absence of growth factors) with those shown here in Figure 45 (in the presence of growth factor, but with exposure to SB202190), shows no significant difference for each cell type ($p>0.05$). Once again (as was the case in section 5.8.2) the inhibitor SB202190 appears only to have prevented the enhanced migration normally produced by growth factors-EGFR stimulation and demonstrated no additional finding in this case. The hypothesis considered, that the p38 MAP kinase might be more critical to keratinocyte migration under conditions that are associated with greater actin-myosin activity and with greater focal adhesion turnover, was unproven by this preliminary experiment. However, this hypothesis may still have validity and further investigation seems warranted.

The significant reduction in migration (MI_{CG}≈25) observed associated with Src-Wt over-expression remains unexplained. It is possible that this reduction results from a situation analogous to that reported in cells expressing v-Src, the oncogenic form of the cellular gene c-Src. In v-Src transformed cells there is over-expression of v-Src that is constitutively kinase active. Cells expressing over-expressing v-Src show activity in the cytoskeleton and focal adhesions that are markedly increased, and a turnover of these elements that is so high that cellular adhesion (and tension) is ultimately lost. These cells show small focal adhesions and a rounded morphology before detaching completely (Fincham and Frame, 1998; Frame et al., 2002). It seems possible that a limited over activity of c-Src secondary to over-expression in Src-Wt cells (Figure 40: B), associated with the observed raised levels of tyrosine phosphorylation of FAK (Figure 41), might reasonably be producing a disparity between adhesion strength (related to focal adhesion turnover which would likely be

increased, reducing adhesion) and activity in cytoskeleton of the cell (related to physical tension which would likely be increased). While in the case of v-Src migration is lost, in a more limited way, over-expression of c-Src might at first reduce migration.

6.6 Discussion

In this chapter experiments were presented that explore the effects of integrin-ECM stimulated signalling in keratinocyte migration on type I collagen mediated via FAK and Src. In particular the relationship of FAK-Src activity to phosphorylation of FAK and paxillin (linked to focal adhesion turnover) and to the activation of the MAP kinase pathways (linked to a number of mediators of migration) are considered as well as potential interactions between these events. Work by other authors has shown integrin binding to ECM leads to clustering and recruitment of FAK and Src followed by their subsequent activation. At a time and place when focal adhesion formation is favoured, such as distally in cellular protrusions of a migrating keratinocyte, assembly of these structures is associated with accumulation of large numbers (over 50 have been identified) of additional proteins. These elaborate structures, which are linked to the cytoskeleton of the cell, are translocated proximally and the majority are disassembled at the base of the protrusion where conditions within the adhesion come to favour its disassembly. While FAK is involved in focal adhesion assembly, disassembly (turnover) of adhesions has been shown to be most closely linked to, and dependent on FAK. The process of focal adhesion assembly, translocation and disassembly results in cellular migration.

The mechanism whereby FAK up-regulates the turnover of integrin-ECM stimulated focal adhesions (Ilić et al., 1995; Wichert et al., 2003; Webb et al., 2004) is still largely unclear and two (overlapping) models linking the activity of FAK and Src to adhesion turnover have been proposed. On the one hand FAK and Src promote a number of tyrosine phosphorylation events (many of which lead to the cytoskeletal changes required for migration), but which also lead over time to degradation of FAK (by largely unknown means) and inactivation of Src. Loss of function of these key proteins from the adhesion is associated with its remodelling and its dissolution proximally. Subsequently, with continued integrin-ECM stimulation new adhesions are formed distally and the process repeats providing for turnover of focal adhesions and migration (Fincham and Frame, 1998). On the other hand, many authors have focused on the role of tyrosine phosphorylation of

FAK, in conjunction with paxillin, (and $p130^{CAS}$) that links to, and feeds back negatively on the activity of the FAK-Src complex (which is responsible for most of the tyrosine phosphorylation). These interactions may be regulated by cycles of tyrosine phosphorylation and de-phosphorylation on a number of proteins under the influence of FAK and Src that leads to cyclical assembly and disassembly (turnover) of focal adhesions. Both explanations likely contain elements of the actual reality and in both cases the process is linked to tyrosine phosphorylation of FAK and paxillin, and to cyclical changes in the activation of the MAP kinase pathways, particularly that involving Erk. While these responses have been studied extensively in many different cell lines notably fibroblasts; more limited work has looked at their role during the migration of human keratinocytes.

Having created the necessary constructs ("wild-type" and dominant negative transgenes) of FAK and Src and successfully transfected human keratinocytes with them (Figure 40), initially it was asked what if any effect on levels of tyrosine phosphorylation of FAK resulted from over-expression of these genes and their products during keratinocyte migration on type I collagen. The levels of tyrosine phosphorylation of FAK in cells transfected with FAK and Src transgenes (and in un-transfected cells) were evaluated by Western blot. It was hypothesized that the changes in FAK phosphotyrosine levels (and paxillin phosphorylation-Tyr-118, discussed later) would be predictably altered and directly linked to changes in keratinocyte migration.

During migration of all cells transfected with FAK and Src transgenes (and including un-transfected cells on non-migratory poly-L lysine) significant levels of tyrosine phosphorylation (p-Tyr) of FAK were seen in the absence of growth factor. Stimulation by growth factor significantly raised those levels in every case to varying degrees (Figure 41). Stimulation by growth factor typically (but not always) was also associated with differing increases migration (Figure 42). Compared to all other cells, cells adherent to poly-L lysine showed the lowest levels of p-Tyr of FAK, and these cells did not migrate. On pro-migratory type I collagen both of the "wild-type" transfected cells (FAK-Wt and Src-Wt) showed a pattern of change in p-Tyr of FAK similar to that seen in un-transfected cells on the same substrate and under the same growth factor exposure. Perhaps not surprisingly, the highest levels of p-Tyr of FAK were shown in those cells over-expressing FAK (the product of the transgene FAK-Wt) in the presence of growth factor. However, the FAK-Wt cells were not associated with increased migration indices under the same growth factor exposure

compared to un-transfected cells (migration was not significantly different, $p>0.05$, between these cells). Interestingly, cells over-expressing Src (Src-Wt transfected) showed the highest levels of p-Tyr of FAK in the absence of growth factor. Reasonably this might be thought to be due to raised levels of Src activity following stimulation by integrin-ECM occurring in the presence of its over-expression. Equally remarkable, while these cells showed unchanged migration compared to un-transfected cells in the absence of growth factor, they showed a relatively reduced migration following stimulation with growth factor again compared to the un-transfected cells. This curious finding was the basis for the additional experiment presented in Section 6.5.4. A hypothesized explanation (which remains unproven) was that the suboptimal augmentation of migration observed following growth factor stimulation of Src-Wt cells might be due to a mismatch between adhesive strength (linked to focal adhesion turnover) and contractile force (linked to actin-myosin activity) stimulated by integrin-ECM and growth factor interactions.

Cells over-expressing the dominant negative transgene products all showed a reduced level of p-Tyr of FAK during migration on type I collagen in both the presence and absence of growth factors, compared to un-transfected cells. FAK-F397 over-expression in particular showed levels of p-Tyr of FAK reduced almost to those of cells adherent to poly-L lysine (they were the same in the absence of growth factor and only slightly higher in its presence). Notably, and as hypothesized these cells (FAK-F397) did not migrate. Both kinase deficient cell lines (those over-expressing FAK-R454 and Src-KM) showed a similar though less dramatic response with a reduction in levels of p-Tyr of FAK and in migration. The levels of p-Tyr of FAK demonstrated were reduced and significantly ($p<0.05$) different from each other (being lower in Src-KM compared to FAK-R454 over-expressing cells), while migration indices were also reduced, but not significantly ($p>0.05$) different. These findings were true in the presence and absence of growth factors.

Western blot was also used to evaluate the effects of over-expression of FAK and Src transgenes on tyrosine phosphorylation of the downstream mediator of migration paxillin. During migration paxillin phosphorylation levels are known to change in relation to those of FAK and together with FAK, paxillin phosphorylation levels influence focal adhesion formation and turnover, and hence migration. Phosphorylation of paxillin at Tyr-118 (and Tyr-31) stimulates focal adhesion assembly, yet can also induce their turnover (probably through the subsequent activation of Erk as will be described below) (Zaidel-Bar et al.,

2007). The paxillin-phospho-paxillin ratio present in focal adhesions depends on the balance of kinase/phosphates activity and also on direct recruitment of paxillin (phosphorylated or un-phosphorylated) into focal adhesions via interactions with integrin clusters and proteins such as talin and FAK. While many factors influence this ratio it is known to depend on the mechanical force experience by the cell (which can be demonstrated by shear tension applied experimentally that likely also occurs with the same forces generated within the cell). Such physical stress regulates changes in the paxillin-phospho-paxillin ratio by a mechanism that remains unclear (Wichert et al., 2003; Zaidel-Bar et al., 2005). In general increasing focal adhesion turnover and greater cytoskeletal tension are both associated with a reduction in phospho-paxillin-(Tyr-118) and in most instances of cells showing greater migration (such as following growth factor stimulation) (Wichert et al., 2003; Zaidel-Bar et al., 2005).

In cells over-expressing the selected transgenes ("wild-type" and dominant negative forms) migrating on type I collagen it was hypothesized that the changes in paxillin phosphorylation (at Tyr-118) would be predictably altered and directly linked to changes in keratinocyte migration and also related to levels of p-Tyr on FAK. Again this was hypothesized to be likely to occur in the presence and absence of growth factors and was shown to be the case. Western blot was performed on un-transfected keratinocytes plated on poly-L lysine as a non-migratory control (Figure 44: C). All cells that migrated on type I collagen at a level (MI_{CG} and MI_{IV}) significantly above that demonstrated by un-transfected cells adherent to poly-L lysine, showed the same pattern of change in tyrosine phosphorylation of paxillin (at Tyr-118). This pattern showed high levels of phospho-paxillin-(Tyr-118) in the absence of growth factor and a significant reduction in levels following growth factor stimulation. This was true for cells over-expressing the "wild-type" transgene products (FAK-Wt and Src-Wt) and for cells over-expressing the kinase inactive transgene products (FAK-R454 and Src-KM). (It was also true for un-transfected cells migrating on type I collagen, although this data is not shown in Figure 44).

Src-Wt cells showed the highest levels of phospho-paxillin-(Tyr-118) in the absence of growth factors and the greatest reduction following growth factor stimulation. Src-Wt cells also demonstrated the highest levels of p-Tyr of FAK in the absence of growth factors. Combined these findings might further contribute to an understanding of the lower than anticipated migration seen in these cells in the presence of growth factor stimulation. (As

mentioned in general, increasing focal adhesion turnover and greater cytoskeletal tension are both associated with a reduction in phospho-paxillin-(Tyr-118), suggesting one or both might be occurring in this case).

Generally (excluding Src-Wt that were discussed above and in Section 6.5.4) keratinocytes migrate on type I collagen at a level greater (significantly larger MI's) than that of un-transfected keratinocytes on poly-L lysine, demonstrated a greater reduction in the levels of phospho-paxillin-(Tyr-118) following growth factor stimulation. This change appeared to correlate well with both higher levels of tyrosine phosphorylation on FAK and with greater migration in the presence of growth factor stimulation for each cell type. For example, cells over expressing FAK-Wt showed a 2.2-fold reduction in phospho-paxillin-(Tyr-118) (4.0-fold in the absence and 1.8-fold in the presence of growth factors: Figure 44: C) and demonstrated 2.5-fold levels of p-Tyr of FAK (in the presence of growth factors: Figure 41) and migration on type I collagen in the presence of growth factors of MI_{CG}≈30 (Figure 42: A). The same calculations for FAK-R454 and Src-KM over-expressing cells and for un-transfected keratinocytes are summarized in (Table 9). (Note: Un-transfected keratinocytes showed the same response in terms of phospho-paxillin-(Tyr-118) as FAK-Wt cells as noted in Section 6.5.3 although the results were not shown).

(Table 9)

	Phospho-paxillin-(Tyr-118) change (GF+ to GF-)	FAK Phosphotyrosine levels (GF+)	Migration index (MI_{CG}) (GF+)
Un-transfected	2.2	2.0	32
FAK-Wt	2.2	2.5*	30
Src-KM	1.1	1.9	19
FAK-R454	1.0	1.6	17
FAK-F397	0.3	0.6	5

Table 9: Correlation between the change in keratinocyte phospho-paxillin-(Tyr-118) levels following growth factor stimulation, keratinocyte FAK phosphotyrosine levels in the presence of growth factor stimulation and keratinocyte migration (MI_{CG}) on type I collagen in the presence of growth factors. (GF+) indicates the presence of growth factors and (GF-) their absence. *As

described in the text, the raised level of phosphotyrosine on FAK in this case most likely results from the high levels of FAK present secondary to its over-expression following transfection with FAK-Wt.

Observations for keratinocytes over-expressing the transgene product of FAK-F397 cells are also shown in (Table 9) and appear to fit well with the data from other transfected cells. FAK-F397 transfected cells did not migrate at a greater level than un-transfected cells on poly-L lysine in the presence or absence of growth factor stimulation, rather they showed the same level of migration ($MI_{CG} \approx 5$) (Figure 42). As well as demonstrating no significant difference in migration compared to keratinocytes on poly-L lysine, FAK-F397 transfected cells also showed a number of other similarities to poly-L lysine adherent cells; such as a low (0.3-fold) change in phospho-paxillin-(Tyr-118) following growth factor stimulation (Figure 44: C).

These findings have confirmed the hypothesis that the changes in paxillin phosphorylation (at Tyr-118) would be predictably altered and directly linked to keratinocyte migration and also to levels of p-Tyr on FAK under similar conditions. It seems reasonable to suppose that the basis for this association lies in the levels of expression and activity of FAK and Src that are present in each of the cells studied under different conditions. (Src-Wt demonstrated some differences, a deviation that is perhaps explicable and was discussed separately above). In addition, it also seems probable that levels of focal adhesion turnover and cytoskeletal tension that are occurring during migration of these cells are reflections of the changes in phospho-paxillin-(Tyr-118) observed in each case although that was not investigated directly here. (An increase in either turnover or tension leads to a reduction in phospho-paxillin-(Tyr-118) as paxillin dissociates from focal adhesions along with FAK).

The results presented and discussed so far have varied in most instances in the presence and absence of growth factors, while the explanations proposed for these observations could reasonably be applied in either case. To better understand the differences underlying keratinocyte migration without growth factor (integrin-ECM stimulation only) and with growth factors present (integrin-ECM and growth factor stimulation) the role of the MAP kinases were considered. The levels of expression of Erk, p38 and JNK and their phosphorylated counterparts were evaluated in cells transfected with the FAK and Src transgenes (and in un-transfected cells) by Western blot. During keratinocyte migration on

type I collagen it was hypothesized that activity in the Erk MAP kinase pathway (and possibly in the SAPK pathways, JNK and p38) would prove to be linked to these changes FAK and Src activity and to migration. Again this was hypothesized to be likely to occur in the presence and absence of growth factors.

Western blot was used to evaluate the effects of over-expression of FAK and Src "wild-type" transgenes and of dominant negative inhibitors FAK-F397, FAK-R454 and Src-KM on activity in the MAP kinase pathways (Erk and p38) in the presence and absence of growth factors (Figure 44: A and B). Activity in the JNK MAP kinase pathway in the presence of FAK and Src transgenes was absent, showing the same response to anti-JNK and anti-phospho-JNK as demonstrated in JNK-transfected keratinocytes; Section 5.5 (Figure 27) and in un-transfected keratinocytes; Section 5.6 (Figure 28). Further results for the JNK pathway are not shown.

For all cell types studied during migration on type I collagen, activity in the Erk MAP kinase pathway was demonstrated to be present in the absence of growth factors (Figure 27 and Figure 44: A). However activity in the Erk MAP kinase pathway was absent in keratinocytes adherent to poly-L lysine that is in the absence of growth factor stimulation. Thus activity seen in the Erk MAP kinase pathway in the absence of growth factors in other cases can reasonably interpreted as being due to integrin-ECM stimulation. Similarly for all cell types, activity in the p38 pathway was also shown here to be present in keratinocytes on type I collagen and on poly-L lysine in absence of growth factors (Figure 27 and Figure 44: B). However in this case p38 MAP kinase activity in the absence of growth factors was thought to be due to experimentally induced physical stresses (for example the stress of cell handling during the experiment and the tension generated by cellular of adhesion), rather than due to integrin stimulation.

A summary of the migration index (MI_{CG}) and levels of phospho-Erk1/2 for all transfected cell types that were studied in the absence of growth factors is shown in (Table 10).

(Table 10)

	Phospho-Erk1/2 Levels (GF-)	Migration index (MI_{CG})
Src-Wt	1.3	11
FAK-Wt	1.0	11
FAK-R454	0.8	8
Src-KM	0.4	6
FAK-F397	0.1	5

Table 10: Migration index (MI_{CG}) and levels of phospho-Erk1/2 in the absence of growth factors.

Increasing keratinocyte migration in the presence of integrin-ECM stimulation only (the absence of growth factor stimulation) is associated with increasing levels of phospho-Erk1/2, reflecting activity in the MAP kinase pathway (Table 10) as was hypothesized. This can be interpreted as a reflection of integrin-ECM stimulated signalling during migration in each cell type studied that is linked to a level Erk MAP kinase pathway activity. The Erk MAP kinase pathway leads to activation of calpain-2 (possibly linked to focal adhesion turnover) and to MLCK activity (linked to actin-myosin contractility). Additionally activity in the MAP kinase pathways is also known to be involved in the regulation of migration via links to focal adhesion turnover. Here an intact Erk MAP kinase pathway has been shown to be essential for keratinocyte migration on type I collagen and these additional findings confirm the link between migration of keratinocytes on type I collagen and the Erk MAP kinase pathway.

For all cell types studied on any substrate, exposure to growth factors not surprisingly raised phospho Erk1/2 levels and also raised phospho-p38 (Figure 44: A and B).

As stated above the Erk MAP kinase pathway is linked to the mediators of migration (MLCK) and to focal adhesion turnover (FAK, Src and calpain-2). Although likely to be present, the effectors of migration in the p38 MAP kinase pathway remain unclear, and p38 MAP kinase pathway links to regulation of focal adhesion turnover and migration are likely to be through the effects of cytoskeletal tension and the changes this tension has on paxillin

tyrosine phosphorylation, and subsequently on the integrin-FAK-paxillin interaction. It is therefore reasonable to suppose that the demonstrated increased activity in the Erk MAP kinase pathway observed following growth factor-EGFR stimulation might be translated into increased migration and the same might also be true of the increased activity in the p38 MAP kinase pathway. The responses demonstrated here are consistent with this idea and underlie at least one aspect of the augmentation of keratinocyte migration on type I collagen that follows the addition of growth factors.

Considering FAK-F397 transfected keratinocytes. FAK-F397 cells on type I collagen and un-transfected cells on poly-L lysine did not migrate ($MI_{CG} \approx 5$) in either the absence or in the presence of growth factors and showed almost zero (if not zero) levels of phospho-Erk1/2 (Figure 44: A and Figure 42: B) when growth factors were absent. Stimulation of these cells on their respective substrates with growth factor resulted in significant increases in phospho-Erk1/2, but no increase in migration (Figure 44: A and Figure 42: A). These findings show that the auto-phosphorylation site on FAK, Tyr-397, is necessary for keratinocyte migration on type I collagen with or without growth factor-EGFR stimulation. They also demonstrate that following growth factor-EGFR stimulation, activity in the Erk MAP kinase pathway (not surprisingly) can occur independently of activity of Tyr-397 in FAK, while in the absence of growth factors inhibition of the auto-phosphorylation site on FAK significantly limits if not nearly abolishes Erk MAP kinase pathway activity.

It has already been shown here that a functioning Erk MAP kinase pathway is necessary for keratinocyte migration on type I collagen in the absence of growth factor-EGFR stimulation. Here the Tyr-397 auto-phosphorylation site on FAK was shown to be necessary for Erk MAP Kinase pathway activity under the same conditions. It is reasonable to suppose these two findings might represent dependant interactions, such that in the absence of growth factor-EGFR stimulation, FAK activation (dependant on an intact auto-phosphorylation site) is a prerequisite to activation of the Erk MAP kinase pathway and both the auto-phosphorylation site on FAK and the Erk MAP kinase pathway are necessary for keratinocyte migration.

Considering cells transfected with kinase inactive transgenes FAK-R454 and Src-KM, these cells showed reduced levels of migration and of phospho-Erk1/2 expression that were intermediated to cells transfected with their respective “wild-type” transgenes and cells

transfected with FAK-397 on type I collagen (Figure 42 and Figure 44: A). These findings were true both in the presence and absence of growth factors. Typically Src-KM showed lower values for both migration index and phospho-Erk1/2 expression compared to FAK-R454 (although MI_{CG} for the two cell lines were not significantly different in the presence of growth factors).

These kinase inactive transduced keratinocytes showed a significant impairment of migration, rather than its complete inhibition, in both the presence and absence of growth factors. Also, these cells demonstrated that despite inhibition of either FAK or Src kinase activity in the absence of growth factors, Erk MAP kinase activity is still present but reduced. Furthermore, in FAK and Src kinase deficient cells following growth factor-EGFR stimulation there was an impaired, but not inhibited increase in Erk MAP kinase pathway activity. Inhibition of FAK and to a greater extent Src kinase activity impairs, but does not inhibit the process of migration in the presence integrin-ECM only and also the addition of growth factor-EGFR stimulation in the presence of FAK or Src kinase inhibition is followed by a less than optimal augmentation of migration.

Considered together these findings appear to link cyclical changes in FAK-Src activity, integrin-FAK-paxillin binding and Erk activity that led to focal adhesion turnover and migration. Focal adhesion turnover and migration occurs in the presence of integrin-ECM stimulation alone with lower levels of Erk MAP kinase activity and Erk MAP kinase pathway activity is enhanced (as was observed here) by the addition of growth factors, as would migration.

Finally, migration of keratinocytes on type I collagen in the presence of the chemical inhibition of the Src family members (by the inhibitor PP2) was reduced to the same level as that seen in cells over-expressing FAK-F397 (auto-phosphorylation inactive) in both the presence and absence of growth factors (Figure 43 and Figure 42). PP2 inhibits all Src family members. Thus inhibition of all Src family members (Src, Fyn, and Yes1) resulted in completely inhibited migration of keratinocytes. This can be compared to the reduced but not absent migration observed in Src-KM transfected cells (in which only Src is inhibited). Additionally it shows that as a group Src family kinases are essential to keratinocyte migration on type I collagen.

Chapter 7. Conclusion

Following a break in its integrity through injury, the skin must be able to repair the breach to restore its protective barrier function quickly and efficiently. Healing of an acute cutaneous wound involves a number of cells serving a number of roles. Fibroblasts are central to deposition of the contractile dermal connective tissue and to its remodelling. Keratinocytes from the wound edge proliferate and migrate into the wound to seal the defect. The processes occurring are coordinated by a regulated balance between changing extracellular matrix components, soluble factors, and the cellular elements. Integrins are central to migration *in vitro* and *in vivo,* mediating effects through signalling pathways that critically involve the activities of FAK and Src kinases in the assembly, translocation and significantly in the disassembly of focal adhesions and concomitant changes in the cytoskeleton. Soluble factors, including growth factors, contribute to migration by activation of one or more of the MAP kinase pathways in different cells. These pathways are linked to mediators of migration. Many of the processes involved have been studied in a number of participating cell types, especially fibroblasts. This work investigated human keratinocytes and their migration during wound healing *in vitro*.

Experiments presented here were conceived to test the hypothesis that signalling from both integrin-ECM interactions and those from soluble growth factors and their receptors work in conjunction to facilitate the migration of keratinocytes. Acting together, it was proposed that they would produce optimal (augmented) migration, while acting alone integrin-ECM initiated signalling might still result in migration, albeit at a reduced level. Growth factor signalling acting alone will prove unable to produce migration in keratinocytes. A consistent finding throughout this work has been that in the presence of type I collagen, migration of keratinocytes occurred with or without the addition of soluble growth factors (for example as was shown in Figure 20, Figure 30 and Figure 32), a finding only shared amongst the substrates investigated by another collagen, type IV collagen (Figure 20). As well as being the only substrates to initiate migration in the absence of growth factors, these collagens showed the greatest levels of migration observed in their presence. Acting together, integrin-collagen stimulation demonstrates what was hypothesized, that is augmented migration in the presence of growth factors and continued migration in their absence, albeit at a reduced level. Neither increasing levels of growth factor

supplementation (Figure 18), or increasing concentration of serum (Figure 19) initiated migration in the absence of integrin-ECM stimulation (on poly-L lysine). However, on a number of substrates on which keratinocytes migrated to different degrees (i.e. in the presence of integrin-ECM stimulation) the addition of growth factor was shown repeatedly to significantly enhanced migration (demonstrated in Figure 20, Figure 30 and Figure 32). Notably, each substrate that demonstrated this finding was either a known component of the connective tissue of a healing wound or known to be secreted by migrating keratinocytes or both (respectively, vitronectin, laminin-5 and fibronectin) (Figure 20). Again this confirms what was hypothesized that growth factors would augment migration (compared to their absence), but would prove to be unable to initiate migration in the absence of integrin-ECM stimulation.

In vivo, the pattern of integrin expression on migratory keratinocytes favours movement on type I collagen (both at the leading edge of the healing wound and in the cells migrating behind) and to a lesser extent fibronectin and vitronectin (Clark et al., 1982; Clark et al., 1996; Gilcrease, 2007). These integrins are $\alpha_2\beta_1$, $\alpha_v\beta_6$ and $\alpha_5\beta_1$, and $\alpha_v\beta_3$ respectively (Kiosses et al., 2001; Laukaitis et al., 2001). Additionally, keratinocytes *in vivo* express several integrins with specificity for laminin-5, such as the β_1-subunit associated with α_3 or β_4 associated with α_6 subunits mediates the integrin interaction with laminin-5 that are upgraded in the migrating phenotype (Niessen et al., 1994; Nguyen et al., 2000; Nguyen et al., 2001). These findings are intuitively linked to the keratinocyte migration observed here. Keratinocytes on laminin-1, a component of the basement membrane did not migrate in the presence or absence of growth factors, a finding seen additionally on poly-L lysine. However, the integrin interactions with laminin-1 are unlike those occurring on poly-L lysine where integrin-ECM stimulation is essentially absent. The keratinocyte migratory response to integrin-ECM stimulation seems almost certainly to be related to the substrate that is present and to the integrins expressed on the keratinocyte. While the integrins expressed were not investigated here, the migration demonstrated in the case of laminin-1 and that was shown to different levels amongst the other substrates evaluated is consistent with this, and is an area worthy of further consideration.

Over time in an *in vivo* wound the provisional extracellular matrix becomes progressively more collagenous (Nguyen et al., 2000; Hintermann and Quaranta, 2004), containing increasing amounts of type I collagen, due primarily to the activity of fibroblasts. An

analogous circumstance was evaluated experimentally here. Increasing concentrations of type I collagen deposition solution showed dose-dependent increases in keratinocyte migration to a maximum migration of $MI_{CG}\approx32$ in the presence of growth factors and of $MI_{CG}\approx12$ in their absence (Figure 23 and Figure 24). (Direct correlation with concentration of type I collagen at the migratory surface was not possible). These results also seem to have an intuitive relationship to what might reasonably occur during wound healing *in vivo*; that is as the level of type I collagen increases keratinocyte migration is enhanced, a change that would facilitate wound closure.

A diverse range of soluble factors and mediators are involved in regulating the complex events involved during the process of wound healing. The profile of soluble factors present varies over time. At the cellular level, response to growth factors is mediated via cell surface receptors, leading in many cases to the activation of the MAP kinase pathways. While the role of MAP kinases has been studied extensively in cellular functions including cellular migration, and in many different cell lines, limited work has looked at their role during the migration of human keratinocytes. Which if any of the three MAP kinase pathways, Erk, p38 and JNK were active during keratinocyte migration on type I collagen (representing the presence of integrin-ECM signalling) was investigated, as well as the activity of each pathway in response to integrin-ECM and growth factor stimulation. It was hypothesized that an intact Erk MAP kinase pathway would prove essential for keratinocyte migration in the presence of a pro-migratory substrate such as type I collagen (based upon a review of our present understanding). The relationship of the each of the MAP kinase pathways, Erk, p38 and JNK to the augmentation of migration observed following growth factor stimulation was also investigated. Dominant negative transgenes and chemical inhibitors were used to inhibit elements of each of these pathways during keratinocyte migration in order to answer this question.

Activity in both the Erk and p38 MAP kinase pathways were shown to be present during keratinocyte migration on type I collagen, while activity in the demonstrably intact (Figure 28) JNK pathway was absent with or without growth factor exposure (Figure 27). Significantly, activity in the Erk pathway was demonstrated during keratinocyte migration on type I collagen and following growth factor exposure on poly-L lysine (but not in the absence of growth factors on this substrate). Activity in the p38 pathway in the absence of growth factors, independent of substrate (type I collagen and poly-L lysine) was shown to

be present and unchanging in a number of experiments (Figure 27: B) and (Figure 44: B). This finding for the p38 MAP kinase pathway seemed most likely to represent a stress response to experimental handling, while the increased activity in the p38 pathway observed in the presence of growth factor was thought to be more meaningful, as will be made clear. Inhibition of the Erk and p38 MAP kinase pathways by either method reduced migration to different degrees in each pathway (Figure 29, Figure 30 and Figure 32). Migration of keratinocytes on type I collagen was abolished in the presence of Erk pathway inhibition at the level of MEK1/2 (the MAP kinase kinase) and showed a dose-dependent reduction in migration and activity downstream in the pathway (at the level of Erk kinase activity) in response to increasing exposure to the MEK1/2 inhibitor that was used (Figure 32: A and Figure 33). Following p38 MAP kinase pathway inhibition at the level of the p38 kinase, keratinocyte migration on type I collagen could no longer be enhanced by the addition of growth factors. A dose-dependent reduction in migration in response to increasing exposure to the p38 inhibitor was demonstrated in the presence of growth factors (Figure 32: B). With maximal inhibition of p38, migration on type I collagen still occurred but only at a level otherwise seen in the absence of growth factors.

In the presence of Erk MAP kinase inhibition, a pro-migratory substrate is no longer sufficient to produce migration in keratinocytes with or without growth factor stimulation. Further, these results confirm that an intact Erk MAP kinase pathway is essential for keratinocyte migration in the presence of a pro-migratory substrate such as type I collagen. It seems reasonable to interpret these findings as reflecting involvement of the Erk MAP kinase pathway in signalling mediating both integrin-ECM stimulated migration alone, as well those mediating the augmentation of migration that follows from soluble growth factor exposure. As was previously described, growth factor signalling alone could not induce keratinocyte migration in the absence of a pro-migratory substrate. However, addition of growth factors significantly enhances keratinocyte migration on a number of pro-migratory substrates, notably type I collagen. This augmentation of migration was shown here to require functionally intact and active Erk and p38 MAP kinase pathways, but apparently does not depend on the JNK MAP kinase pathway in keratinocytes.

The process of cellular migration as it occurs in wound healing can be considered to be a complex, coordinated and repeating series of changes in the actin cytoskeleton linked to concomitant changes in cellular adhesion (focal adhesions formation and dynamics).

Integrins interact directly with the extracellular matrix providing attachment (adhesion) to the underlying substrate. Interactions with extracellular matrix are also linked to signalling pathways that lead to the formation and maintenance of the polarized morphology, and to a number of other important changes in the actin cytoskeleton, in a process that enables the cell to move forwards. Central to many of these processes during migration in a number of cells are integrin interactions with FAK, Src kinase activity and the Erk MAP kinase pathway, a well-known target of integrin mediated signalling by FAK and Src. FAK and Erk interact with paxillin in a complex manner that is linked both to assembly and disassembly of focal adhesions during migration. A key interaction relating to focal adhesion disassembly is the binding of activated Erk to paxillin phosphorylated by Src at Tyr-118. Cyclical changes in FAK-Src activity, integrin-FAK-paxillin binding and Erk MAP kinase activity result in focal adhesion turnover and migration. Additionally, during this process the level of tyrosine phosphorylation of FAK (largely due to activity of Src family kinases) has been shown in a number of cells (notably fibroblasts) to be linked to the rate of disassembly of focal adhesions that is most directly linked to migration. And repeated cycles of phosphorylation and de-phosphorylation of FAK and paxillin are thought to underlie the formation, translocation and disassembly of focal adhesions that, along with coordinated changes in the actin cytoskeleton and actin-myosin activity, enables cells to migrate.

Inhibition of the auto-phosphorylation site (tyrosine 397) on FAK prevented keratinocyte migration on type I collagen in both the presence and absence of growth factors (Figure 42). The downstream signalling in these cells was also affected, notably Erk MAP kinase pathway activity was reduced to levels seen in un-transfected cells on non-migratory substrate in the absence of growth factors (Figure 44: A). Stimulation of either un-transfected cells or FAK-F397 cells on their respective substrates with growth factor resulted in equal and significant increases in phospho-Erk1/2, but no increase in migration (Figure 44: A and Figure 42: A). These findings demonstrate that the auto-phosphorylation site on FAK, Tyr-397, is necessary for keratinocyte migration on type I collagen with or without growth factor stimulation. They also demonstrate that Erk MAP kinase pathway can occur independently of FAK-F397 activity (stimulated by growth factor and no doubt via the EGFR), but in the presence of integrin-ECM signalling only, the auto-phosphorylation site on FAK is required for Erk MAP kinase pathway activity in keratinocytes on type I collagen. In summary, taken together with previous findings

regarding the Erk MAP kinase pathway it can be said that an intact auto-phosphorylation site on FAK is necessary for Erk MAP kinase activation (in the presence of integrin-ECM stimulation only), and both an intact auto-phosphorylation site on FAK and an intact Erk MAP kinase pathway are necessary for keratinocyte migration on type I collagen.

Inhibition of all Src family members (by the chemical inhibitor PP2) also abolished keratinocyte migration on type I collagen in both the presence and absence of growth factors (Figure 43). Demonstrating kinase function of these non-receptor tyrosine kinases as a group was also essential for keratinocyte migration. Evaluation of downstream pathways (Erk and p38 MAP kinase pathways and paxillin) in this setting (PP2 inhibition) was not undertaken but would prove valuable for further investigation. Inhibition of either c-Src kinase activity (by the dominant negative inhibitor Src-KM) or FAK kinase activity (by the dominant negative inhibitor FAK-R454) showed a significant impairment of migration, rather than its complete inhibition, in both the presence and absence of growth factors (Figure 42). Additionally these transfected cells (Src-Km and FAK-R454) also demonstrated that inhibition of either FAK or Src kinase activity reduced Erk MAP kinase pathway activity in both the presence and absence of growth factors (Figure 44: A). This observation provides evidence for a link between integrin-ECM signalling (related to FAK and Src activity) and growth factor signalling via the Erk MAP kinase pathway.

The findings presented here showed that changes in paxillin phosphorylation (at Tyr-118) were qualitatively predictably altered in a manner directly linked to keratinocyte migration and also to levels of p-Tyr on FAK under similar experimental conditions (of growth factor exposure and substrate). An explanation was proposed for this association that connects levels of expression and kinase activity of FAK and Src that were present in each of the cells studied to these changes. ("Wild-type" Src demonstrated some noteworthy differences in these findings that were considered separately). In addition, it also seemed probable from the results presented that levels of focal adhesion turnover and cytoskeletal tension that are occurring during migration of these cells are reflections of the changes in phospho-paxillin-(Tyr-118) observed in each case although that was not investigated directly here but would also be worthy of further evaluation.

The findings demonstrated by over-expression of "wild-type" Src were particularly intriguing although an explanation was proposed, the reduction in migration on type I

collagen demonstrated by these transfected keratinocytes in the presence of growth factors remains unexplained. This too is worthy of further evaluation beyond the limited experiment that was performed here which was inconclusive.

In conclusion signalling from both integrin-ECM interactions and those from soluble growth factors and their receptors has been shown to work in conjunction to facilitate the migration of keratinocytes. Acting together, they produce optimal (augmented) migration, while acting alone integrin-ECM initiated signalling can result in migration, albeit at a reduced level. Growth factor signalling acting alone could not. FAK and Src have been shown to be central to keratinocyte migration in the presence and absence of growth factors, the auto-phosphorylation site on FAK is essential and the kinase activity of each playing an important part of the underlying processes occurring although neither alone is indispensable. The augmentation of migration that was repeatedly demonstrated as being linked to the action of growth factors acting at their cell surface receptors which act to enhance downstream mediators of migration through the MAP kinase pathways Erk and p38. Interestingly the MAP kinase pathway JNK did not appear to be involved in keratinocyte migration. The augmentation of migration although initiated by growth factors at their receptor is also linked to FAK and Src key focal adhesion proteins in a manner suggesting that growth factor stimulation also enhances processes central to focal adhesion dynamics.

A simplified image of the system underlying these findings (and consistent with them) is summarized in two brief models described below:

A mechanism (model) consistent with the findings presented here, whereby integrin-ECM stimulation (in the absence of growth factor-EGFR stimulation) leads to assembly of focal adhesions, recruitment and activation of the Erk MAP kinase pathway and to focal adhesion disassembly can be summarized as: Following integrin-ECM stimulation FAK and Src are recruited and Src activated as focal adhesion assembly begins. Src phosphorylates paxillin at Tyr-118 that binds Erk. Erk is activated by Shc/Grb2-SOS-Ras interactions within the focal adhesion (described previously). Activated Erk bound to Tyr-118 in turn leads to phosphorylation of FAK at Ser-910. Phosphorylation at this site on FAK is known to result in the dissociation of FAK from paxillin and dissociation of both proteins away from the focal adhesion, leading to focal adhesion disassembly. In the presence of continued integrin-ECM stimulation this process could be repeated. As observed, “low” levels of Erk

MAP kinase activity would be present resulting from the process of focal adhesion turnover. Also as observed, “low” levels of migration could be facilitated by activity in the Erk MAP kinase pathway that is linked to downstream mediators of migration and by FAK and Src activity that are linked to changes in the actin cytoskeleton and to cellular adhesion regulation (focal adhesion turnover).

Similarly a mechanism (model) consistent with the findings presented here whereby the addition of growth factors might result in augmented migration can be summarized as: Keratinocytes on a pro-migratory substrate exposed to growth factors experience both integrin-ECM stimulation and additional growth factor-EGFR stimulation. EGFR is located within focal adhesion sites (recruited by FAK) and is also present elsewhere on the cells surface. Activation of the Erk MAP kinase pathway by growth factor stimulation of EGFR occurs following Grb2-SOS interactions and subsequent Ras/Raf activation (described previously). In this case with the addition of growth factor it is reasonable to assume “higher” levels of Erk MAP kinase activity and a significant increase in both the downstream effectors of migration (e.g. MLCK) and also in the interactions described above that stimulate focal adhesion disassembly (calpain-2 activity and Ser-910 phosphorylation of FAK). Continuing integrin-ECM and growth factor stimulation would be associated with continuing enhanced migration, as was observed.

These models include cyclical changes in FAK-Src activity, integrin-FAK-paxillin binding and Erk activity that lead to focal adhesion turnover and migration. Turnover and migration would occur in the presence of integrin-ECM stimulation alone and would be enhanced (as was observed here) by the addition of growth factors these findings were shown here. In addition this outline makes clear the central role of FAK and Src as well as the necessity of an intact Erk MAP kinase pathway for migration in both the presence and absence of growth factors (again a finding that has been demonstrated here)

List of References

Abercrombie M, Heaysman JE, and Pegrum SM (1970). "The locomotion of fibroblasts in culture. 3. Movements of particles on the dorsal surface of the leading lamella " Experimental Cell Research 62(2): 389-398.

Abercrombie M, Heaysman JE, and Pegrum SM (1971). "The locomotion of fibroblasts in culture. IV. Electron microscopy of the leading lamella." Experimental Cell Research 67: 359-367.

Albrecht-Buehler G (1977). "The phagokinetic tracks of 3T3 cells." Cell 11: 395-404.

Albrecht-Buehler G, and Goldman RD (1976). "Microspike-mediated particle transport towards the cell body during early spreading of 3T3 cells." Experimental Cell Research 97(2): 329-339.

Almeida EAC, Ili D, Han Q, Hauck CR, Jin F, Kawakatsu H, Schlaepfer DD, and Damsky CH (2000). "Matrix survival signaling: From fibronectin via focal adhesion kinase to c-Jun NH2-terminal kinase." Journal of Cell Biology 149: 741-754.

Amano S, Akutsu N, Matsunaga Y, Nishiyama T, Champliaud MF, Burgeson RE, and Adachi E (2001). "Importance of balance between extracellular matrix synthesis and degradation in basement membrane formation." Experimental Cell Research 271(2): 249-263.

Aplin AE, Hogan BP, Torneu J, and Juliano RL (2001). "Integrin-mediated adhesion regulates ERK nuclear translocation and phosphorylation of Elk-1." Journal of Cell Biology 153(2): 273-282.

Arnold F, and West D (1991). "Angiogenesis in Wound Healing." Pharmacology and Therapeutics 52(407-422).

Bagrodia S, Derijard B, Davis RJ, and Cerione RA (1995). "Cdc42 and PAK-mediated signaling leads to Jun kinase and p38 mitogen-activated protein kinase activation." Journal of Biological Chemistry 270: 27995-27998.

Bain J, McLauchlan H, Elliott M, and Cohen P (2003). "The specificities of protein kinase inhibitors : an update." Biochemical Journal 371(1): 199-204.

Bandyopadhyay B, Fan J, Guan S, Li Y, Chen M, Woodley D, and Li W (2006). "A traffic control role for TGF beta3: Orchestrating dermal and epidermal cell motility during wound healing." Journal of Cell Biology 172(7): 1093-1105.

Barandon Y, and Green H (1987). "Cell migration is essential for sustained growth of keratinocyte colonies: the roles of transforming growth factor-alpha and epidermal growth factor." Cell 50: 1131-1137.

Bennett BL, Saskai DT, Murray BW, O'Leary EC, Sakata ST, and Xu W (2001). "SP 600125, an anthrapyrazolone inhibitor of Jun N-terminal kinase." Procedures of the National Academy of Science. USA 98: 13681-13686.

Bereiter-Hahn J (1986). Epidermal cell migration and wound repair. In: Biology of the integument 2 vertebrates Berlin, Springer-Verlag.

Bladt F, Aippersbach E, Gelkop S, Strasser GA, Nash P, Tafuri A, Gertler FB, and Pawson T (2003). "The murine Nck SH2/SH3 adaptors are important for the development of mesoderm-derived embryonic structures and for regulating the cellular actin network." Molecular & Cellular Biology 23(13): 4586-4597.

Bompard G, and Caron E (2004). "Regulation of WASP/WAVE proteins. Making a long story short." Journal of Cell Biology 166(7): 957-962.

Borradori L, and Sonnenberg A (1999). "Structure and function of hemidesmosomes: more than simple adhesion complexes." Journal of Investigative Dermatology 112: 411-418.

Bowersox JC, and Sorgente N (1982). "Chemotaxis of aortic endothelial cells in response to fibronectin." Cancer Research 42: 2547-2551.

Brakebusch C (2005). "Keratinocyte Migration in Wound Healing." Cell Migration in Development and Disease: 275-298.

Brigham PA, and McLoughlin E (1996). "Burn incidence and medical care use in the United States: estimate, trends, and data sources." Journal of Burn Care and Rehabilitation 17: 95-107.

Brown MC, and Turner CE (2004). "Paxillin: adapting to change." Physiological Reviews(84): 1315-1339.

Burridge K, Chrzanowska-Wodnicka M, and Zhong C (1997). "Focal adhesion assembly." Trends in Cell Biology 7: 342-347.

Callam MJ, Ruckley CV, and Harper DR (1985). "Chronic ulceration of the leg: extent of the problem and provision of care." British Journal of Dermatology 290: 1855-1856.

Cano E, and Mahadevan LC (1995). "Parallel signal processing among mammalian MAPKs." Trends in Biochemical Science 20(3): 117-122.

Carlier MF, Ressad F, and Pantaloni D (1999). "Control of actin dynamics in cell motility. Role of ADF/cofilin." Journal of Biological Chemistry 274: 33827-33830.

Carragher NO, Westhoff AM, Fincham VJ, Schaller DS, and Frame MC (2003). "A Novel Role for FAK as a Protease-Targeting Adaptor Protein Regulation by p42 ERK and Src." Current Biology 13(16): 1442-1450.

Carter SB (1967). "Haptotaxis and the mechanism of cell motility." Nature 213(5073): 256-260.

Cary LA, Chang JF, and Guan JL (1996). "Stimulation of cell migration by over expression of focal adhesion kinase and its association with Src and Fyn." Journal of Cell Science 108: 1787-1794.

Catling AD, Reuter CW, Cox ME, Parsons SJ, and Weber MJ (1994). "Partial purification of a mitogen-activated protein kinase kinase activator from bovine brain. Identification as B-Raf or a B-Raf-associated activity." Journal of Biological Chemistry 269(47): 30014-30021.

Cha D, O'Brien P, O'Toole EA, Woodley DT, and Hudson LG (1996). "Enhanced modulation of keratinocyte motility by transforming growth factor-alpha (TGF-alpha) relative to epidermal growth factor (EGF)." Journal of Investigative Dermatology 106: 590-597.

Chang SH, Yu KN, Lee YS, An GH, Beck GR, Colburn NH, Lee KH, and Cho MH (2006). "Elevated inorganic phosphate stimulates Akt-ERK1/2-Mnk1 signaling in human lung cells." American Journal of Respiratory Cell and Molecular Biology 35: 528-539.

Chelberg MK, Tsilibary EC, Hauser AR, and McCarthy JB (1989). "Type IV Collagen-mediated melanoma cell adhesion and migration: Involvement of multiple, distinct domains of the collagen molecule." Cancer Research(48): 4796-4802.

Chen JD, Kim JP, Zhang K, Sarret Y, Wynn KC, Kramer RH, and Woodley DT (1993). "Epidermal growth factor (EGF) promotes human keratinocyte locomotion on collagen by increasing the alpha 2 integrin subunit." Experimental Cell Research 209: 216-223.

Chen M, Li W, Fan J, Kasahara N, and Woodley D (2003). "An efficient gene transduction system for studying gene function in primary human dermal fibroblasts and epidermal keratinocytes " Experimental Dermatology 28: 193-199.

Chen M, She H, Kim A, Woodley D, and Li W (2000). "Nckbeta adapter regulates actin polymerization in NIH 3T3 fibroblasts in response to platelet-derived growth factor bb." Molecular & Cellular Biology 20(21): 7867-7880.

Choma DP, Milano V, Pumiglia KM, and DiPersio CM (2007). "Integrin a3b1-dependent activation of FAK/Src regulates Rac1-mediated keratinocyte polarization on Laminin-5." Journal of Investigative Dermatology 127(31-40).

Choma DP, Pumiglia K, and DiPersio CM (2004). "Integrin a3b1 directs the stabilization of a polarized lamellipodium in epithelial cells through activation of Rac1." Journal of Cell Science 117: 3947-3959.

Clark EA, King WG, Brugge JS, Symons M, and Hynes RO (1998). "Integrin-mediate signal regulated by members of the Rho-family of GTPases." Journal of Cell Biology 142: 573-586.

Clark RAF, Ashcroft GS, Spencer M-J, Larjava H, and Ferguson MWJ (1996). "Re-epithelialization of normal human excisional wounds is associated with a switch from avb5 to avb6 integrins." British Journal of Dermatology 135: 46-51.

Clark RAF, Lanigan JM, DellaPelle P, Manseau E, Dvorak HF, and Colvin RB (1982). "Fibronectin and fibrin provide a provisional matrix for epidermal cell migration during wound re-epithelialization." Journal of Investigative Dermatology 79: 264-269.

Coulombe PA (1997). "Towards a molecular definition of keratinocyte activation after acute injury to stratified epithelia." Biochemical and Biophysical Research Communications 236: 231-238.

Coulombe PA (2003). "Wound epithelialisation: accelerating the pace of discovery." Journal of Investigative Dermatology 121: 219-230.

Cross MJ, Lu L, Magnusson P, Nyqvist D, Holmqvist K, Welsh M, and Claesson-Welsh L (2002). "The Shb adaptor protein binds to tyrosine 766 in the FGFR-1 and regulates the Ras/MEK MAPK pathway via FRS2 phosphorylation in endothelial cells." Molecular Biology of the Cell 13(8): 2881-2893.

Cuevas BD, Abell AN, Witowsky JA, Yujiri T, Johnson NL, Kesavan K, Ware M, Jones PL, Weed SA, DeBiasi RL, Oka Y, Tyler KL, and Johnson GL (2003). "MEKK1 regulates calpain-dependent proteolysis of focal adhesion proteins for rear-end detachment of migrating fibroblasts." EMBO Journal 22(13): 3346-3355.

Daniel RJ, and Groves RW (2002). "Increased migration of murine keratinocytes under hypoxia is mediated by induction of urokinase plasminogen activation." Journal of Investigative Dermatology 119: 1304-1309.

Davies SP, Reddy H, Caivano M, and Cohen P (2000). "Specificity and mechanism of action of some commonly used protein kinase inhibitors " Biochemical Journal 351(1): 95-105.

Derynck R (1986). "Transforming growth factor-alpha: structure and biological activities." Journal of Cell Biology 32(4): 293-304.

DeSilva DR, Jones EA, Favata MF, Jaffee BD, Magolda RL, Trzaskos JM, and Scherle PA (1999). "Inhibition of mitogen-activated protein kinase blocks T cell proliferation but does not induce or prevent anergy." Journal of Immunology 160: 4175-4181.

Desmouliere A, Redard M, Darby I, and Gabbiani G (1995). "Apoptosis mediates the decrease in cellularity during the transition between granulation tissue and scar." American Journal of Pathology 146: 56-66.

Dourdin N, Bhatt AK, Dutt P, Greer PA, Arthur JS, Elce JS, and Huttenlocher A (2001). "Reduced cell migration and disruption of the actin cytoskeleton in calpain-deficient embryonic fibroblasts." Journal of Biological Chemistry 276: 48382-48388.

Ellenbroek S, and Collard J (2007). "RhoGTPases: functions and association with cancer." Clinical and Experimental Metastasis 24(8): 657-672.

Epstein B, Epstein JH, and Fukuyama K (1893). "Autoradiographic study of colchicine inhibition of DNA synthesis and cell migration in hairless mouse epidermis in vivo." Cell and Tissue Kinetics 16: 313-319.

Etienne-Manneville S, and Hall A (2002). "Rho GTPases in cell biology." Nature 420(6916): 629-635.

Eyers PA, Craxton M, Morrice N, Cohen P, and Goedert M (1998). "Conversion of SB 203580-insensitive MAP kinase family members to drug-sensitive forms by a single amino-acid substitution." Chemical Biology 5: 321-328.

Favata MF, Horiuchi KY, Manos, EJ, Daulerio AJ, Stradley DA, Feeser WS, Van Dyk DE, Pitts WJ, Earl RA, and Hobbs F (1998). "Identification of a novel inhibitor of mitogen-activated protein kinase kinase ." Journal of Biological Chemistry 273: 18623-18632.

Fincham VJ, and Frame MC (1998). "The catalytic activity of Src is dispensable for translocation to focal adhesions but controls the turnover of these structures during cell motility." EMBO Journal 17: 81-92.

Fincham VJ, Frame MC, and Winder S (2000). "Active ERK/MAP kinase is targeted to newly forming cell–matrix adhesions by integrin engagement and v-Src." EMBO Journal 19: 2911-2923.

Frame MC, Fincham VJ, Carragher NO, and Wyke JA (2002). "v-Src's hold over actin and cell adhesions." Nature Reviews Molecular Cell Biology 3(4): 233-245.

Frank DE, and Carter WG (2004). "Laminin 5 deposition regulates keratinocyte polarization and persistent migration." Journal of Cell Science 117: 1351-1363.

Friedl P, and Wolf K (2003). "Tumour-cell invasion and migration: diversity and escape mechanisms." Nature Reviews Cancer 3: 362-374.

Fuchs E (1990). "Epidermal differentiation: The bare essentials." Journal of Cell Biology 111(6): 2807-2814.

Funamoto S, Meili R, Lee S, Parry L, and Firtel RA (2002). "Spatial and temporal regulation of 3-phosphoinositides by PI 3-kinase and PTEN mediates chemotaxis." Cell 109(5): 541-544.

Gabbiani G, Chaponnier C, and Huttner I (1978). "Cytoplasmic filaments and gap junctions in epithelial cells and myofibroblasts during wound healing." Journal of Cell Biology 76(561-568).

Galiacy S, Planus E, Lepetit H, Fereol S, Laurent V, Ware L, Isabey D, Matthay M, Harf A, and d'Ortho MP (2003). "Keratinocyte growth factor promotes cell motility during alveolar epithelial repair in vitro." Experimental Cell Research 287(2): 199-208.

Ghosh M, Song X, Mouneimne G, Sidani M, Lawrence DS, and Condeelis JS (2004). "Cofilin promotes actin polymerization and defines the direction of cell motility." Science 304: 743-746.

Gilcrease M (2007). "Integrin signals in epithelial cells." Cancer Letters 247(1): 1-25.

Glading A, Bodnar RJ, Reynolds IJ, Shiraha H, Satish L, Potter DA, Blair HC, and Wells A (2004). "Epidermal growth factor activates m-calpain (calpain II), at least in part, by extracellular signal-regulated kinase-mediated phosphorylation." Molecular & Cellular Biology 24: 2499-2512.

Goldfinger LE, Hopkinson SB, deHart GW, Collawn S, Couchman JR, and Jones JC (1999). "The α3 laminin subunit, α6β4 and α3β1integrin coordinately regulate wound healing in cultured epithelial cells and in the skin." Journal of Cell Science 112: 2615-2629.

Goncharova EA, Vorotnikov AV, Gracheva EO, Wang CL, Panettieri RA, Stepanova VV, and Tkachuk VA (2002). "Activation of p38 MAP-kinase and caldesmon phosphorylation are essential for urokinase induced human smooth muscle cell migration." Biological Chemistry 383: 115-126.

Green KJ, and Jones JC (1996). "Desmosomes and hemidesmosomes: structure and function of molecular components." FASEB Journal 10(8): 871-881.

Grinnell F (1992). "Wound repair, keratinocyte activation and integrin modulation." Journal of Cell Science 101: 1-5.

Guan JL, and Shalloway D (1992). "Regulation of focal adhesion-associated protein tyrosine kinase by both cellular adhesion and oncogenic transformation." Nature 358(6388): 690-692.

Guo L, Degenstein L, and Fuchs E (1996). "Keratinocyte growth factor is required for hair development but not for wound healing." Genes & Development 10(2): 165-175.

Han J, Lee JD, Bibbs L, and Ulevitch RJ (1994). "A MAP kinase targeted by endotoxin and hyperosmolarity in mammalian cells." Science 265(5173): 801-811.

Han J, Lee JD, Jiang Y, Li Z, Feng L, and Ulevitch RJ (1996). "Characterization of the structure and function of a novel MAP Kinase Kinase (MKK6) " Journal of Biological Chemistry and Molecular Biology 271(6): 2888-2891.

Han Z, Boyle DL, Chang L, Bennett B, Karin M, Yang L, Manning AM, and Firestein GS (2001). "c-Jun N-terminal kinase is required for metalloproteinase expression and joint destruction in inflammatory arthritis." Journal of Clinical Investigation 108(1): 73-81.

Hanke JH, Gardner JP, Dow RL, Changelian PS, Brissette WH, Weringer EJ, Pollok BA, and Connelly PA (1996). "Discovery of a novel, potent, and Src family-selective tyrosine kinase inhibitor. Study of Lck-and FynT-dependent T cell activation." Journal of Biological Chemistry 271(2): 695-701.

Hanks SK, Calalb MB, Harper MC, and Patel SK (1992). "Focal adhesion protein-tyrosine kinase phosphorylated in response to cell attachment to fibronectin." Procedures of the National Academy of Science. USA 89(18): 8487-8491.

Harper EG, Alvares SM, and Carter WG (2005). "Wounding activates p38 map kinase and activation transcription factor 3 in leading keratinocytes." Journal of Cell Science 118: 3471-3485.

Harui A, Suzuki S, Kochanek S, and Mitani K (1999). "Frequency and stability of chromosomal integration of adenovirus vectors." Journal of Virology 73: 6141-6146.

Hashizume R, Koizumi H, Ihara A, Ohta T, and Uchikoshi T (1996). "Expression of beta-catenin in normal breast tissue and breast carcinoma: a comparative study with epithelial cadherin and alpha-catenin." Histopathology 29: 139-146.

Hauck CR, Hsia DA, Puente XS, Cheresh DA, and Schlaepfer DD (2002a). "FRNK blocks v-Src-stimulated invasion and experimental metastases without effects on cell motility or growth." EMBO Journal 21(23): 6289-6302.

Hauck CR, Hsia DA, and Schlaepfer DD (2002b). "The focal adhesion kinase - Regulator of cell migration and invasion." IUBMB Life 53(2): 115-119.

Hayashi I, Vuori K, and Liddington RC (2001). "The focal adhesion targeting (FAT) region of focal adhesion kinase is a four-helix bundle that binds paxillin." Nature Structural Biology 9(2): 839-849.

Henry G, Li W, Garner W, and Woodley D (2003). "Migration of human keratinocytes in plasma and serum and wound re-epithelialisation." The Lancet 361: 574-576.

Hettiaratchy S, and Dziewulski P (2005). "ABC of Burns." British Journal of Dermatology 328(7452): 1366-1368.

Hildebrand JD, Schaller MD, and Parsons JT (1993). "Identification of sequences required for the efficient localization of the focal adhesion kinase, pp125FAK, to cellular focal adhesions." Journal of Biological Chemistry(123): 4.

Hintermann E, and Quaranta V (2004). "Epithelial cell motility on laminin-5, regulation by matrix assembly, proteolysis, integrins and erbB receptors." Matrix Biology 23: 75-85.

Holly S, Larson M, and Parise L (2000). "Multiple roles of integrins in cell motility." Experimental Cell Research 261: 69-74.

Horesh D, Sapir T, Francis F, Wolf SG, Caspi M, Elbaum M, Chelly J, and Reiner O (1999). "Doublecortin, a stabilizer of microtubules." Human Molecular Genetics 8: 1599-1610.

Hsieh T, Gordon RE, Clemmons DR, Busby WH Jr, and Duan C (2003). "Regulation of vascular smooth muscle cell responses to insulin-like growth factor (IGF)-I by local IGF-binding proteins." Journal of Biological Chemistry 278(44): 42886-42892.

Huang C, Borchers CH, Schaller MD, and Jacobson K (2004a). "Phosphorylation of paxillin by p38 is involved in the neurite extension of PC-12 cells." Journal of Cell Biology 164: 593-602.

Huang C, Rajfur Z, Borchers C, Schaller MD, and Jacobson K (2003). "JNK phosphorylates paxillin and regulates cell migration." Nature 424: 219-223.

Huang C, Schaller MD, and Jacobson K (2004b). "A role of JNK-paxillin signaling in cell migration." Cell Cycle 3: 4-6.

Hubner G, Brauchle M, Smola H, Madlener M, Fassler R, and Werner S (1996). "Differential regulation of pro-inflammatory cytokines during wound healing in normal and glucocorticoid-treated mice." Cytokine 8(7): 548-556.

Hunger-Glaser I, Fan RS, Perez-Salazar E, and Rozengurt E (2004). "PDGF and FGF induce focal adhesion kinase (FAK) phosphorylation at Ser-910: dissociation from Tyr-397 phosphorylation and requirement for ERK activation." Journal of Cellular Physiology 200(2): 213-222.

Hunger-Glaser I, Salazar EP, Sinnett-Smith J, and Rozengurt E (2003). "Bombesin, lysophosphatidic acid, and epidermal growth factor rapidly stimulate focal adhesion kinase phosphorylation at Ser-910: requirement for ERK activation." Journal of Biological Chemistry 278: 22631-22643.

Hunt TK, Hopf H, and Hussain Z (2000). "Physiology of wound healing." Advances in Skin and Wound Care.

Huttenlocher A, Sandborg RR, and Horwitz FA (1995). "Adhesion in cell migration." Current Opinion in Cell Biology 7: 697-706.

Hynes R (2002). "Integrins: Bidirectional allosteric signalling machines." Cell 110: 673-687.

Ilić D, Furuta Y, Kanazawa S, Takeda N, Sobue K, Nakatsuji N, Nomura S, Fujimoto J, Okada M, and Yamamoto T (1995). "Reduced cell motility and enhanced focal adhesion contact formation in cells from FAK deficient mice." Nature 377(6549): 539-344.

Iwasaki T, Nakata A, Mukai M, Shinkai K, Yano H, Sabe H, Schaefer E, Tatsuta M, Tsujimura T, Terada N, Kakishita E, and Akedo H (2002). "Involvement of phosphorylation of Tyr-31 and Tyr-118 of paxillin in MM1 cancer cell migration." International Journal of Cancer 97(3): 330-335.

Jaffe AB, and Hall A (2002). "Rho GTPases in transformation and metastasis." Advanced Cancer Research 84: 57-80.

Javelaud D, Laboureau J, Gabison E, Verrecchia F, and Mauviel A (2003). "Disruption of basal JNK activity differentially affects key fibroblast functions important for wound healing." Journal of Biological Chemistry 278(27): 24624-24628.

Jiang CK, Connolly D, and Blumenberg M (1991). "Comparison of methods for transfection of human epidermal keratinocytes." Journal of Investigative Dermatology 97.

Jiang Y, Chen C, Li Z, Guo W, Gegner J, Lin S, and Han J (1996). "Characterization of the structure and function of a new mitogen-activated protein kinase (p38)." Journal of Biological Chemistry and Molecular Biology 271(30): 17920-17926.

Johnson GL, and Lapadat R (2002). "Mitogen-activated protein kinase pathways mediated by ERK JNK and p38 protein kinases." Science 298: 1911-1912.

Jussila T, Kauppila S, Risteli L, Risteli J, and Stenback F (2002). "Collagen formation in extracellular matrix of transplants of human transformed keratinocyte cell lines." Anticancer Research 22(3): 1705-1711.

King WG, Mattaliano MD, Chan TO, Tsichlis PN, and Brugge JS (1997). "Phosphatidylinositol 3-kinase is required for integrin-stimulated AKT and Raf-1/mitogen-activated protein kinase pathway activation." Molecular & Cellular Biology 17: 4406-4418.

Kiosses WB, Shattil SJ, Pampori N, and Schwartz MA (2001). "Rac recruits high-affinity integrin alphavbeta3 to lamellipodia in endothelial cell migration." Nature Cell Biology 3(316-320).

Kirchner J, Kam Z, Tzur G, Bershadsky AD, and Geiger B (2003). "Live-cell monitoring of tyrosine phosphorylation in focal adhesions following microtubule disruption." Journal of Cell Science 116(6): 975-986.

Kiyokawa E, Hashimoto Y, Kobiashi S, Sugimura H, Kurata T, and Matsuda M (1998a). "Activation of Rac1 by a Crk SH3-binding protein, DOCK180." Genes & Development 12: 3331-3336.

Kiyokawa E, Hashimoto Y, Kurata T, Sugimura H, and Matsuda M (1998b). "Evidence that DOCK180 up-regulates signals from the CrkII-p130(Cas) complex." Journal of Biological Chemistry 372: 24497-24484.

Klingbeil CK, Hauck CR, Hsia D, Jones KC, Reider SR, and Schlaepfer DD (2001). "Targeting Pyk2 to beta 1-integrin-containing focal contacts rescues fibronectin-stimulated signaling and haptotactic motility defects of focal adhesion kinase-null cells." Journal of Cell Biology 152(1): 97-110.

Kornberg LJ (1998). "Focal adhesion kinase and its potential involvement in tumor invasion and metastasis." Head & Neck 20(8): 745-752.

Krawczyk WS (1971). "A pattern of epidermal cell migration during wound healing." Journal of Cell Biology 49: 247-263.

Laing W (1992). "Chronic venous diseases of the leg." London Office of Health Economics.

Larjava H, Salo T, Haapasalmi K, Kramer RH, and Heino J (1993). "Expression of integrins and basement membrane components by wound keratinocytes." Journal of Clinical Investigation(92): 14.

Lauffenburger DA, and Horwitz AF (1996). "Cell migration: a physically integrated molecular process." Cell 84: 359-369.

Laukaitis CM, Webb DJ, Donais K, and Horwitz AF (2001). "Differential dynamics of alpha 5 integrin, paxillin, and alpha-actinin during formation and disassembly of adhesions in migrating cells." Journal of Cell Biology 153: 1427-1440.

Li W, Fan J, Chen M, Guan S, Sawcer D, Bokoch GM, and Woodley DT (2004a). "Mechanism of human dermal fibroblast migration driven by type I collagen and platelet derived growth factor-BB." Molecular Biology of the Cell 15: 294-309.

Li W, Henry G, Fan J, Bandyopadhyay B, Peng K, Gardner W, Chen M, and Woodley DT (2004b). "Signals that initiate, augment and provide directionality for human keratinocyte motility." Journal of Investigative Dermatology 123: 622-633.

Li W, Nadelman C, Henry G, Fan J, Muellenhoff M, Medina E, Gratch NS, Chen M, Han J, and Woodley DT (2001). "The p38-MAPK/SAPK pathway is required for human keratinocyte migration on dermal collagen." Journal of Investigative Dermatology 117(6): 1601-1611.

Li Y, Fan J, Chen M, Li W, and Woodley D (2006). "Transforming growth factor-alpha Is a major stimulus in human serum that promotes human keratinocyte migration." Journal of Investigative Dermatology 126: 2096-2105.

Lin LL, Wartmann M, Lin AY, Knopf JL, Seth A, and Davis RJ (1993). "cPLA2 is phosphorylated and activated by MAP kinase." Cell 72(2): 269-278.

Liu ZX, Yu CF, Nickel C, Thomas S, and Cantley LG (2002). "Hepatocyte growth factor induces ERK-dependent paxillin phosphorylation and regulates paxillin-focal adhesion kinase association." Journal of Biological Chemistry 277(10452-10458).

Loisel TP, Boujemaa R, Pantaloni D, and Carlier MF (1999). "Reconstitution of actin-based motility of Listeria and Shigella using pure proteins." Nature 401: 613-616.

Lu X, Nemoto S, and Lin A (1997). "Identification of c-Jun NH2-terminal protein kinase (JNK)-activating kinase 2 as an activator of JNK but not p38." Journal of Biological Chemistry 272(40): 24751-24754.

Mackay DJ, and Hall A (1998). "Rho GTPases." Journal of Biological Chemistry 273: 20685-20688.

Madlener M, Parks WC, and Werner S (1998). "Matrix metalloproteinases (MMPs) and their physiological inhibitors (TIMPs) are differentially expressed during excisional skin wound repair." Experimental Cell Research 242(201-210).

Martin P (1997). "Wound healing-Aiming for perfect skin regeneration." Science 276: 75-81.

Massague J (1990). "The transforming growth factor-beta family." Annual Review Cell Biology 6: 597-641.

Matthay MA, Thiery JP, Lafont F, Stampfer M, and Boyer B (1993). "Transient effect of epidermal growth factor on the motility of an immortalized mammary epithelial cell line." Journal of Cell Science 106(3): 869-878.

McAllister SS, Becker-Hapak M, Pintucci G, Pagano M, and Dowdy SF (2003). "Novel p27(kip1) C-terminal scatter domain mediates Rac-dependent cell migration independent of cell cycle arrest functions." Molecular & Cellular Biology 23(1): 216-228.

McLaughlin MM, Kumar S, McDonnell PC, van Horn S, Lee JC, Livi GP, and Young PR (1996). "Identification of mitogen-activated protein (MAP) kinase-activated protein kinase-3, a novel substrate of CSBP p38 MAP kinase." Journal of Biological Chemistry 271: 8488-8492.

Mignatti P, Rifkin DB, Welgus HG, and Parks WC (1996). Proteinases and tissue remodeling. In: The molecular and cellular biology of wound repair. New York, Plenum Press.

Miranti CK, and Brugge JS (2002). "Sensing the environment: A historical perspective on integrin signal transduction." Nature Cell Biology 4(4): E83-90.

Mitra SK, Hanson DA, and Schlaepfer DD (2005). "Focal adhesion kinase: In control of cell motility." Nature Reviews Molecular Cell Biology 6(1): 56-68.

Miyamoto S, Teramoto H, Coso OA, Gutkind JS, Burbelo PD, Akiyama SK, and Yamada KM (1995). "Integrin Function: Molecular Hierarchies of Cytoskeletal and Signaling Molecules." Journal of Cell Biology 131: 791-805.

Mukhopadhyay D, Tsiokas L, Zhou XM, Foster D, Brugge JS, and Sukhatme VP (1995). "Hypoxic induction of human vascular endothelial growth factor expression through c-Src activation." Nature 375(6532): 577-581.

Nahas N, Molski TF, Fernandez GA, and Sha'afi RI (1996). "Tyrosine phosphorylation and activation of a new mitogen-activated protein (MAP)-kinase cascade in human neutrophils stimulated with various agonists." Biochemical Journal 318: 247-253.

Nguyen BP, Gil SG, and Carter WG (2000). "Deposition of Laminin 5 by keratinocytes regulates integrin adhesion and signaling." Journal of Biological Chemistry 275(41): 31896-31907.

Nguyen BP, Ren XD, Schwartz MA, and Carte WG (2001). "Ligation of integrin alpha 3beta 1 by laminin 5 at the wound edge activates Rho-dependent adhesion of leading keratinocytes on collagen." Journal of Biological Chemistry 276(47): 43860-43870.

Niessen CM, Hogervorst F, Jaspers LH, and de Melker AA (1994). "The α6β4 integrin is a receptor for both laminin and kalinin." Experimental Cell Research 211: 360-367.

Nobes CD, and Hall A (1999). "Rho GTPases control polarity, protrusion, and adhesion during cell movement." Journal of Cell Biology 144(6): 1235-1244.

O'Keefe EJ, Woodley DT, Falk RJ, Gammon WR, and Briggaman RA (1987). "Production of fibronectin by epithelium in a skin equivalent." Journal of Investigative Dermatology 88(5): 634-639.

O'Toole EA (1997). "Laminin-5 inhibits human keratinocyte migration." Experimental Cell Research 233: 330–339.

Odland GF (1977). Progression of events of epidermal differentiation in wound healing. In: Biochemistry of cutaneous epidermal differentiation. Baltimore, London, Tokyo, University Park Press.

Ono K, and Han JH (2000). "The p38 signal transduction pathway – activation and function." Cellular Signal 12: 1-13.

Otto IM, Raabe T, Rennefahrt UEE, Bork P, Rapp UR, and Kerkhoff E (2000). "The p150-Spir protein provides a link between c-Jun N-terminal kinase function and actin reorganization." Current Opinion in Cell Biology(10): 345-348.

Parsons T (2003). "Focal adhesion kinase: The first ten years." Journal of Cell Science 116: 1409-1416.

Patel GK, Wilson CH, Harding KG, Finlay AY, and Bowden PE (2006). "Numerous keratinocyte subtypes involved in wound re-epithelialisation." Journal of Investigative Dermatology 126: 497-502.

Petit V, Boyer B, Lentz D, Turner CE, Thiery JP, and Valles AM (2000). "Phosphorylation of tyrosine residues 31 and 118 on paxillin regulates cell migration through an association with CRK in NBT-II cells." Journal of Cell Biology 148: 957-970.

Raingeaud J, Gupta S, Rogers S, Dickens M, Han J, Ulevitch J, and Davis J (1995). "Pro-inflammatory cytokines and environmental stress cause p38 mitogen-activated protein kinase activation by dual phosphorylation on tyrosine and threonine." Journal of Biological Chemistry 270: 7420-7426.

Rappolee DA, Mark D, Banda MJ, and Werb Z (1988). "Wound macrophages express TGF-alpha and other growth factors in vivo: analysis by mRNA phenotyping." Science 241(4866): 708-712.

Ray RM, Vaidya RJ, and Johnson LR (2007). "MEK/ERK regulates adherens junctions and migration through Rac1." Cell Motility and the Cytoskeleton 64(3): 143-156.

Riches DW, Chan ED, and Winston BW (1996). "TNF-alpha-induced regulation and signaling in macrophages." Immunobiology 195(4): 477-490.

Ridley AJ, Comoglio PM, and Hall A (1995). "Regulation of scatter factor/hepatocyte growth factor responses by Ras, Rac, and Rho in MDCK cells." Molecular & Cellular Biology 15(2): 1110-1122.

Ridley AJ, Schwartz MA, Burridge K, Firtel RA, Ginsberg MH, Borisy G, Parsons JT, and Horwitz AR (2003). "Cell migration, integrating signals from front to back." Science 302: 1704-1709.

Roberts AB, and Russo A (2000). "Smad3: A key player in pathogenetic mechanisms dependent on TGF-beta." Annual N.Y Academy of Science 995: 1-10.

Robinson MJ, and Cobb MH (1997). "Mitogen-activated protein kinase pathways." Current Opinion in Cell Biology 9(2): 180-186.

Rodriguez OC, Schaefer AW, Mandato CA, Forscher P, and Bement WM (2003). "Conserved microtubule-actin interactions in cell movement and morphogenesis." Nature Cell Biology 5(7): 599-609.

Rousseau S, Houle F, Landry J, and Huot J (1997). "p38 MAP kinase activation by vascular endothelial growth factor mediates actin reorganization and cell migration in human endothelial cells." Oncogene 15: 2169-2177.

Rousselle P, Lunstrum GP, Keene DR, and Burgeson RE (1991). "Kalinin: an epithelium-specific basement membrane adhesion molecule that is a component of anchoring filaments." Journal of Cell Biology 114(3): 567-576.

Russell AJ, Fincher E, Millman L, Smith R, Vela V, Waterman EA, Dey C, Guide S, Weaver V, and Marinkovich P (2003). "α6β4 integrin regulates keratinocyte chemotaxis through differential GTPase activation and antagonism of α3β1 integrin." Journal of Cell Science 116: 3543-3556.

Sakoda T, Kasahara N, Hamamori Y, and Kedes L (1998). "A high-titer lentiviral production system mediates efficient transduction of differentiated cells including beating cardiac myocytes." Journal of Molecular and Cellular Cardiology 31: 2037-2047.

Sarret Y, Stamm C, Jullien D, and Schmitt D (1992a). "Keratinocyte migration is partially supported by the cell-binding domain of fibronectin and Is RGDS-dependent." Journal of Investigative Dermatology 99: 656-659.

Sarret Y, Woodley D, Grigsby K, Wynn K, and O'Keefe E (1992b). "Human keratinocyte locomotion: The effect of selected cytokines." Journal of Investigative Dermatology 98: 12-16.

Satish L, Yager D, and Wells A (2003). "Glu-Leu-Arg-negative CXC chemokine interferon gamma inducible protein-9 as a mediator of epidermal-dermal communication during wound repair." Journal of Investigative Dermatology 120(6).

Satoh T, Nakafuku M, and Kaziro Y (1992). "Function of Ras as a molecular switch in signal transduction." Journal of Biological Chemistry 267(24149-24152).

Schagen FH, Rademaker HJ, Fallaux FJ, and Hoeben RC (2000). "Insertion vectors for gene therapy." Gene Therapy 7(4): 271-272.

Schaller MD, Borgman CA, Cobb BS, Vines RR, Reynolds AB, and Parsons JT (1992). "pp125FAK a structurally distinctive protein-tyrosine kinase associated with focal adhesions." Procedures of the National Academy of Science. USA 89(11): 5192-5196.

Schaller MD, Hildebrand JD, Shannon JD, Fox JW, Vines RR, and Parsons JT (1994). "Autophosphorylation of the focal adhesion kinase, pp125FAK, directs SH2-dependent binding of pp60src." Molecular & Cellular Biology 14(3): 1680-1688.

Schaller MD, and Parsons JT (1995). "pp125FAK-dependent tyrosine phosphorylation of paxillin creates a high- affinity binding site for Crk." Molecular & Cellular Biology 15(5): 2635-2645.

Schaub S, Meister JJ, and AB, Verkhovsky (2007). "Analysis of actin filament network organization in lamellipodia by comparing experimental and simulated images." Journal of Cell Science 120: 1491-1500.

Schlaepfer DD, and Hunter T (1996). "Evidence for In Vivo phosphorylation of the Grb2 SH2-domain binding site on focal adhesion kinase by Src-family protein-tyrosine kinases." Molecular & Cellular Biology 16(10): 5623-5633.

Schlaepfer DD, and Hunter T (1998). "Integrin signaling and tyrosine phosphorylation: just the FAKs?" Trends in Cell Biology 8(4): 151-157.

Schlaepfer DD, Mitra SK, and Ilic D (2004). "Control of motile and invasive cell phenotypes by focal adhesion kinase." Biochimica et Biophysica Acta 1692(2-3): 77-102.

Schwartz MA, Schaller MD, and Ginsberg MH (1995). "Integrins: emerging paradigms of signal transduction." Annual Review of Cell & Developmental Biology 11: 549-599.

Schwartz MA, and Shattil SJ (2000). "Signaling networks linking integrins and Rho family GTPases." Trends in Biochemical Science 25: 388-391.

Schwertz DW (1994). "Cellular communication through signal transduction: the background." Journal Cardiovascular Nursing 8(3): 1-27.

Scott WN, McCool K, and Nelson J (2000). "Improved method for the production of gold colloid monolayers for use in the phagokinetic track assay for cell motility " Analytical Biochemistry 287: 343-344.

Seger R, and Krebs EG (1995). "The MAPK signaling cascade." FASEB Journal 9(9): 726-735.

Sieg DJ, Hauck CR, Ilic D, Klingbeil CK, Schaefer E, Damsky CH, and Schlaepfer DD (2000). "FAK integrates growth-factor and integrin signals to promote cell migration." Nature Cell Biology 2: 249-256.

Sieg DJ, Ili D, Jones KC, Damsky CH, Hunter T, and Schlaepfer DD (1998). "Pyk2 and Src-family protein-tyrosine kinases compensate for the loss of FAK in fibronectin stimulated signaling events but Pyk2 does not fully function to enhance FAK- cell migration." EMBO Journal 17(20): 5933-5847.

Singer A, and Clarke R (1999). "Cutaneous wound healing." New England Journal of Medicine 341(10): 738-746.

Singh RP, Dhawan P, Golden C, Kapoor GS, and Mehta KD (1999). "One-way cross-talk between p38(MAPK) and p42/44(MAPK). Inhibition of p38(MAPK) induces low density lipoprotein receptor expression through activation of the p42/44(MAPK) cascade." Journal of Biological Chemistry and Molecular Biology 274(28): 19593-19600.

Slack JK, Adams RB, Rovin JD, Bissonette EA, Stoker CE, and Parsons JT (2001). "Alterations in the focal adhesion kinase/Src signal transduction pathway correlate with increased migratory capacity of prostate carcinoma cells." Oncogene 20(10): 1152-1163.

Small JV, Stradal T, Vignal E, and Rottner K (2002). "The lamellipodium: where motility begins." Trends in Cell Biology 12(3): 112-120.

Staedel C, Remy JS, Hua Z, Broker TR, Chow LT, and Behr JP (1994). "High-efficiency transfection of primary human keratinocytes with positively charged lipopolyamine:DNA complexes." Journal of Investigative Dermatology 102: 768-772.

Stossel TP (1993). "On the crawling of animal cells." Science 260: 1086-1094.

Tang G, Minemoto Y, Dibling B, Purcell NH, Z, Li, Karin M, and Lin A (2001). "Inhibition of JNK activation through NF-{kappa}B target genes." Nature 414: 313-317.

Torimura T, Ueno T, Kin M, Harada R, Nakamura T, Sakamoto M, Kumashiro R, Yano R, Kojiro M, and Sata M (2001). "Laminin deposition to type IV collagen enhances haptotaxis, chemokinesis, and adhesion of hepatoma cells through β1-integrins." Journal of Hepatology 35(2): 245-253.

Tsuboi R, Sato C, Kurita Y, Ron D, Rubin JS, and Ogawa H (1993). "Keratinocyte growth factor (FGF-7) stimulates migration and plasminogen activator activity of normal human keratinocytes." Journal of Investigative Dermatology 101(1): 49-53.

Tsubouchi A, Sakakura J, Yagi R, Mazaki Y, Schaefer E, Yano H, and Sabe H (2002). "Localized suppression of RhoA activity by Tyr31/118-phosphorylated paxillin in cell adhesion and migration." Journal of Cell Biology 159: 673-683.

Vanhaesebroeck B, Stein RC, and Waterfield MD (1996). "The study of phosphoinositide 3-kinase function." Cancer Surveillance 27: 249-270.

Vasioukhin V, Bauer C, Yin M, and Fuchs E (2000). "Directed actin polymerization is the driving force for epithelial cell-cell adhesion." Cell 100(209-219).

Wadman M (2005). "Scar prevention: The healing touch." Nature 436: 1079-1080.

Webb DJ, Donais K, Whitmore LA, Thomas SM, Turner CE, Parsons JT, and Horwitz AF (2004). "FAK-Src signaling through paxillin, ERK and MLCK regulates adhesion disassembly." Nature Cell Biology 6: 154-161.

Weijland A, Neubauer G, Courtneidge SA, Mann M, Wierenga RK, and Superti-Furga G (1983). "Structure and sequence of the cellular gene homologous to the RSV src gene and the mechanism for generating the transforming virus." Cell 32(3): 881-890.

Welch MD, and Mullins RD (2002). "Cellular control of actin nucleation." Annual Review of Cell & Developmental Biology 18: 247-288.

Welch MP, Odland GF, and Clark RAF (1990). "Temporal relationships of F-actin bundle formation, collagen and fibronectin matrix assembly, and fibronectin receptor expression to wound contraction." Journal of Cell Biology 110: 133-145.

Werner S, Peters KG, Longaker MT, Fuller-Pace F, Banda MJ, and Williams LT (1992). "Large induction of keratinocyte growth factor expression in the dermis during wound healing." Procedures of the National Academy of Science. USA 89(15): 6896-6900.

Werner S, Smola H, and Liao X (1994). "The function of KGF in morphogenesis of epithelium and re-epithelialisation of wounds." Science 266: 819-822.

Wichert G, Haimovich B, Feng GS, and Sheetz MP (2003). "Force-dependent integrin–cytoskeleton linkage formation requires downregulation of focal complex dynamics by Shp2." EMBO Journal 22(19): 5023-5035.

Woodley DT (1996). Re-epithelialisation: The molecular and cellular biology of wound repair. London, Plenum Press.

Woodley DT, Bachmann PM, and O'Keefe EJ (1988). "Laminin inhibits human keratinocyte migration." Journal of Cellular Physiology 136: 140-146.

Xu J, and Clarke RAF (2000). Integrin regulation in wound healing. In: Scarless wound healing, Informa Healthcare.

Yamada KM, and Miyamoto S (1995). "Integrin transmembrane signaling and cytoskeletal control." Current Opinion in Cell Biology 7: 681-689.

Zaidel-Bar R, Cohen M, Addadi L, and Geiger B (2004). "Hierarchical assembly of cell matrix adhesion complexes." Biochemical Society Transactions 32(3): 416-420.

Zaidel-Bar R, Kam Z, and Geiger B (2005). "Polarized downregulation of the paxillin-p130CAS-Rac1 pathway induced by shear flow." Journal of Cell Science 118: 3997-4007.

Zaidel-Bar R, Milo R, Kam Z, and Geiger B (2007). "A paxillin tyrosine phosphorylation switch regulates the assembly and form of cell-matrix adhesions " Journal of Cell Science 120: 137-148.

Zamir E, and Geiger B (2001). "Molecular complexity and dynamics of cell-matrix adhesions." Journal of Cell Science 114: 3583-3590.

Zetter BR (1980). "Migration of capillary endothelial cells is stimulated by tumor derived factors." Nature 285: 41-43.

Zhang K, and Kramer R (1996). "Laminin-5 deposition promotes keratinocyte motility." Experimental Cell Research 227: 309-322.

Zhang L, Deng M, Parthasarathy R, Wang L, Mongan M, Molkentin J, Zheng Y, and Xia Y (2005). "MEKK1 transduces activin signals in keratinocytes to induce actin stress fiber formation and migration." Molecular & Cellular Biology 25(1): 60-65.

Zheng CF, and Guan KL (1993). "Cloning and characterization of two distinct human extracellular signal-regulated kinase activator kinases, MEK1 and MEK2." Journal of Biological Chemistry 268(15): 11435-11439.

Zhu X, and Assoian RK (1995). "Integrin-dependent activation of MAP kinase: A link to shape-dependent cell proliferation." Molecular Biology of the Cell 6: 273-282.

Zigmond SH (1974). "Mechanisms of sensing chemical gradients by polymorphonuclear leukocytes." Nature 249: 450-452.