UNIVERSITY OF SOUTHAMPTON

BONE TISSUE ENGINEERING: BIOMIMETIC STRUCTURES FOR HUMAN OSTEOPROGENITOR GROWTH

XUEBIN YANG M.D., MS.c.

Doctor of Philosophy

University Orthopaedics

Faculty Of Medicine, Health & Biological Sciences

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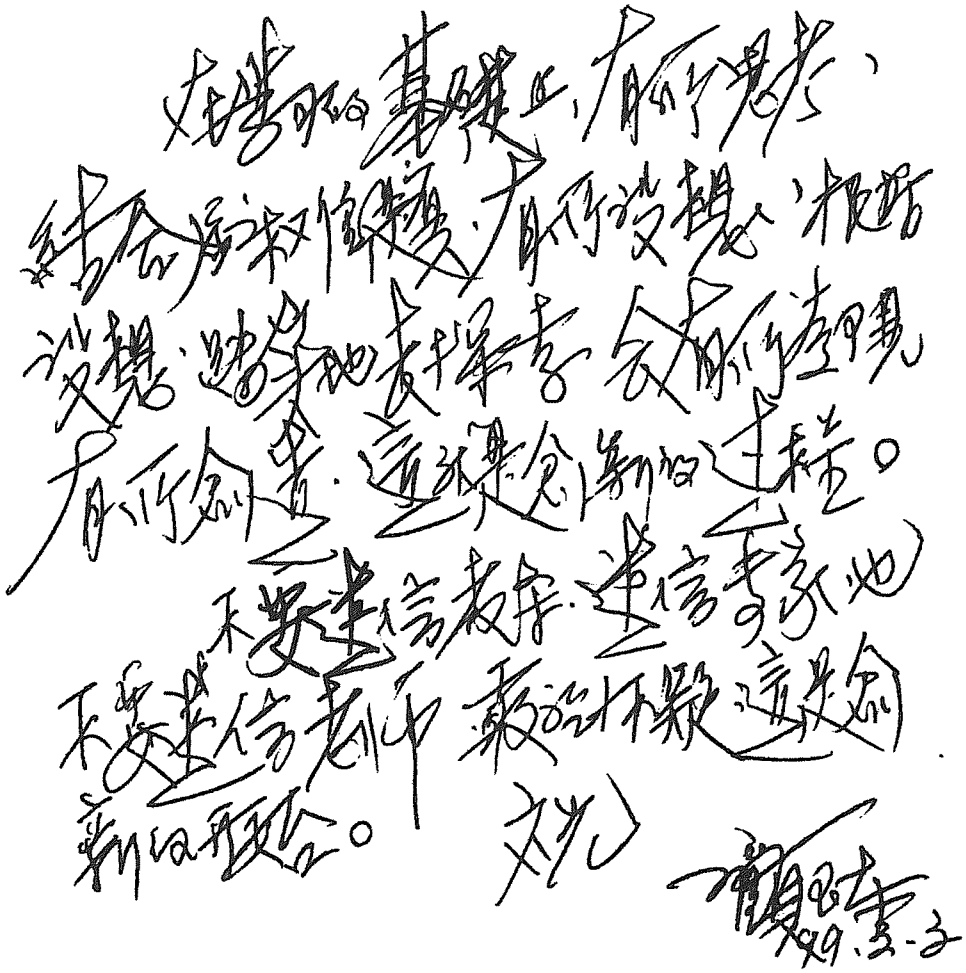
This thesis is specially dedicated to my family. To my father for his encouragement, to my mother for her support, to my wife for her understanding and to my son for his tolerance, all of which made this thesis possible.



Dr. Yang,

Based on studies, you should keep thinking, presuming a combination of clinical difficulties and then steadfastly exploring and leading to some discoveries and creations – this is the way to innovation.

Don't rely on blind faith from textbooks, experts and supervisors, question often – This is the approach to invention.

Professor Yudong Gu
March 3rd, 1999

Professor and Director
Shanghai Institute of Hand Surgery
Shanghai Medical University
PR China

Director
Key Laboratory of Hand Function
Chinese State Ministry of Health

President of
Hand Surgery Society
Chinese Medical Association
PR China

Member
Chinese Academy of Engineering,
Medical and Health Division

UNIVERSITY OF SOUTHAMPTON

ABSTRACT

FACULTY OF MEDICINE, HEALTH & BIOLOGICAL SCIENCES

UNIVERSITY ORTHOPAEDICS

Doctor of Philosophy

BONE TISSUE ENGINEERING: BIOMIMETIC STRUCTURES FOR HUMAN OSTEOPROGENITOR GROWTH

by Xuebin Yang M.D., MS.c.

The ability to generate new bone for skeletal use is a major clinical need. Biomimetic scaffolds that interact and promote human osteoprogenitor differentiation and osteogenesis offer a promising approach to generate skeletal tissue to resolve this major healthcare issue.

This thesis has investigated a tissue-engineering approach to bone regeneration combining a source of bone cells with biodegradable porous polymer scaffolds and selective osteoinductive growth factors.

In vitro and *in vivo* approaches to investigate the ability to generate biomimetic scaffolds for bone tissue engineering included the use of 1) surface-modification of an biodegradable polymer scaffold with GRGDS peptide, fibronectin (FN), 2) Saos-2 'retentate' extracts – bone morphogenetic protein (BMP's), 3) recombinant human pleiotrophin (rhPTN) and, 4) recombinant human bone morphogenetic protein-2 (rhBMP-2). In addition, the potential of growth factor encapsulation and gene therapy to deliver growth factors to modulate human osteoprogenitor adhesion and differentiation was examined.

Human osteoprogenitors were shown to adhere, differentiate and to mineralise on FN and GRGDS coupled polymer films as well as on three-dimensional (3-D) biodegradable scaffolds indicating the potential to generate porous scaffolds with the ability to mimic the biological microenvironments for cell growth. Saos-2 cell 'retentate' extract, which contains a mixture of BMP's, stimulated human osteoprogenitor adhesion, growth and differentiation on poly(-lactic acid *co*÷glycolic acid) (PLGA) (75:25) porous scaffolds. 3-D biomimetic structures adsorbed with rhPTN maintained the ability to modulate human osteoprogenitor activity resulting in new cartilage and bone matrix formation both *in vitro* and *in vivo.* Human BMP-2 gene adenoviral transfer into human bone marrow stromal cells confirmed the possibility of using gene therapy to engineer bone tissue. Furthermore, rhBMP-2 encapsulated within PLA porous scaffolds by a supercritical fluid mixing method, stimulated C2C12 cell differentiation and promoted human osteoprogenitor proliferation, differentiation as well as new cartilage and bone formation on 3-D PLA scaffolds *in vivo.*

In conclusion, these studies indicate the ability to generate osteoinductive and osteoconductive biomimetic scaffold using biodegradable polymers in combination with osteogenic growth factors to create a biomimetic microenvironment for human osteoprogenitor growth and to generate templates for the development of a living tissue substitute for bone.

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CONTENTS

ABBREVIATIONS

μg	Microgram
μl	Microlitre
μM	Micromolar
μm	Micrometer
μm^3	Micrometer cubed
α-MEM	Minimum Essential Medium Eagles (α modification)
$1,25(OH)_2D_3$	1,25-dihydroxy vitamin D_3
2-D	Two dimensional
3-D	Three dimensional
A/S	Alcian blue and Sirius red staining
AA-2-P	Ascorbic acid-2-phosphate
AB	Avidin and Biotin
AEC	3-amino 9-ethyl-carbazole
$AgNO_3$	Silver nitrate
ANOVA	ANalysis Of VAriance between groups
AP	Alkaline phosphatase
AxCALacZ	A replication-deficient adenoviral vector carrying Escherichia coli (E. coli) LacZ gene
AxCAOBMP-2	A replication-deficient adenoviral vector carrying the human BMP-2 gene
Balb/3T3 cells	Mouse embryo fibroblast-like cell line
Basal media	10% Foetal calf serum in Minimum Essential Medium Eagles (α modification)
BMP	Bone morphogenetic protein
BSA	Bovine serum albumin
BSP	Bone sialoprotein
C2C12 cell	Mouse promyoblast cell line
Ca^{+2}	Calcium ions
$Ca_{10}[PO_4]_6[OH]_2$	Hydroxyapatite
CAM	Chorioallantoic membrane

cbfa1	Core-binding factor 1
CFU-F	Colony forming unit-fibroblastic
cm	Centimetre
cm^2	Centimetre square
CMFDA	Cell Tracker™ green (5-chloromethylfluorescein diacetate)
CO_2	carbon dioxide
Dex	Dexamethasone
DL-PLA	Poly(D,L-lactic acid)
D-MEM	Dulbecco's Minimum Essential Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
ds DNA	Double stranded deoxyribonucleic acid
E. coli	*Escherichia coli*
ECM	Extracellular matrix
EDTA	Ethylenediamine tetraacetic acid
EH-1	Ethidium Homodimer-1
-EILDV-	-Glu-Ile-Leu-Asp-Val- peptide sequence
EM	Electron microscopy
FCS	Fetal calf serum
FDA	U.S. Food and Drug Administration
FGF	Fibroblast growth factor
-FHRRIKA-	-Phe-His-Arg-Arg-Ile-Lys-Ala- peptide sequence
Fig.	Figure
FN	Fibronectin
FN-PLA	Fibronectin adsorbed poly(lactic acid)
g	Gram
GH	Growth hormone
GRGDS	Gly-Arg-Gly-Asp-Ser peptide sequence
H_2O_2	Hydrogen peroxide
HA	Hydroxyapatite
HB-GAM	Heparin-binding growth-associated molecule
hr	Hour
HSC	Hematopoietic stem cell

IGF	insulin-like growth factor
IgG	immunoglobulin G
kDa	Kilo Dalton
kg	Kilogram
KH_2PO_4	Potassium dihydrogen phosphate
-KQAGDV-	-Lys-Gln-Ala-Gly-Asp-Val- peptide sequence
-KRSR-	-Lys-Arg-Ser-Arg- peptide sequence
L	litre
LacZ	*LacZ* gene
LN	laminin
m	Meter
M	Mineralisation
MC3T3-E1	Mouse osteoblastic cell
MF1-nu/nu	Immunodeficient nude mice
mg	Milligram
Mg^{2+}	Magnesium ions
mins	Minutes
MK	Midkine
ml	Millilitre
mm	Millimetre
mM	Millimolar
MOI	Multiplicities of infection
mRNA	Messenger ribonucleic acid
MSC	Mesenchymal stem cell
MyoD	Myoblast determination protein
n	Number of samples in a group
ng	Nanogram
ng/ml	Nanogram/millilitre
NIH	National institute of health, USA
NIH 3T3 cells	NIH Swiss mouse embryo fibroblast-like cell line
nM	Nanomolar
nm	Nanometer
nmol NP/hr/ng DNA	Nanomoles of p-Nitrophenol/hour/nanogram DNA

NP	p-Nitrophenol
°C	Degree centigrade
OCN	Osteocalcin
ON	Osteonectin
OPN	Osteopontin
OS35	Osteocalcin specific monoclonal antibody
OSF-1	Osteoblast-stimulating factor-1 (the protein)
Osteogenic media	10% Fatal calf serum in Minimum Essential Medium supplement with dexamethasone (10nM), ascorbic acid-2-phosphate (100μM).
P	P value
P-15	The cell binding domain of type I collagen (15 amino acid fragment).
PBS	Phosphate-buffered saline
PCL	Poly(∈-caprolactone)
PEBP2αA	*cbfa1* primary antibody
PFA	Paraformaldehyde
pg	Picogram
PG	Prostaglandin
pg/ml	Picogram/millilitre
PGA	Poly(glycolic acid)
Pi	Inorganic phosphate
PLA	Poly(lactic acid)
PLGA	Poly(l-lactic-co-glycolic acid)
PLL	Poly(L-Lysine)
PMMA	Polymethylmethacrylate
PPARγ	peroxisome proliferator-activated receptor gamma
PTH	Parathyroid hormone
PTN	Pleiotrophin
RGD	The integrin amino acid recognition sequence Arg-Gly-Asp
rhBMP-2	Recombinant human bone morphogenetic protein-2
rhPTN	Recombinant human pleiotrophin
Saos-2	Human osteogenic sarcoma cell line

$scCO_2$	Supercritical CO2
SD	Standard deviation
SEM	Scanning electron microscope
T/B	Toluidine blue
TGF-β	Transforming growth factor-β
Tris	Tris(hydroxymethyl)methylamine
$^{v}/_{v}$	Volume by volume
$^{w}/_{v}$	Weight by volume
$\bar{X}$	Mean

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CHAPTER 1

INTRODUCTION

1.1. Bone Anatomy, Physiology and Histology

Bone is a specialized connective tissue that makes up, together with cartilage, the skeletal system. It consists of cells (*osteoblasts*, which synthesize the organic components of the matrix; *osteocytes*, which are osteoblasts entrapped within their matrix; and *osteoclasts*, which are involved in the resorption and remodeling of bone tissue) and bone matrix. However, unlike the most of other connective tissues, the extracellular components of bone are calcified, making it a hard, unyielding substance ideally suited for its supportive and protective function in the skeleton. Although a relatively lightweight material, bone has a high tensile and compressive strength, with a degree of elasticity. Despite this strength and hardness, bone is a dynamic living material, constantly being renewed and reconstructed through out the lifetime of the individual (Junqueira et al, 1995).

The microscopic examination of bone dates back to the earliest days of microscopy - in 1674, Anton van Leeuwenhoek read a letter to the Royal Society on the topic. Soon afterwards, in 1691, Clopton Havers published his Osteologia Nova in which he described the pores in the cortical bone that we now refer to as Haversian canals. Since then, major contributions to the study of bone anatomy and histology have been made by many of the most famous names in pathology and medicine. In 1772, Hunter elucidated the mechanism of bone growth, particularly the appositional mechanism rather than that of interstitial growth such as occurs in other organ systems. In 1776, Windlow's Anatomical Exposition systematized the approach to bone anatomy (Bullough et al, 1997).

1.1.1. Functions of skeletal system

The skeletal system performs several basic functions including (Marieb, 1998):

1) Support: As a hard framework, providing a mechanical support and site of muscle attachment for locomotion.
2) Movement: As a facilitation to allow the movement of body and its parts.
3) Protection: As a protective environment, for vital organs such as those in the cranial and thoracic cavities.
4) Storage: As a metabolic reserve of lipids, ions, especially calcium and phosphate, for the maintenance of serum homeostasis, which is essential to life.
5) Blood cell formation: As a source of the blood-forming elements of marrow (Haemopoiesis).

1.1.2. Classification of bones

Bone can be classified according to:

1) shape: long, short, flat, and irregular bone.
2) gross appearance: cortical (compact: dense areas without cavities) or cancellous (spongy or trabecular: with numerous interconnecting cavities) bone.
3) developmental origin: intramembranous or endochondral bone.

1.1.3. Structure of a typical long bone

Epiphysis

The epiphysis comprises the distal and proximal ends of the bone. In mature mammals, they are composed of spongy bone covered by a thin layer of compact bone or a thin layer of articular (hyaline) cartilage, which cushions the opposing bone ends during joint movement and absorbs stress (Fig. 1-1) (Brooker, 1998).

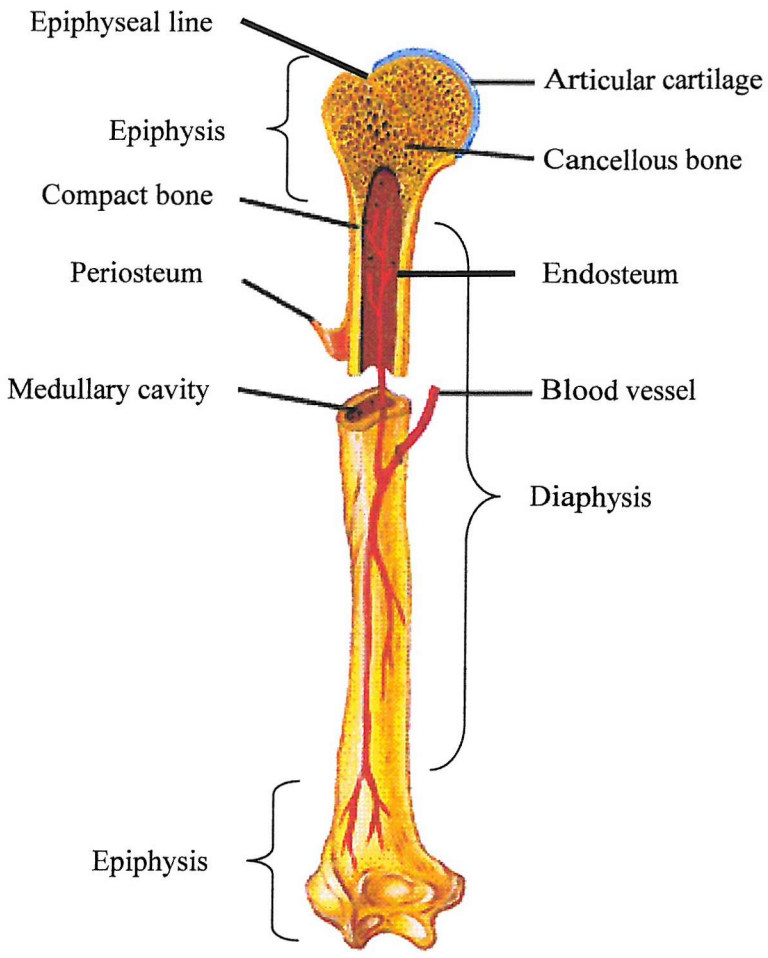


Figure 1-1 Structure of a long bone (Adapted from www.uml.edu)

Diaphysis

The cylindrical part (midshaft) of the long bone, is predominately composed of compact bone, with a small component of spongy bone on its inner surface around the central bone marrow (or medullary) cavity which contains yellow marrow in adult (Fig. 1-1).

Metaphysis

This is the developmental zone between the epiphysis and diaphysis. In a growing long bone, the epiphysis and the metaphysis originate from two independent ossification

centres and are separated by a layer of epiphyseal cartilage (growth plate – a disc-like region of hyaline cartilage) which is responsible for the longitudinal growth of bones during childhood and forms the epiphyseal line in adulthood.

Periosteum

The periosteum is a double-layered membrane, which consists of an outer (fibrous) layer of collagen fibres, fibroblasts and an inner (osteogenic) layer of mesenchymal stem cells, which can differentiate into osteoblasts. It is richly supplied with nerve fibres, lymphatic vessels, and blood vessels, which enter the bone of the shaft via a nutrient foramen.

Endosteum

The endosteum lines all of the internal bone surfaces and is composed of a single layer of flattened osteoprogenitor cells, osteoclasts and a small amount of connective tissue (Junqueira et al, 1995).

Marrow cavity

The marrow (medullary) cavity is the space within the diaphysis containing the bone marrow. The cell population of the bone marrow consists of stem (haemopoietic and stromal) cells with the ability to differentiate along diverse lineages (haemopoietic cells, osteoclasts as well as the stromal lineage, which give rise to osteoblasts, chondrocytes, myoblasts or adipocytes).

1.1.4. Microscopic structure of bone

Compact bone

Compact bone characteristically shows collagen fibres running in a single direction, parallel to each other or concentrically organized around a vascular canal in particular lamellae and always running in opposite directions in adjacent lamellae. This alternating pattern is beautifully designed to withstand torsion stresses -- the adjacent lamellae reinforce one another to resist twisting. In compact bone, the lamellae exhibit a typical organisation consisting of Haversian systems (or osteons) with an outer circumferential lamellae, an inner circumferential lamellae, and an intermediate lamellae (Marieb, 1998). Each Haversian system consists of a central canal surrounded by 4-20 concentric lamellae. Each endosteum-lined canal contains blood vessels, nerves and loose connective tissue. The central canals communicate with the marrow cavity, the periosteum, and each other through transverse or oblique Volkmann's canals which perforate the lamellae (Fig. 1-2) (Junqueira et al, 1995).

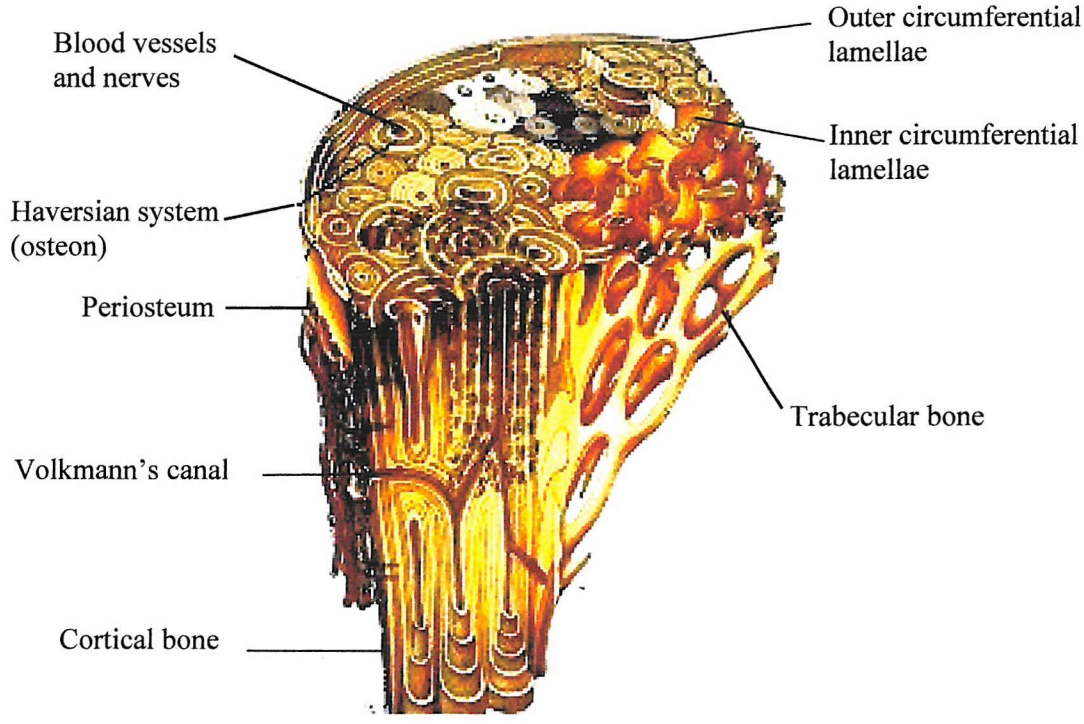


Figure 1-2 Microscopic structure of bone (Adapted from Medes, France)

Between the lamellae are small spaces called lacunae, which contain osteocytes. The osteocytes are connected via radiating small channels, called canaliculi, which connect the lacunae to each other and to the central canal. The canaliculi allow nutrients and waste products to be easily transported throughout the osteon.

Spongy bone

In contrast to compact bone, spongy bone consists of lamellae in an irregular lattice network called trabeculae without the presence of osteons. However, the trabeculae usually align precisely along lines of stress and help the bone to resist that stress as much as possible. The nutrients reach the osteocytes by diffusion through the canaliculi from the marrow spaces between the trabeculae. Spongy bone comprises most of the bone tissue in short, flat and irregularly shaped bones as well as the epiphyses of long bones.

1.1.5. Composition of bone

Bone matrix consists of 15-25% water, 25-35% organic matter and 50-65% inorganic or mineral salts dependent on age and type of the bone tissue. The hardness of bone depends on its inorganic constituents, while its toughness and resilience reside in its organic matrix, particularly within the collagen. Thus, the organic and inorganic matrices provide a highly ordered, remarkably resistant tissue, adapted for its chemical and mechanical functions.

1.1.5.1. Organic components

The organic components include the cells of bone (osteoprogenitors, bone lining cells, osteoblasts, osteocytes, osteoclasts) and osteoid secreted by osteoblasts. In adult mammals, about 90% of the organic matrix is type I collagen and minor constituents include bone sialoprotein (BSP), osteocalcin, osteopontin, osteonectin, proteoglycans and glycoproteins (Junqueira et al, 1995).

Collagen

Collagen is the most abundant protein in vertebrates and provides the structural framework for connective tissues (Fig. 1-3). The preferential orientation of the collagen fibres alternates in adult bone from layer to layer, giving bone a typical lamellar structure. The lamellae can be parallel to each other (trabecular bone and periosteum), or concentric if centered on a blood vessel (Haversian system). However, during development and fracture healing, the collagen fibres are not organized resulting in woven bone (Baron, 1999).

The family of collagen molecules has a triple helical region in which every third amino acid is glycine. The general structure of collagen is a repetition of a triplet of amino acids GLY-X-Y where X is usually proline and Y hydroxyproline.

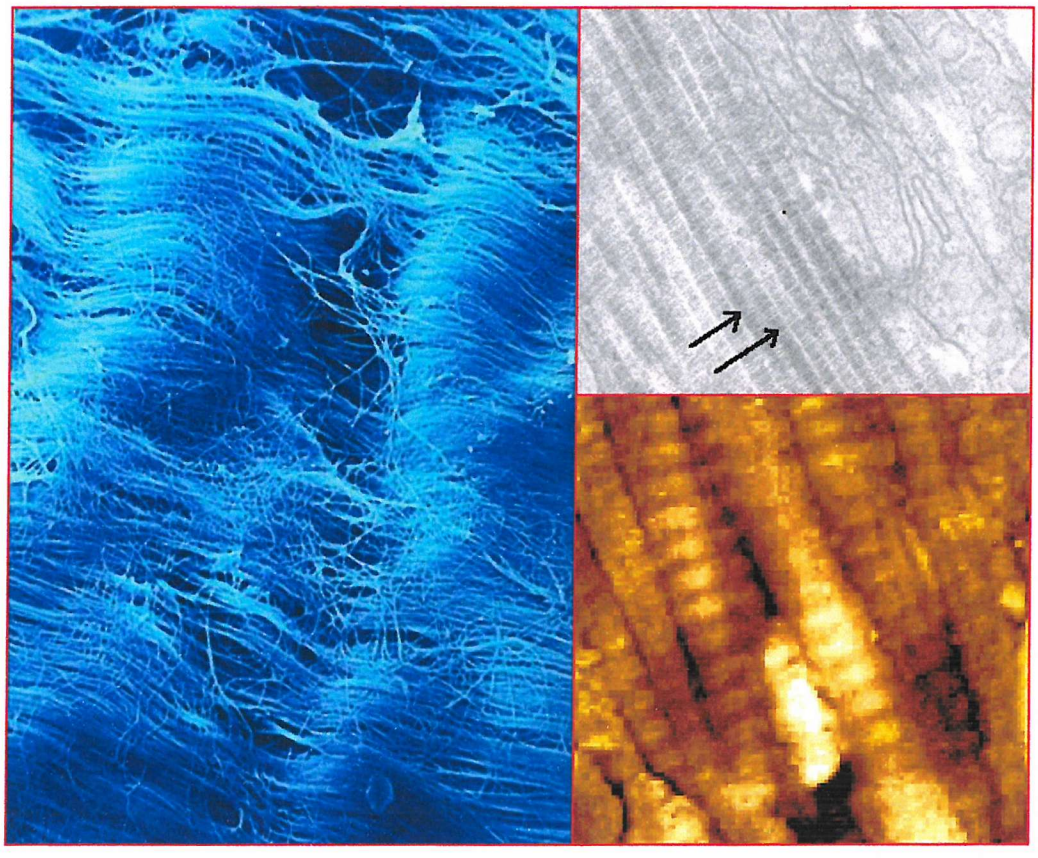


Figure 1-3 Collagen

Non-collagenous constituents of matrix

The non-collagenous proteins represent about 10% by weight of the organic matrix and include sialoprotein (rich in sialic acid), osteocalcin, osteonectin, chondroitin sulphate, albumin, glycoprotein, lipids, laminin, and fibronectin. Bone sialoprotein and osteocalcin contain several γ−carboxyglutamic acid residues; this enables them to bind calcium and may be important in promoting calcification of bone matrix. Other tissues containing type I collagen are not normally calcified and do not contain these glycoproteins.

1.1.5.2. Inorganic matrix

The inorganic components of bone (50% by mass) consist of hydroxyapatite ($Ca_{10}[PO_4]_6[OH]_2$) (Fig. 1-4 and 1-5), or mineral salts, predominantly calcium, phosphates and carbonate with smaller amounts of sodium, magnesium, and fluoride. Calcium salts are present in the form of small crystals that lie in and around the collagen fibres in the extra-cellular matrix (Junqueira et al, 1995).

Figure 1-4 Crystal hydroxyapatite

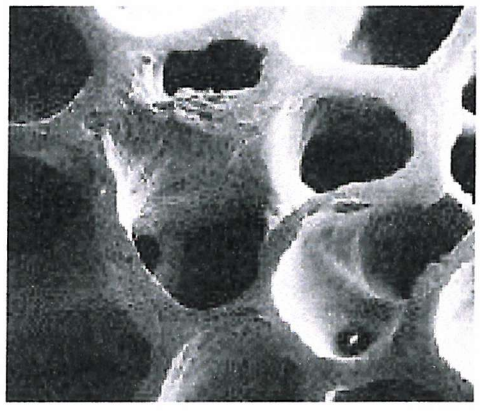


Figure 1-5 Structure of trabecular bone

1.1.6. Cells of bone

Bone, the main calcified component of the skeleton, contains metabolically active cells. These cells can be morphologically classified as osteoprogenitor cells, bone lining cells, osteoblasts, osteocytes and osteoclasts (Triffitt, 1996; Triffitt and Oreffo, 1998b).

1.1.6.1. Osteoprogenitor cells

Like other connective tissues, bone develops from embryonic mesenchyme. It retains in postnatal life a population of relatively undifferentiated cells that have the capacity for mitosis and for further structural and functional specialisation. These osteoprogenitor cells are found on or near all the free surfaces of bone: endosteum, inner layer of the periosteum, lining the Haversian canals, and trabeculae of cartilage matrix at the metaphysis of growing bones (Triffitt, 1996; Oreffo and Triffitt, 1999). During normal growth, remodelling and fracture repair, osteoprogenitor cells differentiate into osteoblasts. Mesenchymal stem cells also have the ability to develop into adipocytes, fibroblasts, reticular cells and myoblasts (Fig. 1-6) (Owen and Friedenstein, 1988; Aubin and Liu, 1996; Rubin and Rubin, 1997; Oreffo and Triffitt, 1999; Bianco and Robey, 2001).

1.1.6.2. Bone-lining cells

Bone-lining cells are thin, flattened inactive cells, which cover all endosteal bone surfaces and prevent bone surfaces from being resorbed (Aubin and Liu, 1996).

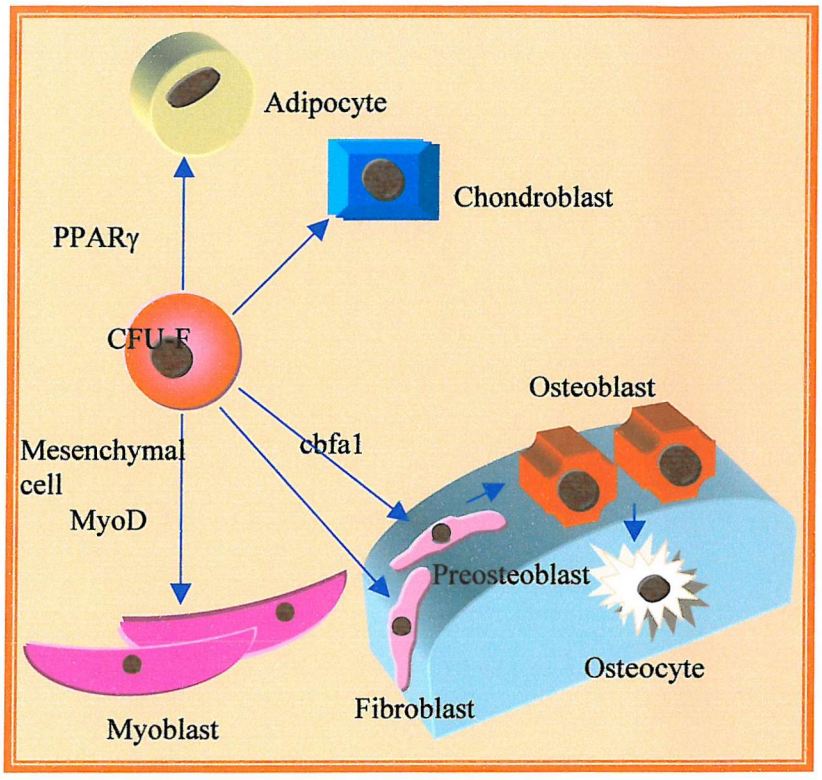


Figure 1-6 Osteoblast maturation. The pluripotent mesenchymal cells, shown here as colony forming unit-fibroblastic (CFU-F) can differentiate into multiple cell types. (Adapted from Rubin et al, 1997).

1.1.6.3. Osteoblasts

The osteoblast is a plump, cuboidal cell (Fig. 1-7), which is located on active bone-forming surfaces, and is responsible for the production of the organic components of bone matrix (collagen, proteoglycans, glycoproteins). Its major function is to lay down osteoid and to control the nucleation and mineralisation of the osteoid matrix. The osteoblast originates from a local mesenchymal stem cell (Owen, 1988; Beresford, 1989; Gao et al, 2001). These precursors are cellular components of the stromal system of bone and marrow and undergo proliferation and differentiation into pre-osteoblasts and then into mature osteoblasts (Friedenstein et al, 1987; Triffitt and Oreffo, 1998a).

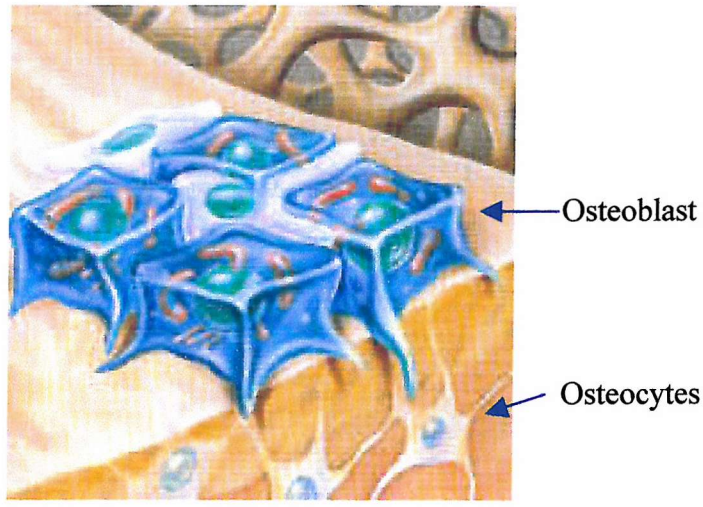


Figure 1-7 Osteoblasts and osteocytes

When actively engaged in matrix synthesis, osteoblasts have a cuboidal to columnar shape and basophilic cytoplasm (rich in alkaline phosphatase-APL). An active human osteoblast produces a seam of osteoid about 15μm thick, at a rate of 0.5 to 1.5μm per day, suggesting the average osteoblast life span in humans to be 15 days. As mineralisation proceeds, the osteoblast may become engulfed in its own calcifying osteoid matrix and is referred as an osteocyte (Fig. 1-8) (Rubin et al, 1997).

1.1.6.4. Osteocytes

The osteocyte is the single most numerous type of bone cell present in all bones (Fig. 1-7 and 1-8). The morphology of the osteocytes varies according to age and functional activity. A young osteocyte has most of the ultra-structural characteristics of the osteoblast. In contrast, an older osteocyte shows a decrease in cell volume and exhibits a significantly reduced rough endoplasmic reticulum and golgi complex and more condensed nuclear chromatin (Baron, 1999). Osteocytes remain connected to each other via canaliculi, which enable cell-cell communication as well as oxygen and nutrition uptake.

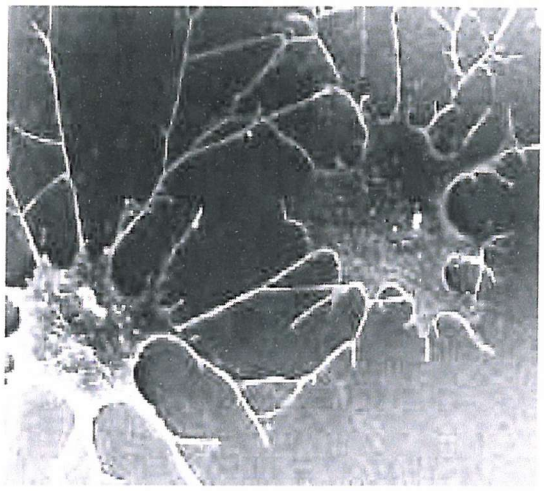


Figure 1-8 Osteocytes

The function of the osteocyte is not clear, it may be concerned with the maintenance of bone matrix and the synthesis of new bone matrix at the surface of the osteocytic lacunae or it may perceive and respond to mechanical stimuli acting as mechanical receptors. In the latter case, the osteocyte then signals to osteoblasts and osteoclasts with a resultant change in bone remodeling as appropriate (Skerry et al, 1989; Burger et al, 1991; Sun et al, 1995; Skerry, 1997).

1.1.6.5. Osteoclasts

Osteoclasts are formed by the fusion of blood-derived mononuclear progenitors of the monocyte-macrophage family and are large (20-100μm in diameter), highly motile, multinucleated cells, which resorb bone. Osteoclasts migrate over the bone surface, creating irregular scalloped shallow cavities called Howship's lacunae (Fig. 1-9). Activated osteoclasts can travel up to 100μm per day, resorbing a cavity 300μm in diameter and excavating 200,000μm^3 of bone (Marks, 1983; Chambers, 1985; Hall and Chambers, 1990; Rubin et al, 1997).

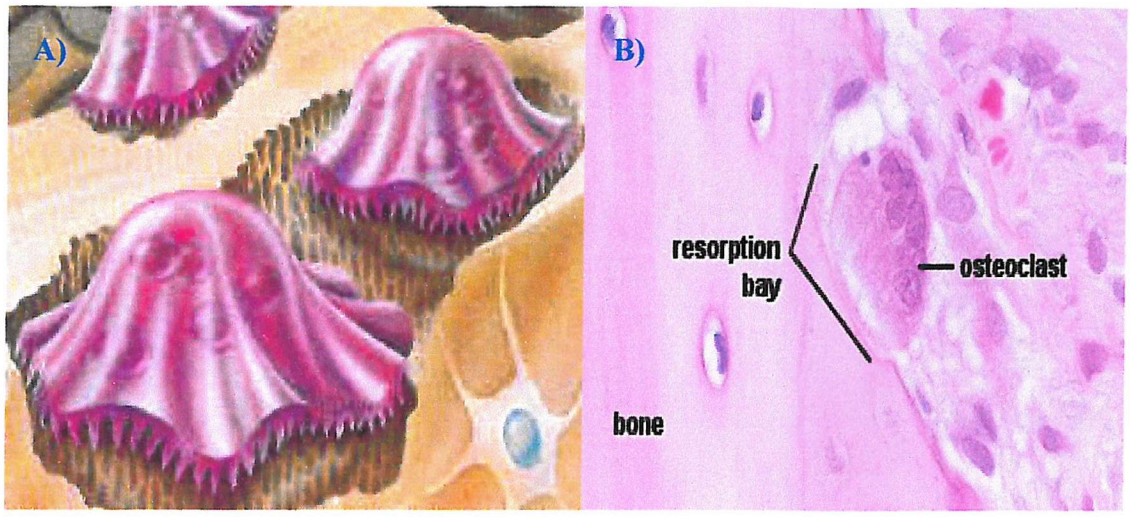


Figure 1-9 Osteoclasts: A) Schemetic illustration of osteoclastic bone resorption, B) Histochemical staining showing the resorption bay in the bone surface by osteoclast (Adapted from Orthoteers, UK).

Osteoclastic bone resorption requires an initial adhesion to the bone surface. For this to occur, osteoclasts attach via the ruffled border (Fig. 1-9). Surrounding the ruffled border is a cytoplasmic zone (clear zone: devoid of organelles), which is rich in actin microfilaments. This zone is the site of adhesion of the osteoclast to the bone matrix and creates a microenvironment for bone resorption. Resorption is by delivery of lysosomal degradative enzymes, collagenase and acid phosphatase to the zone of resorption. At the end of the resorption process (approximately three weeks), the osteoclast is removed by the process of apoptosis (Chambers, 1985; Baron, 1999; Mundy, 1999).

1.1.7. Histogenesis of bone

Bone can be formed by deposition of matrix secreted by osteoblasts onto a membrane **(intramembranous ossification)** or by deposition of bone matrix on a pre-existing cartilage matrix **(endochondral ossification)**. The combination of bone synthesis and removal (remodelling) occurs not only in growing bones but also throughout adult life,

although its rate of change in adult life is much slower (Junqueira et al, 1995; Baron, 1999).

1.1.7.1. Intramembranous ossification

Intramembranous ossification takes place within condensations of mesenchymal tissue (Fig. 1-10) and contributes to the growth of short bones and the thickening of long bones as well as the source of most flat bone. The process is as follows: 1) In the mesenchymal condensation layer, groups of cells differentiate into osteoblasts (about the eighth week of development). 2) These cells form new bone matrix which calcify resulting in the encapsulation of some osteoblasts. These islands of developing bone are known as spicules because of their appearance in histological sections. 3) Several such groups arise almost simultaneously at the ossification centre to give a spongy structure. 4) The connective tissue that remains among the bone spicules is then penetrated by growing blood vessels and undifferentiated mesenchymal cells, giving rise to the bone marrow cells. 5) The ossification centres fuse together, replacing the original connective tissue. The portion of the connective tissue layer that does not undergo ossification gives rise to the endosteum and the periosteum of intramembranous bone (Gilbert, 1997; Baron, 1999)

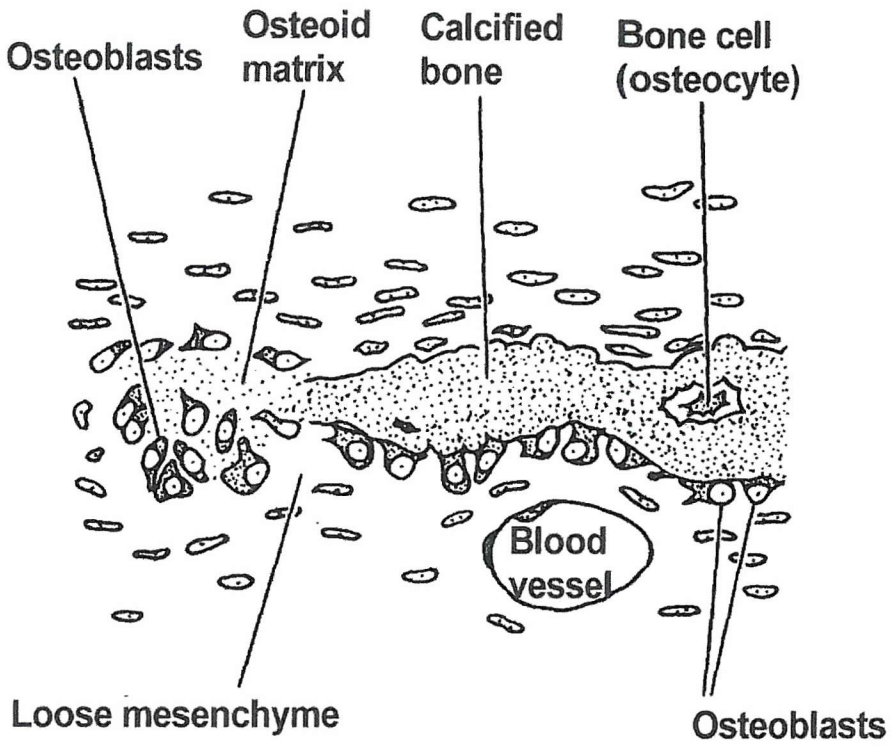


Figure 1-10 Intramembranous ossification (Adapted from Gilbert, 1997)

1.1.7.2. Endochondral ossification

Endochondral ossification (begins late in the second month of development) is responsible for the formation of short and long bones (Fig. 1-11) (Stevens and Lowe, 2000). The following steps have been identified:

1) Chondroblasts develop in primitive mesenchyme and form an early perichondrium and cartilage model.
2) The developing cartilage model assumes the shape of the bone to be formed, and a surrounding perichondrium can be seen.
3) At the mid shaft of the diaphysis, a collar of bone is formed around the hyaline cartilage model: Osteoblasts of the newly converted periosteum secrete osteoid against the hyaline cartilage diaphysis, encasing it externally in a bone collar. The

chondrocytes within the shaft hypertrophy and signal the surrounding cartilage matrix to calcify, which is impermeable to diffusing nutrients. The chondrocytes die and the matrix they maintained begins to deteriorate. However the other cartilage remains healthy and continues to grow rapidly, causing the entire cartilage model to elongate.

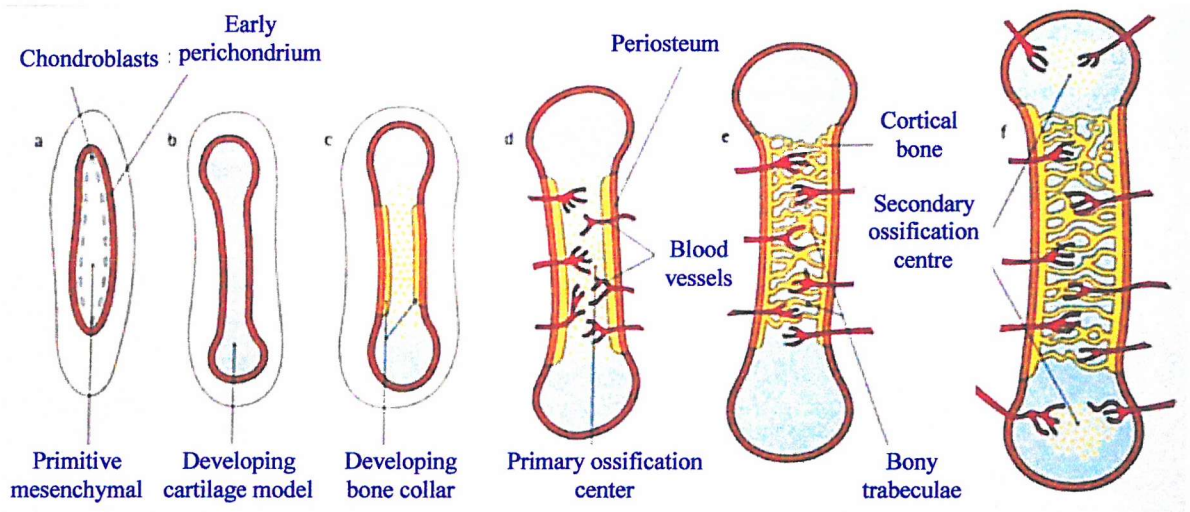


Figure 1-11 Endochondral ossification (Adapted from Stevens and Lowe, 1997)

4) Nutrient blood vessels grow through the periosteum and bone collar (in the third month of development), carrying osteoprogenitor cells within them, which establish a primary ossification centre in the centre of the diaphysis. The entering osteoblasts secrete osteoid around the remaining fragments of hyaline cartilage, forming bone-covered cartilage trabeculae.
5) As the bony trabeculae spread out proximally and distally from the primary ossification center, osteoclasts break down the newly formed spongy bone and open up a medullary cavity in the center of the shaft. The formation of this cavity is the final step in ossification of the shaft.

6) Before or after birth, secondary or epiphyseal ossification centres are established in the centre of each epiphysis by the ingrowth along with blood vessels of mesenchymal cells. The cartilage in the center of the epiphysis calcifies and deteriorates. The entering osteoblast secrete bone matrix around the remaining cartilage fragments.

1.1.7.3. Postnatal bone growth

During infancy and youth, long bones lengthen entirely by interstitial growth of the epiphyseal plates, and all bones grow in thickness by appositional growth. Most bones stop growing during adolescence or in early adulthood (Marieb, 1998). The process of postnatal longitudinal bone growth is through the events of endochondral ossification. As the cartilage of the epiphyseal plate grows, it is replaced continuously by newly formed bone matrix. Epiphyseal cartilage is divided into five zones with each cell population constituting a different maturation state as follows (Fig. 1-12):

1) The resting (or germinal, reserve) zone.
2) The proliferative zone: Proliferating chondrocytes divide rapidly and form columns of stacked cells parallel to the long axis of the bone.
3) The hypertrophic cartilage zone: Contains large chondrocytes whose cytoplasm has accumulated glycogen.
4) The calcified cartilage zone: Simultaneous with the death of chondrocytes and the resorption of non-calcified transverse septa in this zone, the thin longitudinal septa of cartilage matrix become calcified by the deposition of hydroxyapatite.
5) The ossification zone: Endochondral bone tissue appears in this zone, as the osteoblasts deposit bone matrix over the calcified cartilage.

The process of appositional growth results in the widening (thickness) of the bone. Osteoblasts beneath the periosteum secrete bone matrix onto the external bone surface

while osteoclasts on the endosteal surface of the diaphysis remove bone. However, there is normally slightly less breaking down than building up. This unequal process produces a thicker and stronger bone but prevents it from becoming too heavy.

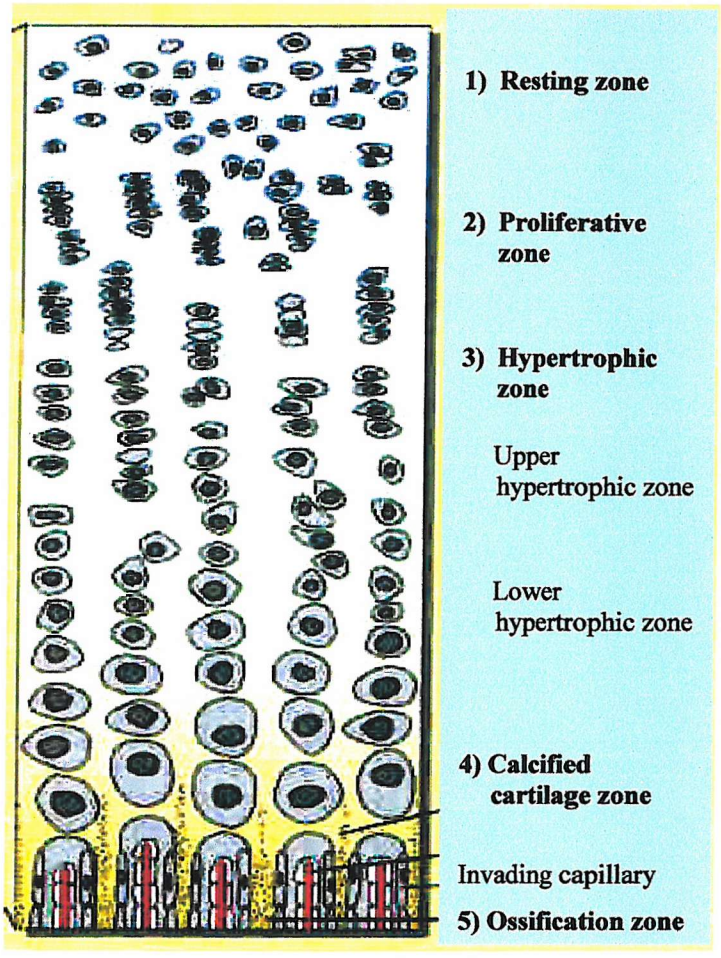


Figure 1-12 Epiphyseal plate (Growth in length of a long bone) (Adapted from Dr Roach - www.soton.ac.uk).

1.1.8. Bone remodelling

Bone remodeling **(bone turnover)** involving bone formation and resorption is necessary for skeletal growth and maintenance, fracture repair, and maintenance of serum calcium. In the adult skeleton, the most common stimulus for this remodeling is physical stress. The process involves bone multicellular units (Frost, 1973; Mundy et

al, 1999). Formation and resorption are closely coupled processes as described below (Fig. 1-13) and it is the uncoupling of this processes that results in the bone disease (osteoporosis):

Figure 1-13 The cellular mechanism of bone remodeling

1) Osteoclast precursors (hematopoietic stem cells) are attracted by chemotaxis to sites where osteoblasts or bone lining cells have moved away from the bone surface. The precursors fuse to become osteoclasts and attach to bone.
2) During osteoclastic bone resorption, osteoclasts secrete lysosomal enzymes that digest the organic matrix, and metabolic acids that convert the calcium salts into soluble forms that pass easily into solution. This is followed by osteoclast apoptosis.
3) Osteoprogenitor cells are attracted to the sites vacated by osteoclasts and differentiate into osteoblasts, which start to synthesize new bone matrix. Sites of new matrix deposit are revealed by the presence of an osteoid seam and the older mineralised bone in between. There is an abrupt transition called the calcification front.

4) Normally, osteoblasts continue to lay down bone matrix until the resorption pit created by osteoclasts is filled in. At the same time, osteoblasts control the maturation of the osteoid (over a period of 10 to 12 day), which is essential for mineralisation.
5) Bone lining cells cover the inactive bone surface. Osteoblasts that did not become osteocytes or bone lining cells undergo apoptosis.

Bone remodelling is related to several factors: 1) strain and stress imposed by muscular contraction and body movements, 2) pregnancy, 3) hormones and, 4) growth factors. The rate of bone remodelling is very active in young children, where it can be 200 times faster than in adults. Normally, the human skeleton is completely replaced via this tightly regulated remodelling process every 10 years and at any one time, one million remodelling units (basic multicellular units, BMUs) are active (Meghji, 1992; Mundy, 1999).

1.1.9. Fracture healing

After reduction, a simple fracture can be described in terms of four major phases (Fig. 1-14) (Marieb, 1998).

Hematoma formation

After fracture, blood vessels are torn and form a hematoma in bone, periosteum and surrounding tissues at the fracture site. Soon after, bone cells die and the tissue swells.

Fibrocartilaginous callus formation

Capillaries grow into the hematoma. Phagocytic cells invade the area and begin to clean up the debris. At the same time, fibroblasts and osteoblasts migrate into the fracture site and begin to reconstruct the bone. The fibroblasts produce collagen fibres to connect the broken bone ends and some of them differentiate into chondroblasts that secret cartilage matrix.

Bony callus formation

Osteoblasts and osteoclasts continue to migrate toward and gradually convert the fibrocartilaginous callus to a bony callus of spongy bone. This phase begins 3-4 weeks after the injury and continues until a firm union is formed 2-3 months later.

Remodelling

During the formation of bony callus, the remodelling takes place to remove the excess matrix on the outside of the bone shaft and within the medullar cavity. At the same time, compact bone is laid down to reconstruct the shaft. The progress of remodelling will continue for a few months. The final structure of the remodelled area resembles that of the original unbroken bony region because it responds to the same set of mechanical stimuli.

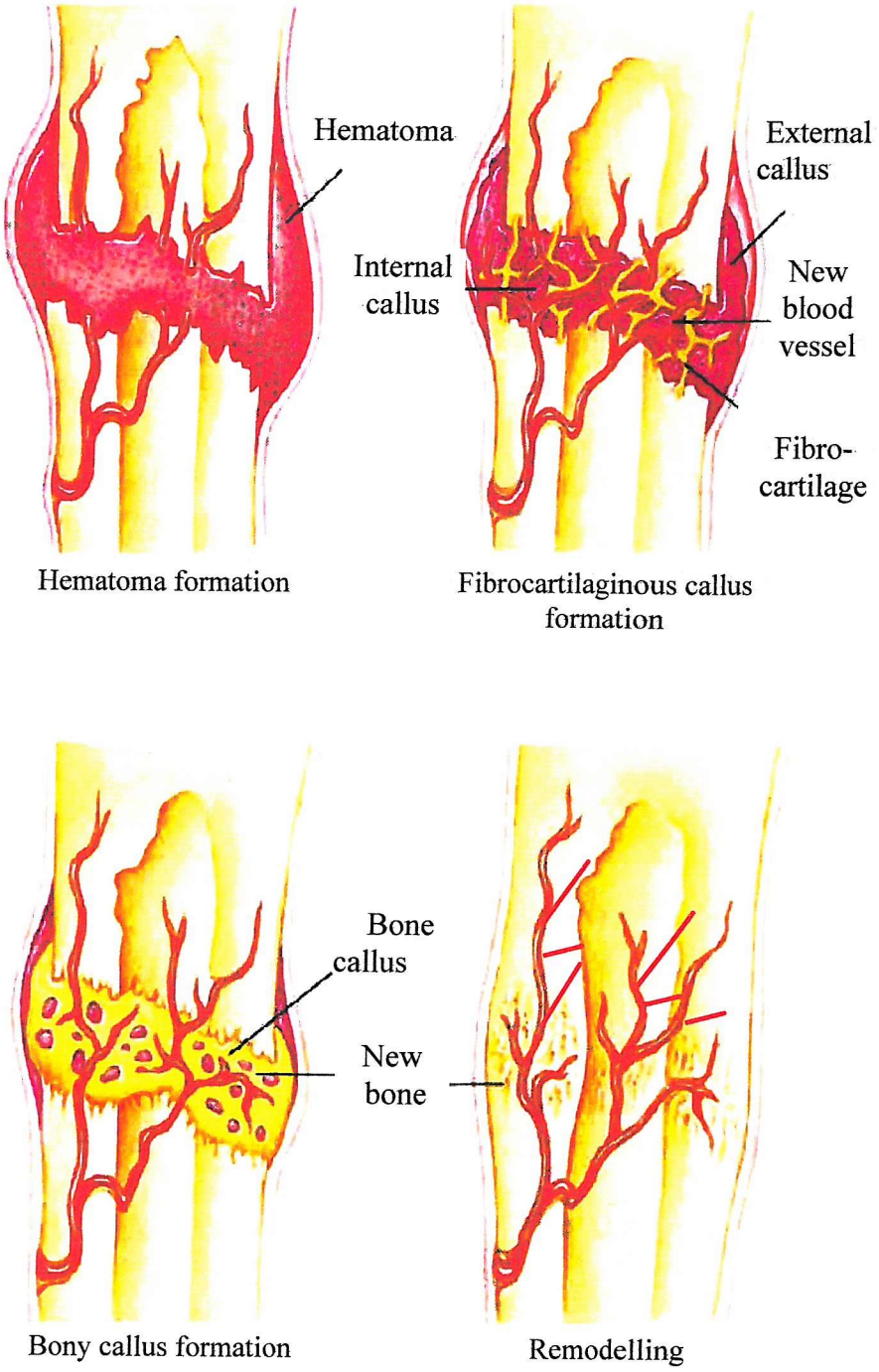


Figure 1-14 Stages of fracture healing (Adapted from Marieb, 1998)

1.2. Skeletal repair – clinical need

Over one million orthopaedic operations annually involve bone repair as a consequence of replacement surgery, trauma, cancer, congenital abnormalities or skeletal deficiency (Chaput et al, 1996; Peter et al, 1998a; Gazit et al, 1999; Bucholz, 2002). Thus the ability to generate new bone for skeletal use is a major clinical need.

Although a number of different methods have been developed to meet such a clinical requirement, to date, the most common procedures still rely on bone grafts (Goldberg and Stevenson, 1993). Fresh autogenous and allogenic bone grafts, both cancellous and cortical provide a source of osteoprogenitor cells, osteoinductive growth factors and a structural scaffold for new bone formation. Furthermore, the three-dimensional framework of both autografts and allografts can function as mechanical supports for angiogenesis and the invasion of osteoprogenitor cells into the bone grafts ('osteoconduction'). However, fresh allografts can induce both local and systemic immune responses that diminish or destroy the osteoinductive and conductive processes (Goldberg and Stevenson, 1993). To circumvent this issue, recent approaches have included the use of freezing or freeze-drying allografts to improve the usability (Goldberg and Stevenson, 1993; Betz, 2002).

Although autogenous or allogenic bone grafts have been used for many years, a number of disadvantages have limited their use including 1) these methods are inappropriate in cases of large bone deficiency, 2) the requirement for surgery from multiple areas, 3) the loss of normal bone structure from donor areas, 4) the risk of infection and secondary deformities at the donor site (Weiland, 1981; Burchardt, 1983; Peter et al, 1998b) and, 5) allogenic bone graft carries potential risks of cell-mediated immune responses and pathogen transmission (Burchardt, 1983; Friedlaender, 1983; Damien and Parsons, 1991; Lenke et al, 2002). In addition, cancellous bone grafts are

completely replaced, in time, by creeping substitution while cortical bone grafts remain an admixture of necrotic and viable bone over time (Goldberg and Stevenson, 1993). The above limitations in the use of autogenous or allogenic bone graft has resulted in the search for alternative bone substitutes.

Synthetic grafting materials eliminate many of the aforementioned risks and these materials do not transfer osteoinductive or osteogenic elements to the host site. However, the advantages of autograft and allograft can be considered using a composite graft for clinical application.

Over the last 10-20 years, bone tissue engineering has emerged as an alternative approach to bone regeneration (Omstead et al, 1998; Peter et al, 1998a; Oreffo and Triffitt, 1999; Anselme, 2000; Khan et al, 2000; Laurencin et al, 2001; Rose and Oreffo, 2002; Muschler and Midura, 2002).

1.3. Bone regeneration – a role for bone tissue engineering

Tissue engineering can be defined as the application of scientific engineering principles to the design, construction, modification, growth and maintenance of living tissue and organs from native or synthetic sources for the human body to restore function based on principles of molecular developmental biology, cell biology and biomaterial sciences (Boyan et al, 1999). Current tissue engineering programmes include skin, cornea, liver, pancreas, kidney, urinary bladder, digestive tract, vessel, muscle, nerve, ligament, bone and cartilage and many other tissues (Fig. 1-15). Among the many tissues in the human body, bone has considerable powers for regeneration and is a prototype model for tissue engineering. More importantly, bone tissue engineering is rapidly developing towards a clinical reality with hope for patients (Reddi, 2000; Stock and Vacanti, 2001; Hutmacher et al, 2001; Muschler and Midura, 2002).

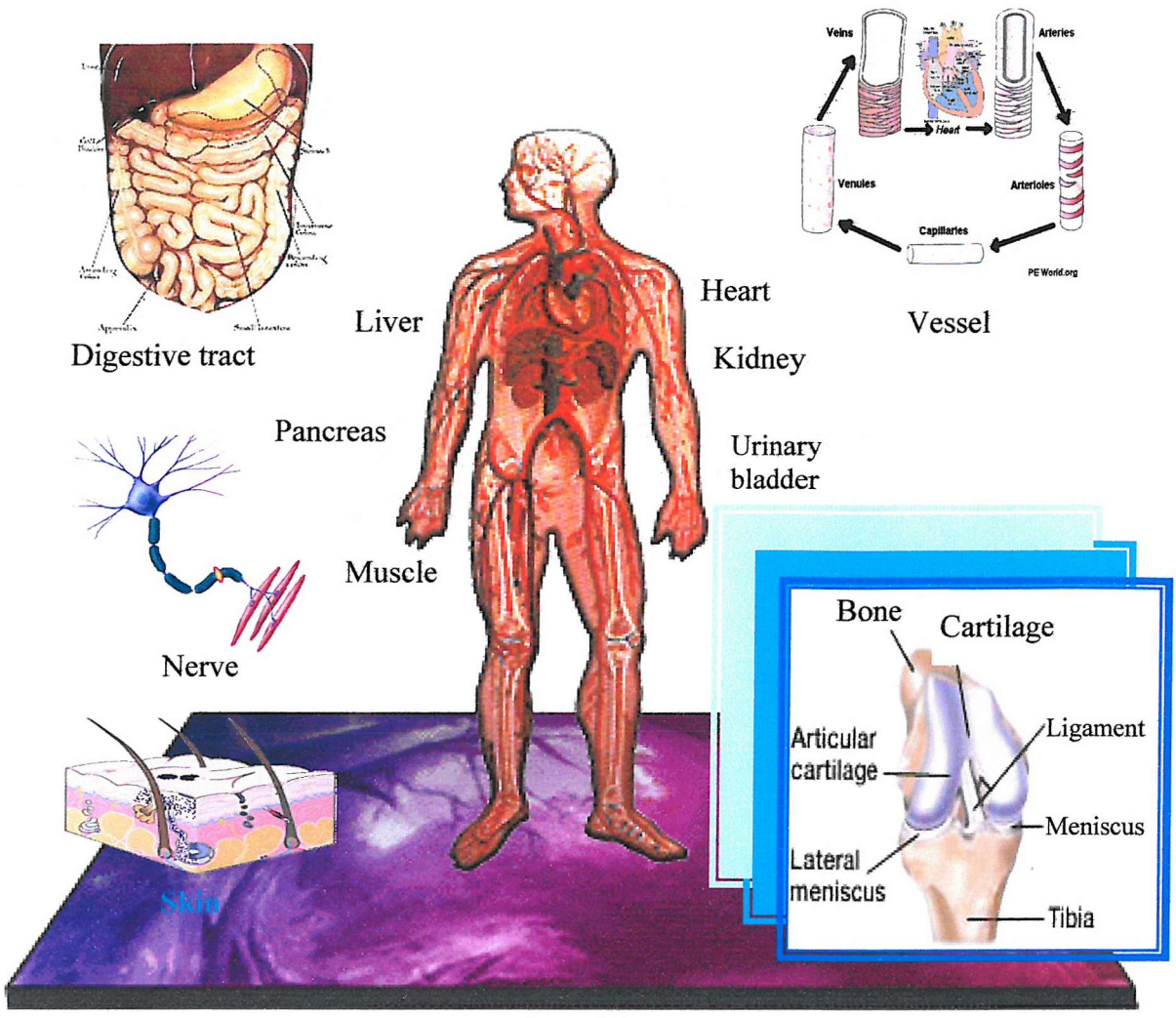


Figure 1-15 Tissues targeted for regeneration by a tissue engineering strategy.

A number of innovative therapeutic approaches for tissue engineering have been developed by scientists and clinicians (Fig. 1-16). Typically, a three step approach has been proposed for tissue engineering including 1) an expansion phase, 2) initiated tissue differentiation phase and, 3) histotypical differentiation phase (Strehl et al, 2002). While recent advances in progenitor cells, novel factors, smart materials, and gene therapy offer much hope and the question of good clinical practice, consistency, reproducibility, validation and appropriate regulation of these new biological treatments remain significant issues (Stock and Vacanti, 2001; Rose and Oreffo, 2002).

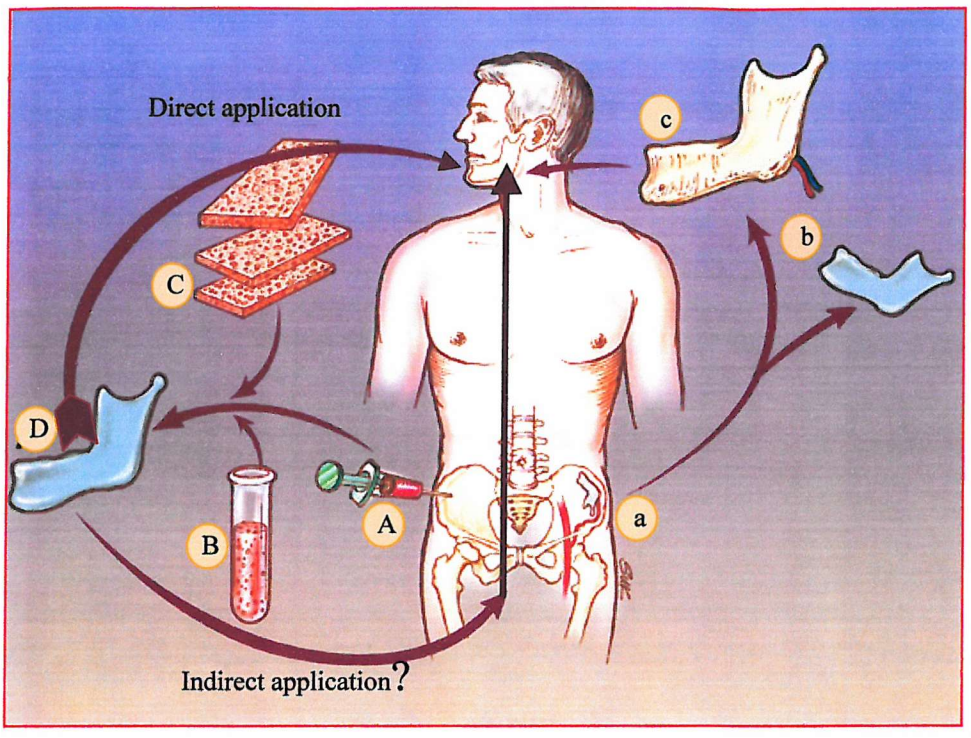


Figure 1-16 The procedures involved in bone tissue engineering (A-D) and the normal procedure of an autogenous bone graft (a-c).

Bone tissue engineering requires three basic elements: 1) a source of osteogenic cells, 2) a source of osteoinductive agents (growth and differentiation factors or genes) and, 3) an osteoconductive scaffold (extracellular matrix scaffolds) (Oreffo and Triffitt, 1999; Bruder and Fox, 1999; Lane et al, 1999; Glowacki, 2001; Rose and Oreffo, 2002).

The symbiosis of bone inductive and conductive strategies is critical for tissue engineering, and is in turn governed by the microenvironment, consisting of the extracellular matrix, which can be duplicated by biomimetic biomaterials such as collagens, hydroxyapatite, calcium phosphates, bioactive glass, proteoglycans, and cell adhesion proteins including fibronectin and bone morphogenetic proteins (Brekke and Toth, 1998; Vogelin et al, 2000; Bauer and Smith, 2002; LeGeros, 2002; Roether et al, 2002; Yang et al, 2002a). Advances in scaffold design, bioactive factors and cell

culture have improved the prognosis for success in orthopaedic tissue engineering (Boyan et al, 1999; Reddi, 2000) and a multidisiplinary approach combining all three key components is likely to result in clinical success in the long term (Lane et al, 1999).

1.3.1. Osteogenic cells

The potential of using stem cells for tissue regeneration has generated tremendous scientific and public interest. This follows the discovery of the reasonably well-characterized hematopoietic stem cell (HSC) from bone marrow and peripheral blood, which has found clinical efficacy. Over the last 20 years, the mesenchymal stem cell (MSC) has been shown to be multi potential (Friedenstein et al, 1987; Owen and Friedenstein, 1988; Beresford et al, 1993; Triffitt, 1996). The MSCs, originally characterized from bone marrow, is capable of differentiating along multiple lineages under the appropriate culture conditions and have significant expansion capability (Fig. 1-17) (Triffitt, 1996; Joyner et al, 1997). Recent information indicates HSCs and MSCs in general may have more universal differentiation abilities than previously thought (Bianco and Robey, 2001; Ballas et al, 2002).

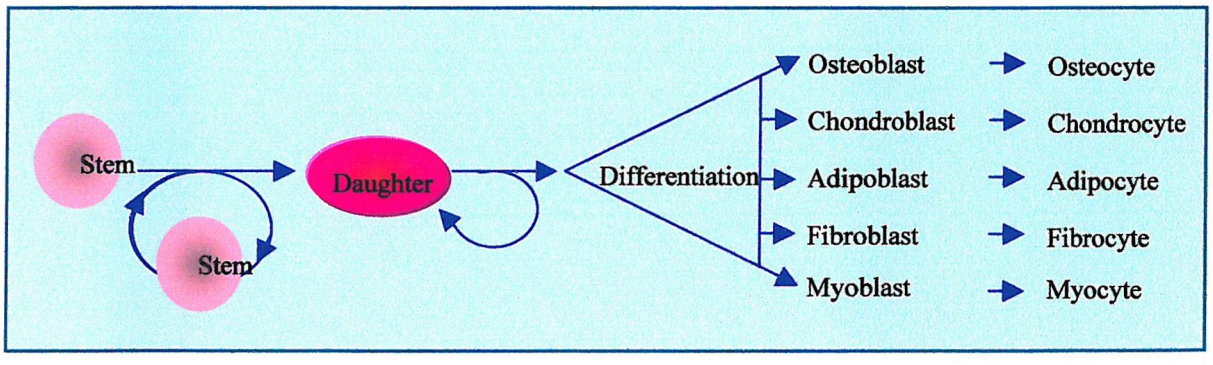


Figure 1-17 Cell differentiation from the hypothetical, pluripotent stem cell. (Adapted from Triffitt, 1996).

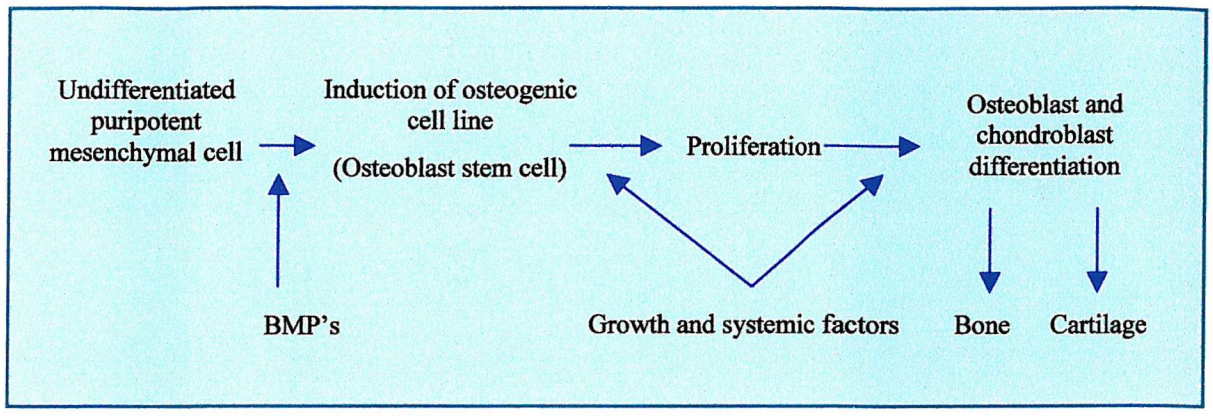


Figure 1-18 Possible stages of differentiation of pluripotent mesenchymal stem cells into skeletal tissues. (Adapted from Triffitt, 1996).

The presence of stem cells for non-hemotapoietic cells in bone marrow was first suggested by the observations of the German pathologist Cohnheim over a century ago (Prockop, 1997). However, the definitive evidence that bone marrow contains multipotential cells came from the pioneering work of Friedenstein (Friedenstein et al, 1976). Mesenchymal stem cells are a rare population of undifferentiated cells that have the capacity to differentiate into mesodermal lineages, including bone, fat, muscle, cartilage, tendon, and marrow stroma (Fig. 1-17) (Friedenstein et al, 1987; Owen and Friedenstein, 1988; Triffitt, 1996). It is now generally accepted that the skeletal mesenchymal cells including osteoblasts, chondroblasts, adipocytes, myoblasts and fibroblasts derived from the common multipotential mesenchymal progenitor (stem) cells within bone marrow (Fig. 1-18 and 1-19) (Grigoriadis et al, 1988; Wlodarski, 1990; Yamaguchi, 1995; Lennon et al, 1995; Triffitt and Oreffo, 1998b; Raisz, 1999). These stem cells have a number of unique properties: 1) the ability to self-renew, 2) they are multipotential and, 3) the capacity to regenerate tissue after injury (Owen and Friedenstein, 1988; Caplan, 1991; Triffitt, 1996; Owen, 1998; Bianco and Robey,

2001; Bianchi et al, 2001). These cell populations may be expanded in culture and subsequently permitted to differentiate into the desired lineage (Fig. 1-3). Understanding the functional potential of these cells and the signaling mechanisms underlying their differentiation should lead to innovative protocols for clinical orthopaedic therapy (Lennon et al, 2001).

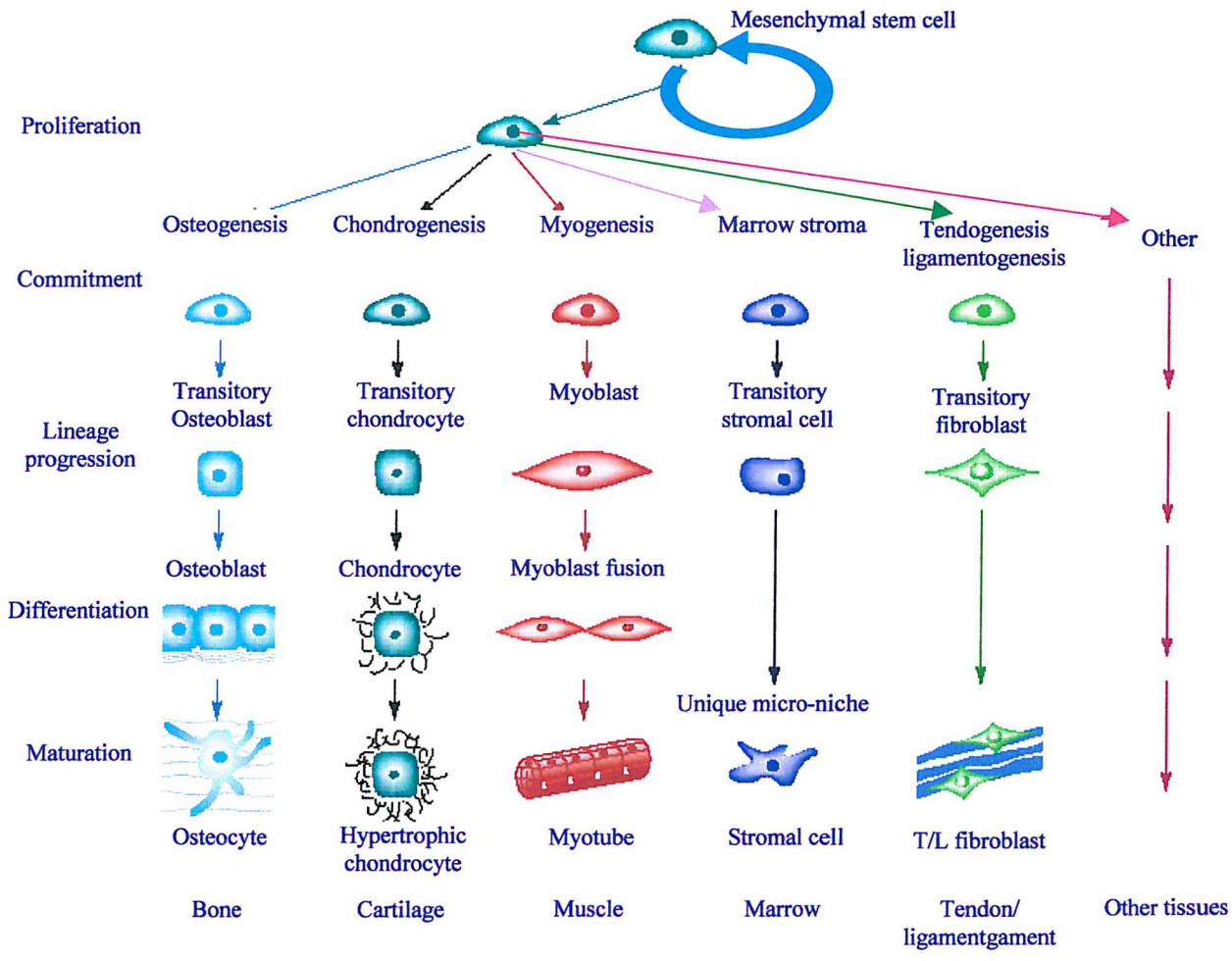


Figure 1-19 The mesengenic process (Adapted from Caplan and Bruder, 2001).

The validity of using stromal cells in tissue engineering arises from a number of previous studies: 1) Marrow stromal cells can be isolated from other cells within marrow by their tendency to adhere to tissue culture plastic (Prockop, 1997) and have been shown to have osteogenic activity (Friedenstein et al, 1987; Charles et al, 1994),

2) Large quantities of human MSCs can be readily obtained following a simple bone marrow aspiration procedure and subsequent expansion over a million-fold in culture, 3) Culture expanded rat and sheep bone marrow cells have been shown to heal the critical-size bone defect following reimplantation (Dennis and Caplan, 1993; Kadiyala et al, 1997; Andrades et al, 1999; Qu et al, 1999; Devine et al, 2001), 4) Connolly (Connolly, 1995) has shown the potential for human bone marrow injection for the treatment of delayed fracture-union and bone defects and, 5) Horwitz et al (1999)(2001) has reported on the therapeutic effects of bone marrow-derived osteoprogenitors transplanted into children with osteogenesis imperfecta.

1.3.2. Osteoinductive agents

Bone has a remarkable capacity for growth, regeneration, and remodelling. This capacity is largely due to the induction of osteoblasts that are recruited to sites of new bone formation. The process of recruitment remains unclear, though the immediate environment of the cells is likely to play a role via cell-matrix-osteoinductive factor-cell interactions (Baylink et al, 1993; Reddi, 2000).

Indeed, bone is a physiological storehouse for a multitude of growth factors including insulin-like growth factors I and II (IGF-I, IGF-II), members of transforming growth factor-beta (TGF-β) super family including BMP's, platelet-derived growth factor, and fibroblast growth factors (FGFs). Osteoblasts have been shown to produce many of these growth factors, many of which are incorporated into the extracellular matrix during bone formation. In addition, small amounts can also be trapped systemically into the matrix. The growth factors are located within the matrix until remodeling or trauma results in their subsequent release (Mundy, 1999). The released growth factors are able to regulate osteoblast and osteoclast metabolism and function during bone remodeling

and may also initiate and regulate bone formation after trauma in an autocrine and paracrine fashion (Fig. 1-20) (Baylink et al, 1993; Lind and Bunger, 2001).

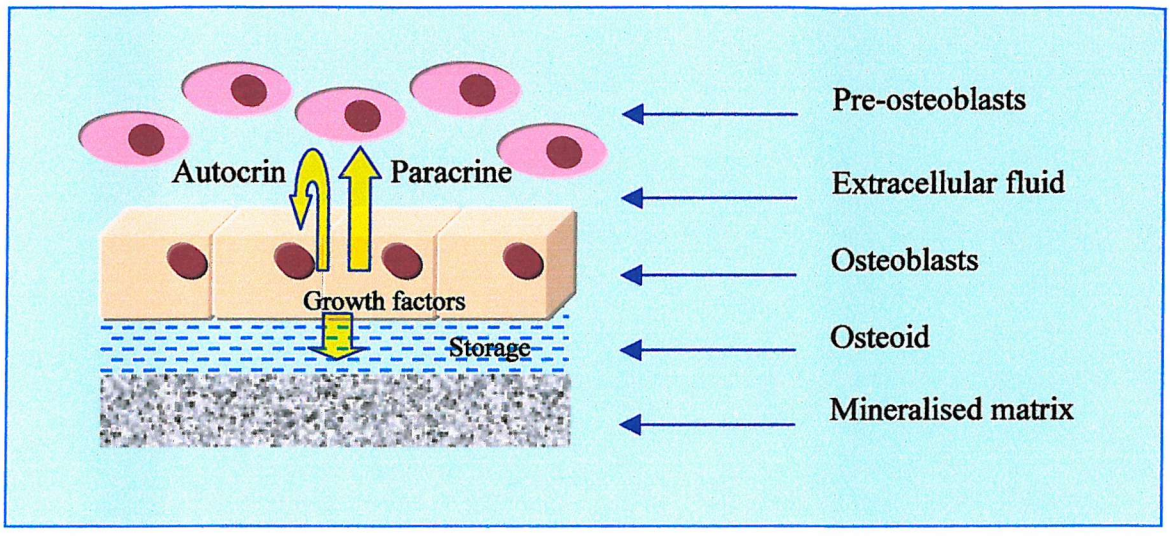


Figure 1-20 Modulation of bone formation by growth factors. (Adapted from Baylink et al, 1993).

1.3.2.1. Bone morphogenetic proteins (BMP's)

Bone morphogenetic proteins were first reported by Marshall Urist (Urist, 1965) who described the isolation of a bone inductive extract from adult bone and demonstrated the ability of this extract to induce new endochondral bone formation at ectopic sites in rodents. Since then, a number of osteogenic proteins have been discovered and added to the BMP family (Wozney and Rosen, 1998). These proteins have been identified as the key signal molecules or stimuli to induce the conversion of mesenchymal stem cells into osteoprogenitor cells and the subsequent development of new bone via endochondral ossification (Ahrens et al, 1993; Yamaguchi, 1995). During the differentiation process from mesenchymal progenitors, various hormones and cytokines regulate osteoblast differentiation. Among these, the BMP's are the most potent inducers and stimulators for osteoblast differentiation from bone marrow stromal cells

(Table 1-1) (Thies et al, 1992; Baylink et al, 1993; Yamaguchi et al, 2000; Miller et al, 2000).

Table 1-1 Action of BMP's on Osteoblasts

Factor	Synonym	KDa	Proliferation	Differentiation
BMP-2	BMP-2A	30-35	↑*	↑
BMP-3	Osteogenin	30-40	↑	↑
BMP-4	BMP-2b	30-35	↑	↑
BMP-5		30-35	↑	?
BMP-6		30-35	↑	↑
BMP-7	Osteogenic protein-1	30-35	↑	↑
BMP-9	Dorsalin-1	60-63	↑	?

*↑: Increase; ?: not known (Adapted from Baylink et al, 1993 and Miller et al, 2000)

The mature BMP monomer consists of about 120 amino acids, with seven cysteine residues (Reddi, 2000; Yamaguchi et al, 2000). A number of studies have shown the bone inductive potential of BMP-2, 4-7, and 9 in the treatment of fracture repair, segmental bone defects and in the fixation of prosthetic implants and their use in ectopic bone induction (Wozney et al, 1988; D'Alessandro et al,1991; Celeste et al,1994; Gitelman et al, 1994; Rubin et al, 1997; Nifuji and Noda, 1999; Anderson et al, 1999). To date, BMP's comprise over 30 members. However, BMP-1 is a cysteine-rich peptidase and is not a member of the TGF-β super family (Yeh et al, 2002).

In vitro experiments have shown that BMP-2 stimulates the formation and mineralisation of bone-like nodules in primary osteoblast cultures (Harris et al, 1994; McCuaig et al, 1995) and promotes the development of an osteoblast-like phenotype in pluripotent mesenchymal stem cell lines (Ahrens et al, 1993). Furthermore,

recombinant human bone morphogenetic protein-2 has been successfully used to promote a greater degree of osseous and periodontal repair (Whang et al, 1998). Following the first report of the healing of large segmental bone defects using BMP-2 implantation, BMP-2 has proven useful in healing critical size defects in rat and sheep femurs (Yasko et al, 1992). Anderson and coworkers (1992, 2002) reported that Saos-2 cells, derived from human osteosarcoma cells, uniquely contain a bone-inducing activity and that components of the Saos-2 cells contain bone morphogenetic proteins (BMP's)-1, -2, - 3, -4, -5, -6, and -7 and the non-collagenous matrix proteins bone sialoprotein, osteonectin (ON), osteopontin (OPN), and osteocalcin (OCN). The combination of BMP-1/tolloid, BMP-3 and BMP-4, and bone sialoprotein was important for the osteoinductive capacity of Saos-2 cells. *In vivo* data has shown the ability of using freeze-dried Saos-2 cells to promote endochondral bone formation (Anderson et al, 1992). To date, the optimal mix of BMP's, the appropriate dosage and carrier remain significant challenges.

1.3.2.2. Pleiotrophin (PTN)

Pleiotrophin, also known as heparin-binding growth-associated molecule (HB-GAM), is a 136 amino acid polypeptide which is widely expressed during embryonic life but whose expression is restricted to bone and brain during adulthood (Masuda et al, 1997; Imai et al, 1998).

PTN was first identified as HB-GAM to processe mitogenic activity in rat and mouse fibroblasts and as a factor that promotes neurite outgrowth in cultures of neonatal rat brain cells (Rauvala, 1989; Milner et al, 1989). In 1990, Tezuka et al (1990) detected the same mRNA in calvarial osteoblast–enriched cells and MC3T3-E1 cells by differential hybridisation screening between osteoblastic and fibroblastic cells, and named the factor osteoblast stimulating factor-1 (OSF-1). In bone and cartilage tissues,

PTN is expressed in developing and regenerating bone as a matrix-bound form and in culture, it stimulates differentiation of osteoblasts and chondrocytes (Zhou et al, 1992; Tapp et al, 1999).

PTN is prominently expressed in the cell matrices that act as target substrates for bone formation, probably by mediating chemotactic recruitment and attachment of osteoblasts/osteoblast precursors to the appropriate matrices (Gieffers et al, 1993; Imai et al, 1998; Souttou et al, 2001; Petersen and Rafii, 2001). In addition, PTN is thought to play a role in the process of angiogenesis in endochondral ossification (Gieffers et al, 1993; Imai et al, 1998; Souttou et al, 2001; Petersen and Rafii, 2001). Studies have shown PTN stimulates *in vitro* proliferation and differentiation of osteoblastic cells (Zhou et al, 1992; Masuda et al, 1997). Furthermore, N-syndecan has been identified as an essential cell surface receptor for PTN, which in turn is immobilized in the extracellular matrices onto which those cells are recruited. The receptor has been found on osteoprogenitors and osteoblasts (Merenmies and Rauvala, 1990; Kim et al, 1994; Szabat and Rauvala, 1996; Kilpelainen et al, 2000). Recently, Sato and co-workers (2002) demonstrated that PTN had a dose-dependent synergistic or inhibitory effect on BMP-2 induced osteogenesis in endochondral ossification in rat (Sato et al, 2002).

1.3.2.3. Other factors

There are a number of other important growth/transcription factors involved in the bone formation process.

Core-binding factor-1 (cbfa1)

Core-binding factor is a helix-loop-helix factor required for the expression of osteoblastic characteristics and bone development (Ducy et al, 1997). A number of studies have shown that *cbfa-1* plays a crucial rule in not only bone cell differentiation but also in the maturation and function of osteoblasts and in osteogenesis, both

intramembranous and endochondral ossification (Fig. 1-21) (Komori et al, 1997; Yamaguchi et al, 2000).

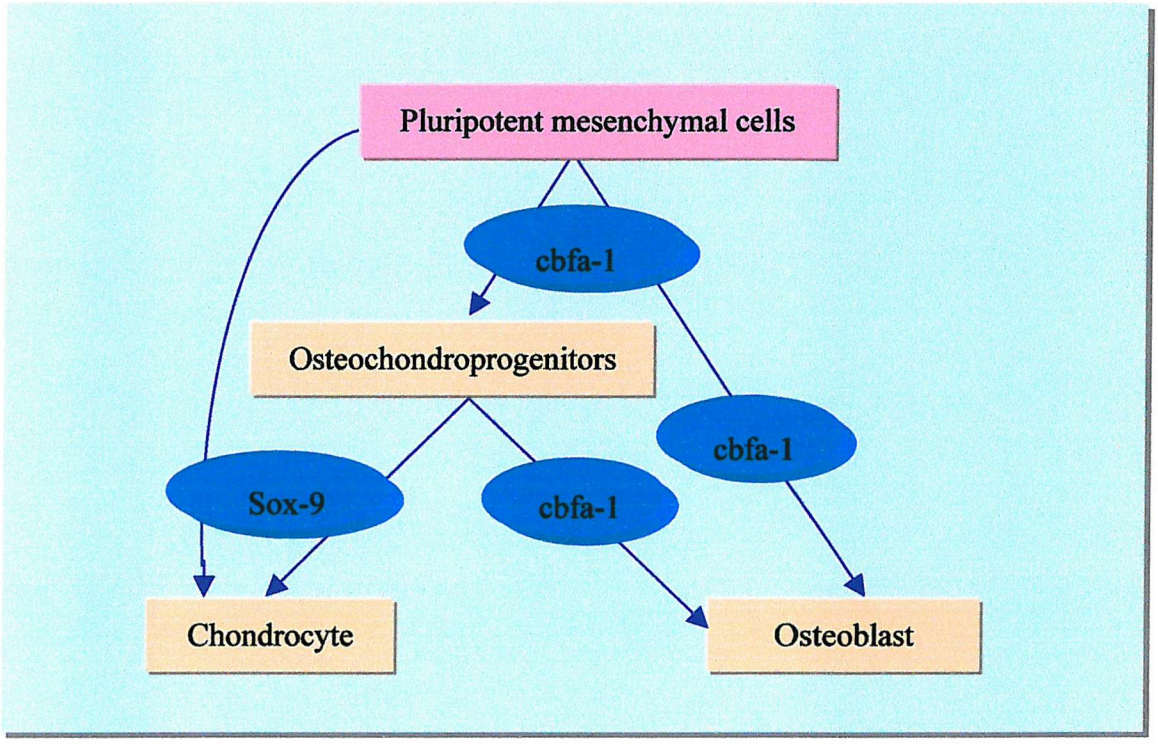


Figure 1-21 Cell lineage-specific transcription factors.
(Adapted from Yamaguchi et al, 2000).

Insulin-like growth factors (IGFs)

Insulin-like growth factors have been shown to not only enhance bone collagen, matrix synthesis and stimulate the replication of osteoblasts (Hock and Gera, 1992), but also decrease collagen degradation, which plays a central role in the maintenance of bone matrix and bone mass (Lian et al, 1999).

Fibroblast growth factors

Fibroblast growth factors have angiogenic properties and are considered important in neovascularisation, wound healing and bone repair. Bones treated with FGFs contain a greater number of cells that synthesize bone collagen matrix. However FGFs do not directly affect osteoblast differentiation (Nakamura et al, 1995; Lian et al, 1999).

Parathyroid hormone (PTH)

Parathyroid hormone has significant effects on osteoblast activity and has been shown to increase the net release of alkaline phosphatase activity. PTH plays a central role in concert with $1,25(OH)_2D_3$ in maintaining serum calcium and phosphate levels (Canalis et al, 1989; Dempster et al, 1993; Anh et al, 1998).

Growth hormone (GH)

Growth hormone is well known to be important in the regulation of longitudinal bone growth. *In vivo* and *in vitro* studies have demonstrated that GH is important in the regulation of both bone formation and bone resorption (Ohlsson et al, 1998).

Estrogen

In vitro studies indicate estrogen can modulate osteoblast proliferation, differentiation and the stimulation of other growth factors. It is the major sex steroid regulating the metabolism and maturation of bone and bone turnover in women and men (Ernst et al, 1989; Saadeh et al, 2001). Qu et al (1998) demonstrated that estrogen stimulated sequential differentiation of osteoblasts and increased deposition and mineralisation of matrix in mouse bone marrow cultures.

Prostaglandins (PGs)

Prostaglandins are important local factors in bone cell metabolism and can stimulate cell proliferation, collagen, non-collagenous protein synthesis and bone formation (Dohi et al, 1992; Kawaguchi et al, 1995). In addition, PGs have been shown to stimulate osteoclast formation in a variety of cell culture systems. The stimulation of osteoclastic bone resorption may be important in mediating bone loss in response to mechanical forces and inflammation (Raisz et al, 1993).

Collagen fragment – P-15

Type I collagen can induce osteoblast-related gene expression of bone marrow cells during osteoblastic differentiation (Mizuno and Kuboki, 2001). Bhatnagar et al (1999) has shown the potential to construct biomimetic environments by immobilizing a collagen-derived high-affinity cell-binding peptide P-15 in 3-D templates to promote attachment of human dermal fibroblasts to anorganic bovine bone mineral phase to enhance expression of the osteoblast phenotype.

A number of strategies have been developed in recent years in the use of bioactive factors for bone tissue engineering including: 1) extraction and partial purification of growth factors, 2) recombinant protein synthesis and, 3) gene therapy (Boden, 1999).

1.3.3. Gene therapy

In the last few years, osteogenic stem cell transplantation has been further developed to incorporate and utilize the principles of gene therapy. The approach is compelling with gene therapy combining endogenous bone stem cells with genes encoding physiological specific osteoinductive growth factors to provide an enhanced and significant bone healing response. Thus, progenitor cells function as growth factor factories with overexpression of a single growth factor maintained, typically, for several weeks (Oakes and Lieberman, 2000; Lind and Bunger, 2001). Boden and co-workers (2000) have suggested that three critical steps are essential in gene therapy for bone formation: 1) An appropriate osteoinductive gene (and effective dose), 2) An appropriate delivery vector (proper transduction time and reliable gene transfer method) and, 3) An appropriate carrier material as scaffold for the new bone formation (Boden, 2000). A number of different methods have been generated for gene therapy with retroviral and adenoviral vectors receiving significant attention.

Previous studies have demonstrated the clinical utility of BMP's in spinal fusion, fracture healing and prosthetic joint stabilisation (Fischgrund et al, 1997; Welch et al, 1998; Boden, 2000; Cheng et al, 2001). Stem cells transfected with bone morphogenetic proteins have, to date, been the most successful gene therapy for bone tissue engineering and offer several theoretical advantages over implantation of the recombinant human BMP, including persistent BMP delivery and eliminating the need for a foreign body carrier (Musgrave et al, 1999).

A number of studies have described successful stimulation of bone formation using adenovirus-mediated direct gene transfer of BMP's. Musgrave et al (1999) constructed a replication defective adenoviral vector to carry the rhBMP-2 gene (AdBMP-2) and showed intramuscular bone formation as early as 2 weeks following injection. Alden and co-workers (1999) demonstrated that BMP-2 adenoviral vector could induce striated muscle cells to produce BMP-2, leading to endochondral bone formation in athymic nude rats. In clinically related animal studies, the BMP-2-expressing adenovirus-transfected marrow cells were subsequently injected into critically sized defects in rat femurs and reached higher healing rates compare to controls (Lieberman et al, 1999). Retroviral BMP-2 gene transfer has been used effectively in combination with a biodegradable matrix (PLGA-HA scaffold) to stimulate the synthesis of bioactive BMP's and promote bone formation in a mouse model (Laurencin et al, 2001).

Thus, gene therapy offers the possibility of gene modification of isolated and expanded cells to produce a population of progenitor cells over-expressing selected signaling molecules. Extensive research is underway to determine the potential advantages and indications for gene therapy. However, concern exists regarding clinical safety (immunogenesis *in vivo*, fate of adenovirus) and the long-term complications of

injecting genetically altered cells into humans (Bianco and Robey, 2001; Lind and Bunger, 2001).

1.3.4. Osteoconductive materials

Bone formation comprises a complex and temporal sequence of events that begins with the recruitment and proliferation of osteoprogenitors from mesenchymal stem cells (Friedenstein et al, 1987; Owen, 1988; Kadiyala et al, 1997; Bruder et al, 1998; Triffitt and Oreffo, 1998b). Central in this process is a material or scaffold for the migration, attachment and growth of stem cells and progenitor cells from the surrounding tissue (Qian and Bhatnagar, 1996).

Table 1-2 Mechanical properties of human tissues

	Tensile strength (MPa)	Compressive strength (MPa)
Cancellous bone	N/A	4-12
Cortical Bone	60-160	130-180
Cartilage	3.7-10.5	N/A

(Adapted from Yang et al, 2001)

Ideally, a scaffold should have properties comparable to normal bone (Table 1-2) with the following characteristics: 1) biocompatibility (i.e., not to provoke any unwanted tissue response and at the same time to possess the right surface chemistry to promote cell attachment and function), 2) bioresorbable with a controllable degradation (Table 1-3 and Fig. 1-23) and resorption rate to match cell/tissue growth *in vitro* and/or *in vivo* (i.e., degradable into nontoxic products, leaving the desired living tissue), 3) suitable surface chemistry for cell attachment, proliferation, and differentiation, 4) appropriate porosity, interconnectivity for cell growth and nutrients/wastes transport

and, 5) mechanical properties to match those of the tissues at the site of implantation (Freed et al, 1994; Hutmacher, 2000; Yang et al, 2001).

Table 1-3 Properties of biodegradable polymers

Polymer type*	Melting point (°C)	Glass trans. Temp. (°C)	Degrading time (months)**	Tensile strength (MPa)
PLGA	Amorphous	45-55	Adjustable***	41.4-55.2
DL-PLA	Amorphous	55-60	12-16	27.6-41.4
L-PLA	173-178	60-65	>24	55.2-82.7
PGA	225-230	35-40	6-12	>68.9
PCL	58-63	-65	>24	20.7-34.5

*PLGA: poly(l-lactic-co-glycolic acid)

DL-PLA: poly(D,L-lactic acid)

L-PLA: poly(L-lactic acid)

PGA: poly(glycolic acid)

PCL: Poly(∈-caprolactone)

**Time to complete mass loss. Time also depends in part on geometry.

***See Figure 1-23

Biodegradable polymers

For a number of years, biocompatible materials such as metals (stainless steels, titanium-based alloys), ceramics (alumina, coralline hydroxyapatite, porous calcium phosphate, calcium phosphate cements, bioglass) and polymethylmethacrylate (PMMA) have been used extensively for surgical implantations. Coralline hydroxyapatite and porous calcium phosphate have also been used as carriers for osteoinductive factors and as osteoconductive matrices for human bone cells and human bone marrow populations in cell transplantation studies (Fig. 1-22) (Burwell, 1994; Wang et al, 2002).

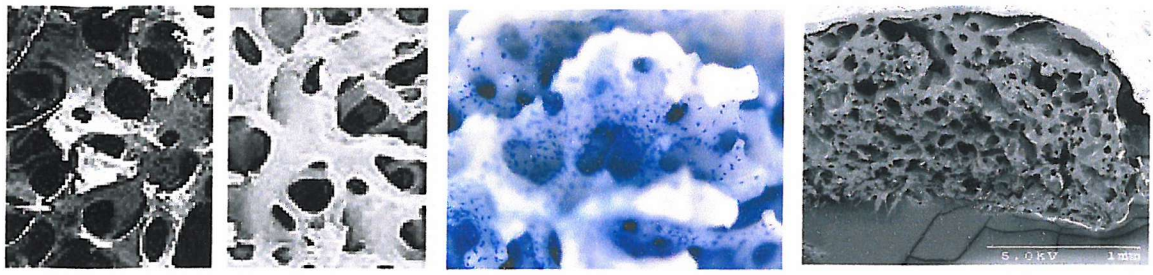


Figure 1-22 Bone and osteoconductive scaffolds. (From left to right: Bone, Calcium phosphate, HA, PLGA).

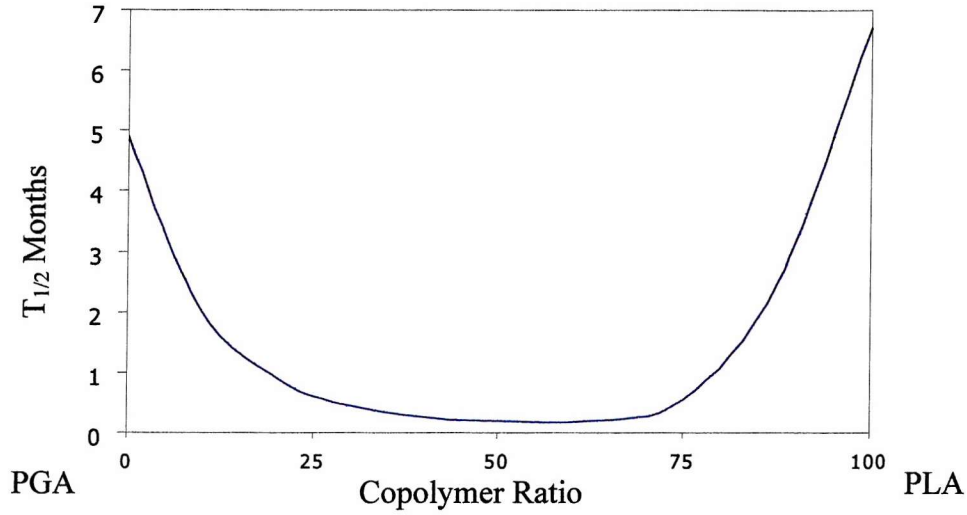


Figure 1-23 Half-life of PLA and PGA homopolymers and copolymers implanted in rat tissue. (Adapted from Miller et al, 1977)

However, these materials are not themselves osteoinductive and are resorbed relatively slowly *in vivo*. To overcome these limitations, natural or synthetic materials such as poly(lactic acid) (PLA), poly(glycolic acid) (PGA) and copolymers of PLA and PGA as well as biodegradable composite scaffolds based on poly(lactic acid-÷-co-glycolic acid) and polypropylene fumarate have been developed (Burwell, 1994; Billotte et al, 1995; Chaput et al, 1996; Oreffo and Triffitt, 1999; Yang et al, 2002b). However, existing three-dimensional scaffolds for tissue engineering are currently less than ideal for clinical applications, not only because they lack mechanical strength, but also because

these scaffolds do not provide the appropriate interconnection porosity critical for cell ingrowth and penetrating the scaffold to generate 3-D tissue (Ishaug et al, 1997; Yang et al, 2001). Thus an understanding of the normal mechanical properties of human tissues (Table 1-2), properties of polymer materials (Table 1-3) and the structure of the 3-D scaffold is central to design and generation of scaffold materials for bone tissue engineering.

Biomimetic scaffolds

The use of the synthetic polymer materials (Yang et al, 2001) as well as collagen sponges (Friess et al, 1999) to generate biomimetic scaffolds for cell transplantation and tissue growth has become a realistic objective (Chaput et al, 1996; Patel et al, 1998; Quirk et al, 2000). To date, Poly(lactic acid), Poly(glycolic acid), polydioxanone and copolymers are the only synthetic, degradable polymers with U.S. Food and Drug Administration (FDA) approval and widely used in the formation of resorbable sutures, meshes, scaffolds and drug delivery devices. They are 1) biocompatible, 2) processable into a three dimensional structure and, 3) eventually degradable. In recent years, procedures for the surface modification of these materials with biological agents have been developed (Shakesheff et al, 1998). Thus, an alternative approach for skeletal repair is the selection, expansion and modulation of osteoprogenitor cells in combination with biodegradable biomimetic scaffolds, which interact and promote osteoblast differentiation and osteogenesis. These structures, when coupled with appropriate factors, can provide positional and environmental information for bone tissue engineering.

1.3.5. Biomaterial surface modification

The central and first step to successful tissue engineering is the ability of cells to adhere to an extracellular material followed by the ability of the cell to differentiate leading to

the production and organisation of an extracellular matrix. The immediate limitation for many polymer materials is the absence of a chemically reactive pendent chain for the easy attachment of cells, drugs, crosslinkers, or biologically active moieties (Yang et al, 2001). Thus, substantial effort and research has centered on the improvement of cell adhesion with a variety of materials. Generally, cell adhesion is a series of interactive events comprising: 1) initial cell attachment, 2) cell spreading, 3) organisation of an actin cytoskeleton and, 4) formation of focal adhesions (LeBaron and Athanasiou, 2000). The attachment of the cell to the extracellular matrix (and biomaterials) is known to be controlled by various families of adhesion receptors, including the integrins, selectins, cadherins and immunoglobulins (Ruoslahti and Pierschbacher, 1987; Hynes, 1992; Anselme, 2000). The integrin superfamily consists of 16 known α-subunits and eight known β-subunits. Each integrin is composed of an association of one α-subunit and one β-subunit, forming a heterodimeric transmembrane receptor. The binding between integrin receptor and ligand is often mediated through an amino acid recognition sequence Arg-Gly-Asp (RGD). The RGD sequence was first found in the tenth type III repeat of fibronectin and has been shown to serve as a primary cell attachment cue (Table 1-4) (Hynes, 1992). In addition, Pierschbacher and Ruoslahti (1984) have shown that synthetic peptides that contain the amino acids RGD, such as GRGDSP, can essentially mimic cell attachment activity of the parental molecule. Thus the RGD peptide cell adhesion ligand provides a simple mechanism of creating polymer surfaces that mimic the extracellular matrix to support osteoblast-like cell adhesion and spreading (Pierschbacher and Ruoslahti, 1984; LeBaron and Athanasiou, 2000). Essentially, all peptides that promote cell adhesion are derived from sequences found in a number of different extracellular matrix molecules. Such molecules in bone include collagen, osteopontin, fibrinogen, fibronectin, vitronectin, thrombospondin,

bone sialoprotein I and other as yet unidentified molecules (Satomura et al, 2000). Fibronectin, vitronectin (VN) and laminin (LN) are believed to play a central role in cellular morphology, migration and the provision of signals that orchestrate cellular proliferation, metabolism, function and differentiation in anchorage dependent cells such as osteoblasts and osteoclasts (Hynes, 1992; Horton, 1997; Moursi et al, 1997; Garcia et al, 1998; Globus et al, 1998; Rezania and Healy, 1999; Villanova et al, 1999). Fibronectin is regarded as not only being a general cell attachment protein but also modulates cell migration and specificity. Most cell types except erythrocytes will adhere to certain surfaces by interacting specifically with the immobilized fibronectin. The binding sites of fibronectin include those for fibrin, heparin, collagen, DNA, cells, amyloid P component and fibrin(ogen) (Robey, 2000). Ito et al (1991) have demonstrated that the cell adhesion onto RGDS- and FN-immobilised film is based not on physical interactions, but on specific ligand/receptor interactions. Therefore, the adhesion proteins together with their receptors constitute a versatile recognition system providing cells with anchorage, traction for migration, and signals for polarity, position, differentiation and possibly growth (Ruoslahti and Pierschbacher, 1987).

Table 1-4 The Integrin receptors in fibronectin

Subunits		Ligands and Counterreceptors	Binding Site
β_1	α_3	Fibronectin, laminin, collagens	RGD
	α_4	Fibronectin, VCAM-1	EILDV
	α_5	Fibronectin	RGD
	α_V	Vitronectin, fibronectin	RGD
β_3	α_{IIb}	Fibrinogen, fibronectin, von Willebrand factor, vitronectin, thrombospondin	RGD, KQAGDV
	α_V	Vitronectin, fibrinogen, von Willebrand factor, thrombospondin, fibronectin, osteopontin, collagen	RGD
β_6	α_V	Fibronectin	RGD
β_7	α_4	Fibronectin, VCAM-1	EILDV

*VCAM-1: immunoglobulin superfamily

RGD: Arg-Gly-Asp sequence

KQAGDV: Lys-Gln-Ala-Gly-Asp-Val sequence

EILDV: Glu-Ile-Leu-Asp-Val sequence. (Adapted from Hynes, 1992)

1.3.6. Growth factor and cell encapsulation

Cell encapsulation techniques have been successfully used for the transplantation of pancreatic islets to treat diabetes (Jaatinen et al, 2002) and for the treatment of Parkinson's disease (Lindner and Emerich, 1998). In the last decade, drugs and vaccines have also been delivered by encapsulation (Widmer et al, 1998; Gao et al, 2002; Chandy et al, 2002). Gao et al (2002) showed that thermoreversible polymers are compatible with rhBMP-2 induced osteogenesis and can serve as novel biomaterials for rhBMP-2 delivery. Thus the encapsulation of osteogenic factors within biodegradable porous polymer scaffold may provide an alternative approach to produce biomimetic osteogenic scaffold for bone regeneration (Li et al, 2000).

In conclusion, the progress of stem cell research, growth factor regulation, smart biomaterial synthesis and the combination of these new techniques will aid research in bone tissue engineering. A biomimetic approach offers great promise to engineer bone, however to achieve such a goal, an understanding of how tissues and organs develop and the normal processes of growth and repair are required.

1.4. Aims

The aims of these studies were to investigate, using 1) a source of human osteoprogenitors, 2) biodegradable porous scaffolds and, 3) osteoinductive growth factors, a tissue engineering approach for the development of a living tissue substitute for bone.

1.5. Objectives

To develop a tissue-engineering approach to bone formation, specifically:

1) To isolate, culture, expand and characterise human osteoprogenitor cells.
2) To demonstrate the growth and differentiation of human osteoprogenitors on biodegradable and biomimetic scaffolds generated using FN and RGD peptides *in vitro*.
3) To demonstrate human osteoprogenitors growth and differentiation on biodegradable scaffolds coupled with PTN and BMP's *in vitro* and *in vivo*.
4) To examine the potential of gene therapy using adenoviral BMP-2 gene transfer to stimulate the synthesis of biologically active BMP-2 to promote human bone marrow stromal cell differentiation leading to cartilage and bone matrix formation *in vitro*, *ex vivo* and *in vivo*.
5) To demonstrate human osteoprogenitor growth and differentiation on biomimetic osteoinductive PLA scaffolds encapsulated with BMP-2 *in vivo*.

The approach adopted in this programme of work is shown in figure 1.24.

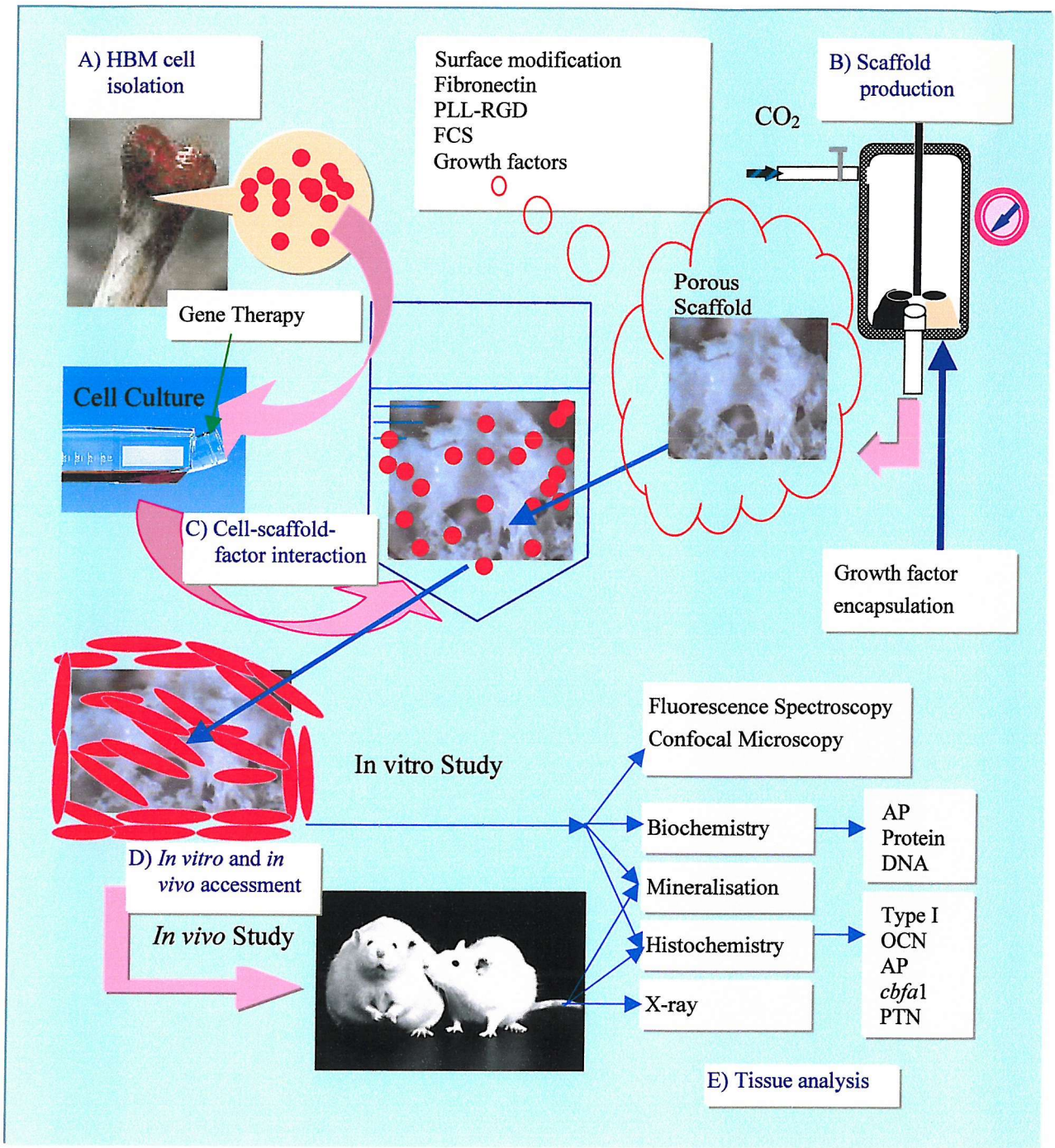


Figure 1-24 Bone tissue engineering scheme: A) Human bone marrow cell isolation, B) Scaffold production, C) Cell-scaffold-growth factor interaction, D) In vitro and in vivo accessment, E) Tissue analysis.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

Unless otherwise stated, tissue culture reagents and biochemical reagents were obtained from Sigma-Aldrich Company Ltd (Poole, Dorset, England). Molecular Biology reagents were purchased from Promega (Southampton, UK). All amino acids and resin support were purchased from Novabiochem (Calbiochem-Novabiochem (UK) Ltd, Beeston, Nottingham).

Fetal Calf Serum was from Meldrum Ltd (Bourne End, UK). $1,25(OH)_2D_3$ was a generous gift from Dr. L Binderup (Leo Pharmaceutical Products Ltd, Ballerup, Denmark). PicoGreen dsDNA Quantitation Reagent, Cell Tracker^{TM} green (5-chloromethylfluorescein diacetate, CMFDA) and Ethidium Homodimer-1 (EH-1) were purchased from Molecular Probes Ltd (Leiden, Netherlands). Diffusion chamber kits were from Millipore (130 µl capacity; UK).

Poly(L-Lysine)-GRGDS (PLL-GRGDS) peptide was provided by Professor Shakesheff (Nottingham, UK). Recombinant human bone morphogenetic protein-2 was a generous gift from Professor Walter Sebald (Wurzburg, Germany). Saos-2 cell extraction ('retentate') was provided by Professor Anderson (Kansas City, USA). Recombinant human pleiotrophin (rhPTN) was purchased from PeproTec Ltd (London, England).

Type I collagen antibody (LF 67) was a gift from Dr Larry Fisher (NIH, USA). Osteocalcin specific monoclonal antibody OS35 was purchased from CisBio (Cedex, France). *Cbfa-1* Staining was carried out using Goat ABC Staining System (Santa Cruz Biotechnology, Inc., Heidelberg, Germany). Anti-PTN antibody was a gift from Professor Rauvala (Helsinki, Finland). Secondary antibodies were purchased from Dako Ltd (Angel Drove, Cambridgeshire, UK).

Poly(D,L-lactic acid) (MW 12,845) was from Alkermes (Cambridge, Massachusetts, USA). Poly(-lactic Acid), Poly(-lactic-co÷glycolic acid) (75:25) (MW 22K) and growth factor encapsulated PLA porous scaffolds (50-200μm) were generated by Professor Shakesheff and Professor Howdle (University Nottingham, UK).

2.2. Methods

For chapter specific methods see individual chapters.

2.2.1. Isolation and culture of human bone marrow cells

Bone marrow samples were obtained from femoral heads and curetted from bone shaft of patients undergoing routine total hip replacement surgery. Only tissue, which would have been discarded, was used with the approval of the Southampton University Hospitals Trust ethics committee (LR194/99). Human bone marrow cells were isolated from fresh trabecular bone marrow samples using Minimal Essential Medium - alpha modification (αMEM). The cell suspension was centrifuged at 1100rpm for 5 minutes at 4°C. The marrow was discarded and the cell pellet resuspended in αMEM and filtered through a 70μm Cell Strainer (Lockertex, Warrington, England). Samples of cell suspension were diluted with 0.5% ($^{w}/_{v}$) trypan blue in 0.16M ammonium chloride and the number and viability of nucleated cells determined. Cells were cultured (2×10^{7}/T80cm^2, 1×10^{8}/T180cm^2) in αMEM supplemented with 10% Fetal Calf Serum (FCS), with or without dexamethasone (Dex., 10nM), ascorbic acid-2-phosphate (AA-2-P, 100μM) (osteogenic or basal media) as required at 37°C and 5%CO_2 for up to 28 days until confluence. Media were changed every 4-5 days. For osteocalcin expression studies, 1,25$(OH)_2D_3$ (10nm) was added during the last 48 hours of the culture period.

2.2.2. Cell trypsinisation

Cells were washed three times with 1× phosphate buffered saline (PBS) and detached by addition of 0.05% trypsin and 0.2% ethylenediaminetetraacetic acid (Trypsin/EDTA

in PBS, pH 7.4) for 5-20 minutes at 37°C. Once detached, the trypsin was inactivated by the addition of αMEM supplemented with 10%FCS and the cells immediately centrifuged at 1100rpm, 4°C for 5 minutes. The pellet was then washed and re-centrifuged in αMEM alone. After resuspension, the cell number was determined using a haemocytometer and cells were passaged at the required density.

2.2.3. Passage of mineralised cultures

The cell layer was rinsed three times with Ca^{2+} and Mg^{2+} free 1× PBS and the cells were cultured in serum free αMEM containing 25Unit/ml purified collagenase IV for 2 hours at 37°C and 5% CO_2. After washing twice with Ca^{2+} and Mg^{2+} free PBS, freshly thawed 1× Trypsin/EDTA solution was added for a further 5-20 minutes. The reaction was stopped by the addition of αMEM supplemented with 10%FCS and the cells collected by centrifugation. Cells were counted and seeded at the required density.

2.2.4. Matrix mineralisation

For examination of osteogenesis, cells were cultured in αMEM supplemented with 10% FCS, 10nM dexamethasone and 100μM ascorbic acid-2-phosphate. After subculture, cells were plated in 25 cm^2 flasks at a density of 1×10^4 cells/cm^2 or on biodegradable porous scaffolds and the media were changed twice weekly for 14 days. During the final 48 hours of culture, the medium was supplement with 5mM inorganic phosphate (Pi) by adding 0.01% (v/v) of a 500mM phosphate solution, pH 7.4 at 37°C. After 48 hours, the cell layers or scaffolds were washed two to three times with serum-free medium and fixed with 95% ethanol at 4°C overnight. Mineralisation was detected by von-Kossa staining.

2.2.5. Cell viability

Cell-Tracker^{TM} green (CFMDA)(25μg) was dissolved in 10μl DMSO and added into 5mls medium together with 25μg of Ethidium Homodimer-1 (EH-1). Cells were incubated at 37°C, 5% CO_2 for 45 minutes and medium replaced with αMEM for a further hour. CMFDA is only incorporated into the cell cytoplasm of viable cells and will remain present through at least four cell divisions (Johnson, 1998). Viable cells (green fluorescence) and necrotic cells (red fluorescence) were detected by fluorescence microscopy.

2.2.6. Colony forming unit-fibroblastic (CFU-F) culture

Primary human bone marrow cells were cultured in 6 well tissue culture plates (2×10^5 cell/well) in the presence and absence of factors in basal or osteogenic medium for up to 12 days (basal media) and 9 days (osteogenic media) respectively. Cells were fixed using 95% (v/v) ethanol prior to examination of CFU-F number. Total colony numbers and alkaline phosphatase-positive colony numbers were counted using an Anderman Colony Counter (Anderman and Co. Ltd, Kingston, UK). The colony was identified if the cell number is over 35 and colonies were determined to be alkaline phosphatase positive if they showed any observable staining by light microscopy. All counts were performed without prior knowledge of the sample characteristics and the counts were repeated to confirm reproducibility of counts obtained. Mean values for each group were derived from 3-6 samples.

2.2.7. Microscopy

Fluorescence microscopy

Images from PLA films and porous scaffolds were taken using an inverted microscope (Leica DMIRB/E), equipped with a fluorescence filter-enabling fluorescence imaging.

Confocal microscopy

After labelling with fluorescence CFMDA and EH-1, samples were examined using a Leica Leitz DM RBE confocal microscopy with a ×50 water immersion objective.

Scanning electron microscopy

Electron microscopy was undertaken using a Hitachi S-800.

2.2.8. Scaffold production using super critical fluid processing

PLGA and PLA porous (50-200μm) scaffolds were used in all studies. The scaffolds were generated by Professor Howdle and Professor Shakesheff (University of Nottingham) using a supercritical carbon dioxide method in which the polymer is plasticised at 35°C under a pressure of 200 atm (Whitaker et al, 2001; Howdle et al, 2001). On release of the pressure, pores are formed in the polymer by the release and escape of the carbon dioxide gas (Fig. 2-1). The PLGA used in these studies will dissolve in vivo in approximately 3-4 months and was selected on the ability of incorporated glycolic acid to allow sufficiently rapid degradation of the copolymer. Scaffolds were characterized using scanning electron microscopy.

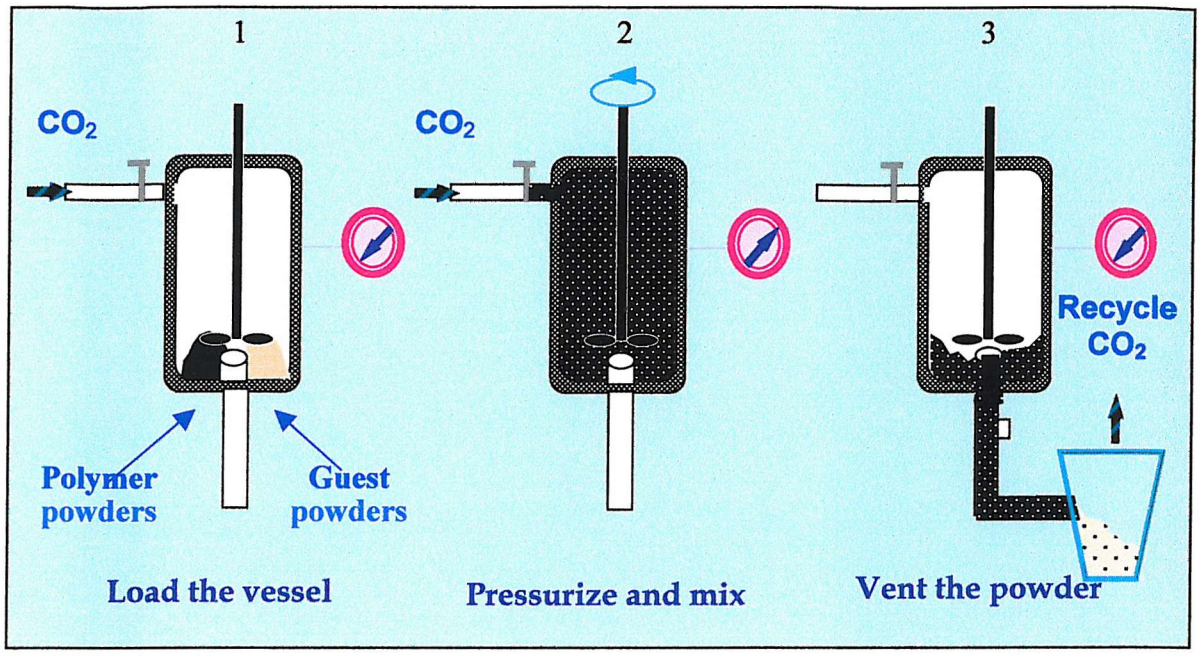


Figure 2-1 Scaffold production using super critical fluid processing (courtesy of Professor Howdle and Professor Shakesheff, Nottingham)

2.2.9. Cell culture on scaffolds

Porous scaffolds were sterilized using 70% ethanol for 3 hours and coated with fibronectin (0.2μM) or PLL-GRGDS (30μM) or Saos-2 'retentate' (50ng/ml) or PTN (50ng/ml), or αMEM alone (serum free) or αMEM supplemented with 20% FCS for 3 hours as required. Human bone marrow cells were incubated with Cell-Tracker™ green (10μg/ml) and EH-1 for 45 minutes to label viable and necrotic cells respectively. The media was then replaced and the cells incubated for a further hour. Following trypsinisation and resuspension in serum-free αMEM, 1×10^6 cells were then added to individual wells of a 24-well plate containing surface-modified and unmodified scaffolds. Fluorescence images were taken after 5 hours and 24 hours. Cells were grown in parallel on PLGA or PLA scaffolds in serum-containing medium as a positive control. After 24 hours, the media was removed and cultures maintained in

αMEM supplemented with 10% FCS, dexamethasone (10nM) and ascorbic acid-2-phosphate (100μM) for up to 6 weeks. Cell-Tracker green and EH-1 were added to cultures at appropriate time points (4 and 6 weeks) to examine cell viability using fluorescence and confocal microscopy. At 4 and 6 weeks, samples were harvested and processed for scanning electron microscopy (SEM) and histology.

2.2.10. Processing

PLA films, PLGA and PLA scaffold samples were fixed with 4% Paraformaldehyde (for Type I collagen, OCN staining) or 95% ethanol (for Alkaline phosphatase, *cbfa-1*, PTN staining), dependent on the staining protocol and, as appropriate, processed through graded alcohols (50%, 90%, 100%, 100%) and 2× 100% Citroclear to paraffin wax and 5μm sections prepared using a microtome (Microm HM 330, Heidelberg, Germany).

2.2.11. Histochemistry and immunohistochemistry

Sections were taken through 100% histoclear, graded alcohols (100%, 100%, 90%, 50%) to water prior to histochemical and immunocytochemical analysis. Antibody controls (omission of primary antibody) were included in all studies.

Alcian Blue/Sirius Red

Sections were stained with Weigert Haematoxylin solutions for 10 minutes and dipped 3 times in acid methanol prior to staining with 0.5% Alcian Blue for 30 minutes. After staining with 1% molybdophosphoric acid, samples were stained by 0.1% Sirius Red for one hour. Sirius red stains collagen within bone matrix and Alcian blue stains the proteoglycans within cartilage.

von-Kossa and Toluidine Blue

Samples were stained with 1% $AgNO_3$ under UV light (wave length 254nm) for approximate 20 minutes until black deposits were visible in sections. After air-drying, slides were counterstained in 1% toluidine blue.

Alkaline phosphatase activity

Cultures were rinsed 3 times in PBS and fixed in 95% ($^{v}/_{v}$) ethanol. 0.4mls of 0.25% Naphthol AS-MX phosphate alkaline solution was mixed with 9.6mls of distilled water and 2.4mg of Fast Violet B salt was added just prior to use. The samples were covered with the mixture solution and incubated for 30-60 minutes in the dark. Samples were rinsed in distilled water.

Type I collagen

Reactivity to Type I collagen antibody (LF 67) was assessed after fixation in 4% Paraformaldehyde for three hours. Endogenous peroxidase activity was blocked using 3% H_2O_2 prior to incubation with LF 67 (1:300 in 1% BSA in PBS) for 3 hours at 4°C. Samples were incubated with peroxidase-conjugated anti-rabbit IgG (1:30 in 1% BSA in PBS) and peroxidase activity was detected using 3-amino-9-ethyl-carbazole (AEC) in acetate buffer containing H_2O_2. Samples were counterstained with Mayer's haematoxylin.

PTN

Reactivity to PTN was assessed using a rabbit polyclonal antibody raised against a synthetic N-terminal peptide of PTN isolated from rat perinatal rat brain. After fixation in 95% ($^{v}/_{v}$) ethanol for three hours, endogenous peroxidase activity was blocked using 3% H_2O_2. Incubation with anti-PTN 1:500 in 1% BSA in PBS was for 3 hours at 4°C. Samples were incubated with peroxidase-conjugated anti-rabbit IgG (1:30 in 1% BSA

in PBS) and peroxidase activity was detected using AEC in acetate buffer containing H_2O_2. Samples were counterstained with Mayer's haematoxylin.

Osteocalcin

Reactivity to the osteocalcin specific monoclonal antibody OS35 was assessed after fixation of the cells in 4% ($^{w}/_{v}$) paraformaldehyde in PBS (pH 7.4). Fixed cultures were permeabilised using 0.05% Nonidet P40 in TBS (15min) pre-blocked with goat serum 1:10 in TBS (15min) and incubated with OS35 (1:100; 30 min at 15°C). Goat anti-mouse alkaline phosphatase conjugate (1:200) was used as secondary antibody and visualized enzymatically with fast red. Endogenous alkaline phosphatase activity was blocked by the addition of 1mM levamisole. Samples were counterstained with Mayer's haematoxylin.

Cbfa-1

After incubation in 1% hydrogen peroxide (10 minutes) and 1.5% blocking serum (one hour), samples were incubated with primary antibody (PEBP2αA) (1:300 dilution) overnight at 4°C and then incubated for 30 minutes with biotinylated donkey anti-goat secondary antibody. Avidin and Biotin (AB) enzyme reagent was added for 30 minutes and samples incubated in peroxidase substrate (10 minutes).

2.2.12. Biochemical Assays

Alkaline phosphatase activity

Primary human bone marrow cells were cultured in 6 well tissue culture plates (5×10^5 cells/well) in the absence or presence of factors at different concentrations using basal or osteogenic medium for 21 days. Cell layers were washed with 1× PBS for three times and stored at -80°C until assayed for alkaline phosphatase activity. For each assay, the cell layer from each well was scraped into 0.5 ml 0.1% (v/v) triton X-100, freeze-thawed twice and total cell lyses in triton X-100 confirmed by light microscopy.

Alkaline phosphatase activity was measured using p-nitrophenyl phosphate as substrate in 2-amino-2-methyl-1-propanol alkaline buffer solution (1.5M, pH 10.3 at 25°C). Hydrolysis of the substrate to para-nitrophenol was measured as a change in absorbance by spectroscopy at 410 nm wavelengths. Alkaline phosphatase specific activity was expressed as nanomoles of p-nitrophenol per hour per μg DNA.

PicoGreen dsDNA quantitation Assay

DNA content was determined using PicoGreen fluorescence reagent according to the manufacture's instructions. The PicoGreen assay is an ultra sensitive fluorescence nucleic acid stain for quantitative double-stranded DNA in solution. Sample fluorescence was measured using a CytoFluor micro-plate reader (excitation ~480nm, emission ~520nm).

2.2.13. Chorioallantoic membrane (CAM) assay

Fertilized eggs were incubated using a Multihatch automatic incubator (Brinsea Products, Sandford, UK) at 37°C in a humidified atmosphere for 10-18 days. Chick femurs were excised using a dissection microscope and a wedge-shaped segmental defect created in the middle of the femur. HBM cells together with scaffold constructs (with appropriate growth factor) were placed to fill the bone defect site (Fig. 2-2). On 10-day-old eggs, a square hole (1×1 cm^2) was cut into the shell with a hacksaw blade. The cut piece was carefully lifted to expose the CAM. The chicken bone, human bone marrow stromal cells and scaffold constructs were placed on the surface of the chorioallantoic membrane, the shell replaced and sealed with adhesive tape and the eggs incubated at 37°C for a further 7 days as previously detailed (Fig. 2-3) (Roach et al, 1998). Samples were harvested, fixed in 95% ethanol and processed for histology. The chick embryo was killed by decapitation after completion of the study.

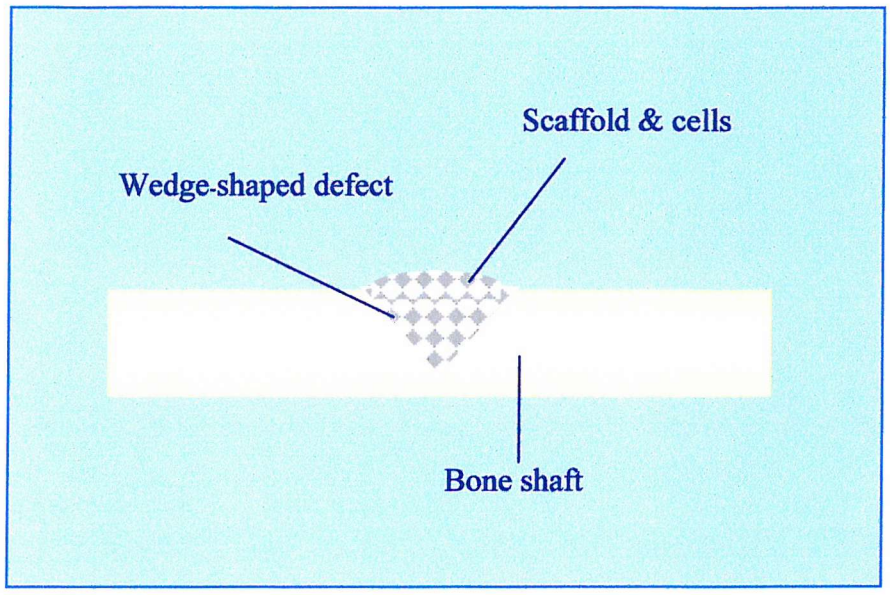


Figure 2-2 Chick bone, HBM cells and scaffold constructs

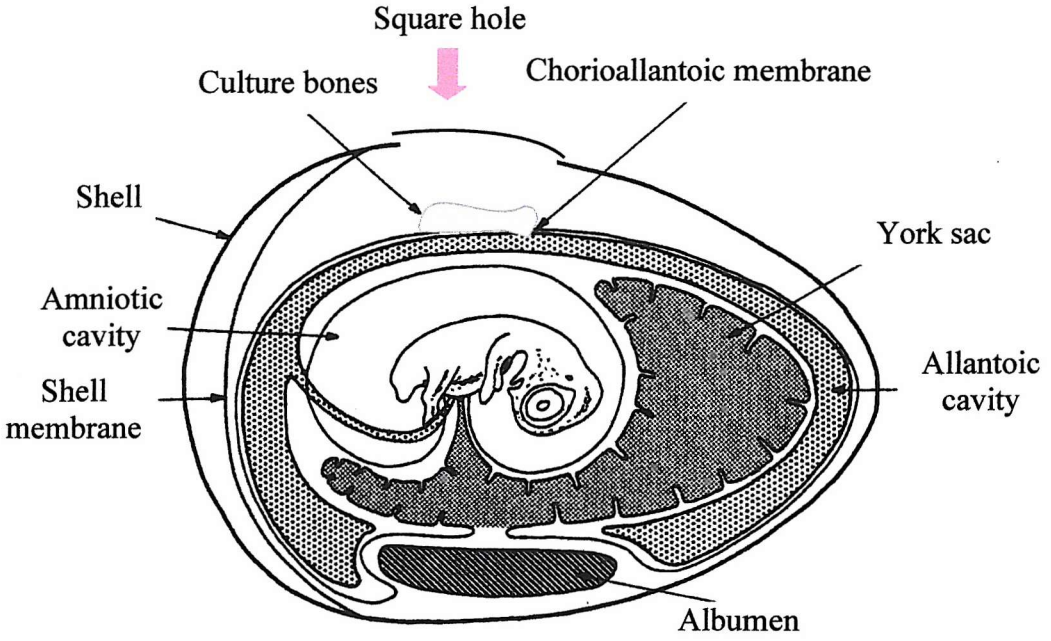


Figure 2-3 Chorioallantoic membrane culture model

2.2.14. *In vivo* studies

Primary human bone marrow cells were cultured in osteogenic media until confluent (day 21) prior to subcutaneous implantation or intra-peritoneal implantation.

Subcutaneous implantation

Confluent primary human bone marrow cells were trypsinized and seeded (2×10^5 cells/sample in serum free αMEM) onto PLGA/PLA scaffolds adsorbed with factors or scaffold alone for 15 hours. After 15 hours, constructs were placed in osteogenic media for a further 3 days, prior to subcutaneous implantation into MF1-nu/nu mice (20-24g, 4-5 weeks old). After 4-6 weeks, the mice were killed and specimens were collected and fixed in 95% ethanol for histochemical analysis (Fig. 2-4).

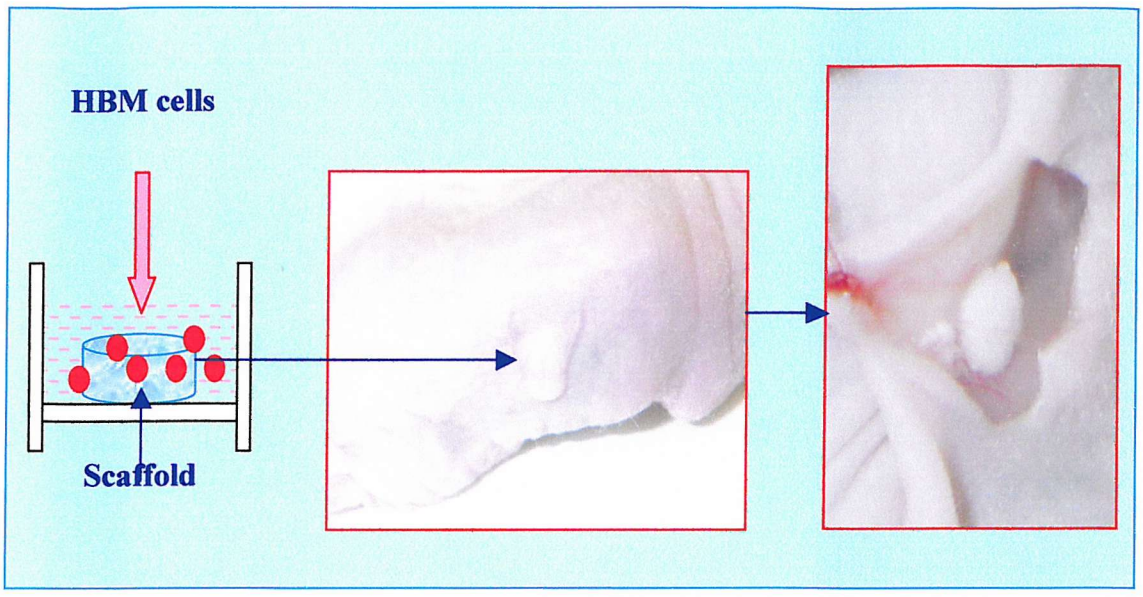


Figure 2-4 Subcutaneous implantation model

Intra-peritoneal implantation

The diffusion chamber (130μl capacity) model provides an enclosed environment within a host animal to study the osteogenic capacity of skeletally derived cell populations, which resolves the problems of host versus donor bone tissue generation (Fig. 2-5). Cells were recovered by collagenase (Clostridium histolyticum, type IV; 25U/ml) and trypsin/EDTA digestion. Human bone marrow cells were sealed in diffusion chambers (2×10^6 cells/chamber) together with PLGA/PLA porous scaffold adsorbed with or without factors in αMEM. Chambers were implanted intra-peritoneally in MF1-nu/nu mice and after 10 weeks the mice were killed, chambers were removed and examined by X-ray analysis prior to fixation in 95% ethanol at 4°C. Polymer samples were processed undecalcified and sectioned at 5 μm and stained for toluidine blue, type I collagen, osteocalcin and mineralisation by von Kossa.

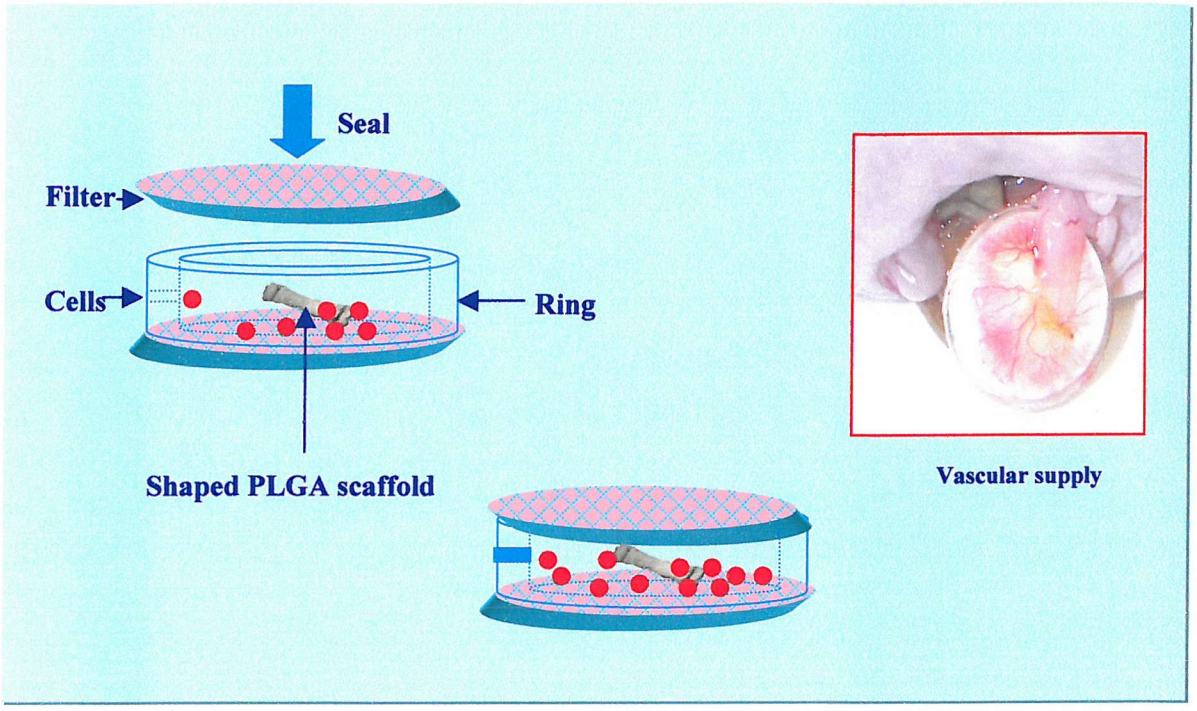


Figure 2-5 Diffusion chamber model

2.2.15. Radiological assessment (X-ray)

Radiological assessment was performed using a HP 43806 X-ray system.

2.2.16. Statistics

Values are expressed as the mean ± SD. Experiments were performed at least three times. Statistical analysis was performed using student t-test, One-Way Analysis of Variance (ANOVA) with Tukey-Kramer Multiple Comparisons post-test using GraphPad Instant Software (GraphPad Software Inc, San Diego, California, USA).

CHAPTER 3

HUMAN OSTEOPROGENITOR GROWTH AND DIFFERENTIATION ON BIOMIMETIC POLYMER FILMS & POROUS SCAFFOLDS

3.1. Introduction

The development and use of synthetic materials, such as poly(lactic acid) and poly(-lactic-co-÷glycolic acid) as scaffolds for cell transplantation and tissue regeneration has made the development of biomimetic scaffolds a realistic objective (Chaput et al, 1996; Patel et al, 1998; Quirk et al, 2000). These synthetic materials have been approved by the FDA and are widely used as resorbable sutures and drug delivery devices over the last 20 years with a long and favorable clinical record. However, these materials themselves are not suitable for cell attachment and growth due to their lack of a chemical reactive pendent chain (Yang et al, 2001). To overcome these limitations, many procedures, using different biological agents, have been developed for the surface modification of these materials (Shakesheff et al, 1998). Thus, the ability to modify the surface chemistry of these polymer materials through the incorporation of signal recognition ligands to mediate the molecular and cellular response of cells provides new approaches to promote osteoblast adhesion, proliferation and differentiation for bone tissue engineering. In essence, biomimetic materials provide microenvironments, which mimic biological environments for cell-matrix interaction (Patel et al, 1998).

A number of matrix proteins such as fibronectin, vitronectin and laminin have been shown to play a central role in cellular morphology, migration and the provision of signals that orchestrate cellular proliferation, metabolism, function and differentiation in anchorage dependent cells such as osteoblasts and osteoclasts (Hynes, 1992; Horton, 1997; Globus et al, 1998; Rezania and Healy, 1999; Villanova et al, 1999; Duong et al, 2000). The attachment between cells and of cells to the extracellular matrix is controlled by members of the various families of adhesion receptors, including the integrins, selectins, cadherins and immunoglobulins (Ruoslahti and Pierschbacher, 1987; Hynes, 1992). The binding between integrin receptor and ligand is often

mediated through an RGD recognition sequence. Within bone, a variety of RGD-containing ligands such as osteopontin, thrombospondin and bone sialoprotein, which are important in bone cell motility, polarity, proliferation and survival have been identified (Horton, 1997; Globus et al, 1998). Furthermore, studies from Rezania and Healy (1999) and Cargill *et al* (1999) indicate the potential to modulate the adhesion of osteoblasts selectively and significantly by immobilized -Arg-Gly-Asp-(-RGD-) and -Phe-His-Arg-Arg-Ile-Lys-Ala-(-FHRRIKA-) and -Lys-Arg-Ser-Arg- (-KRSR-) peptides.

However, the attachment of appropriate biological recognition structures, such as the adhesion motif RGD to the surface of PLA is hampered by the lack of chemical reactivity of the poly(α-hydroxyacids) due to the absence of functional groups to support covalent attachment. In order to overcome this issue and generate biomimetic structures, a number of approaches have been developed including 1) surface hydrolysis, 2) plasma deposition and, 3) the generation of polymer-peptide hybrid molecules in an attempt to guide cell attachment and development (Cannizzaro et al, 1998). Recently, a simple solution to the presentation of peptides to the PLA polymer surface has been used (Quirk et al, 2000) to attach the peptide of interest to poly(L-Lysine) and immobilize the peptide sequence at the PLA surface through adsorption. The aim of this study was to examine the adhesion, spreading, growth, differentiation and phenotypic modulation of human osteoprogenitors on two-dimensional poly(lactic acid) films and three dimensional poly(-lactic-*co*-÷glycolic acid) scaffolds, which have been surface-modified by either fibronectin or the cell adhesion motif RGD, adsorbed using poly(L-lysine).

3.2. Materials and Methods

For general materials and methods see Chapter 2.1 and Chapter 2.2. Poly(L-Lysine)-GRGDS was a gift from Professor Shakesheff (University of Nottingham).

3.2.1. Cell culture

Bone marrow samples (10 patients in total: 4 females and 6 males, aged 52–80, with a mean age of 67.6 years) were obtained from the patients undergoing routine total hip replacement surgery. Primary cultures of bone marrow cells were established as detailed in Chapter 2.2.1.

3.2.2. Polymer film preparation

The PLA films were prepared in collaboration with the University of Nottingham by melting the raw material on a glass tile placed on a hot plate set at 150°C. Glass microscope circular slides were then used to sandwich the melted polymer and form discrete discs (1.3cm diam × 0.3mm). After cooling, the glass plates were immersed in de-ionised water for 20 minutes, enabling removal of the polymer films from the glass.

3.2.3. Cell culture on PLA films

In initial experiments, osteoblast adhesion and spreading on 2-dimensional PLA films was examined. PLA films placed in 24-well plates were sterilized for 15mins using UV irradiation and coated with 1 ml of either the surface-modifying solution PLL-GRGDS conjugate (30nM – 30μM) or fibronectin (20pM – 0.2μM) in PBS or αMEM supplemented with 20% FCS and placed in the incubator for 2 hours (3 repeats per sample). Samples were washed with PBS and 0.5 ml of serum-free αMEM was added to the wells to prevent the films from floating during cell seeding. Cells (2×10^7) were incubated with Cell Tracker^{TM} green for 45 minutes. The media was then replaced and the cells incubated for a further hour. Following trypsinisation and resuspension, cells were centrifuged and resuspended in serum-free αMEM. Cells (20,000) were added to

each well and fluorescence images were taken after 5 hours and 24 hours. Cells were grown simultaneously on tissue culture plastic in serum-containing medium as a positive control. After 24 hours, the media was removed and cultures were maintained with 10% fetal calf serum αMEM containing dexamethasone (10nM), ascorbic acid-2-phosphate (100μM) for up to 28 days. Cultures were fed every 5 days and maintained at 37°C and 5%CO_2. Thereafter, samples were assayed for alkaline phosphatase, type I collagen, *cbfa-1* and osteocalcin immunocytochemistry (See Chapter 2.2.10).

3.2.4. Fluorescence microscopy and image analysis

Images from PLA films and porous scaffolds were taken using an inverted microscope, equipped with a fluorescence filter enabling fluorescence imaging. Three random images were taken per well, producing a total of nine images for each surface (image size covering an area of approx. $1.77\times10^5\mu m^2$). Analysis was performed retrospectively using ScanImage software, which detects variations in image color intensity and calculates individual cell parameters based upon the fluorescence signal per pixel.

3.3. Results

3.3.1. Cell attachment & spreading on PLA films - Histological analysis

Initial dose-response studies were performed using PLA films coated with FN (20pM – 0.2μM) or PLL-GRGDS (30nM – 30μM) to determine an appropriate concentration for the attachment and growth of human bone marrow cells on PLA polymer biomaterial. Enhanced cell adhesion, spreading and differentiation was observed in serum free conditions, on PLA films coated with FN (Fig. 3-1A and Fig. 3-2A) or coupled with PLL-GRGDS (Fig. 3-1B and Fig. 3-2B) compared to the hydrophobic PLA film surface alone (Fig. 3-1C and Fig. 3-2C). Negligible growth, as indicated by poorly attached and rounded cells, was observed on unmodified PLA surfaces in serum-free

conditions (Fig. 3-1C and Fig. 3-2C). Adherent cells were observed at 5 hours on PLA films coated with FN or coupled with GRGDS and after 24 hours comparable cell spreading on these films compared to tissue culture plastic surfaces (TCPS) was observed. In all studies, cell adhesion and spreading on TCPS containing serum, was used as a positive control (Fig. 3-1D and Fig. 3-2D). Cell viability was confirmed by the intense green fluorescence observed as a consequence of incorporation of the Cell Tracker™ green fluorescence probe into the cell cytoplasm.

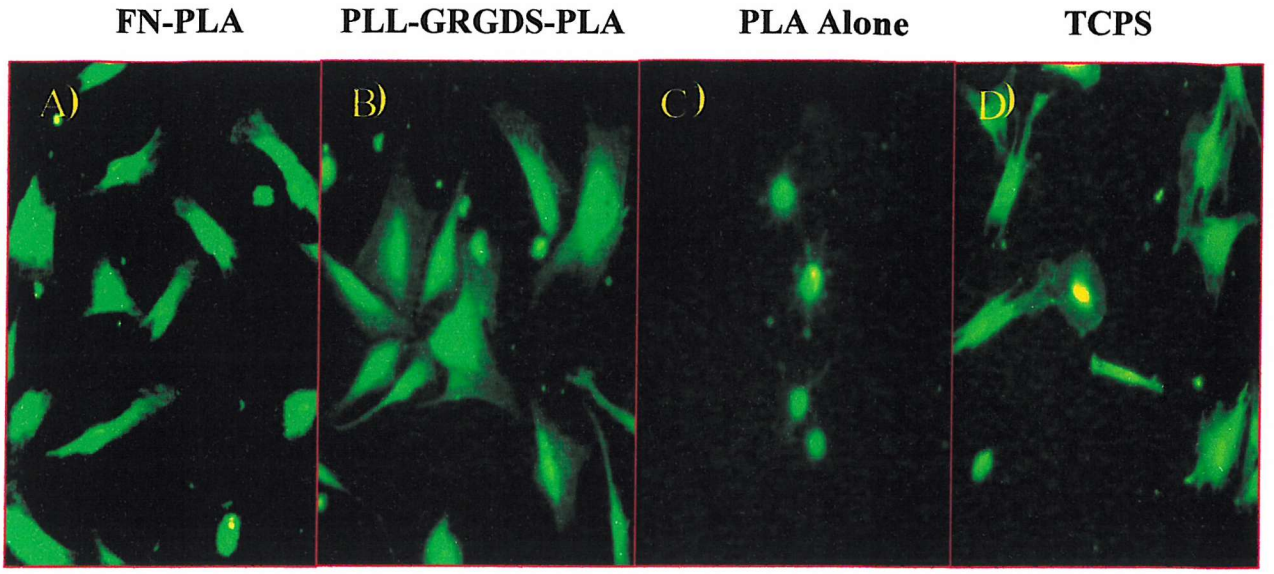


Figure 3-1 Human bone marrow cell attachment and spreading on PLA films:
A) Enhanced cell attachment and spreading were observed in serum free conditions on PLA films coated with FN, or B) coupled with PLL-GRGDS compared to C) PLA film alone. D) tissue culture plastic coated with serum after 5 hours as detected by fluorescence microscopy. Original magnification: ×200

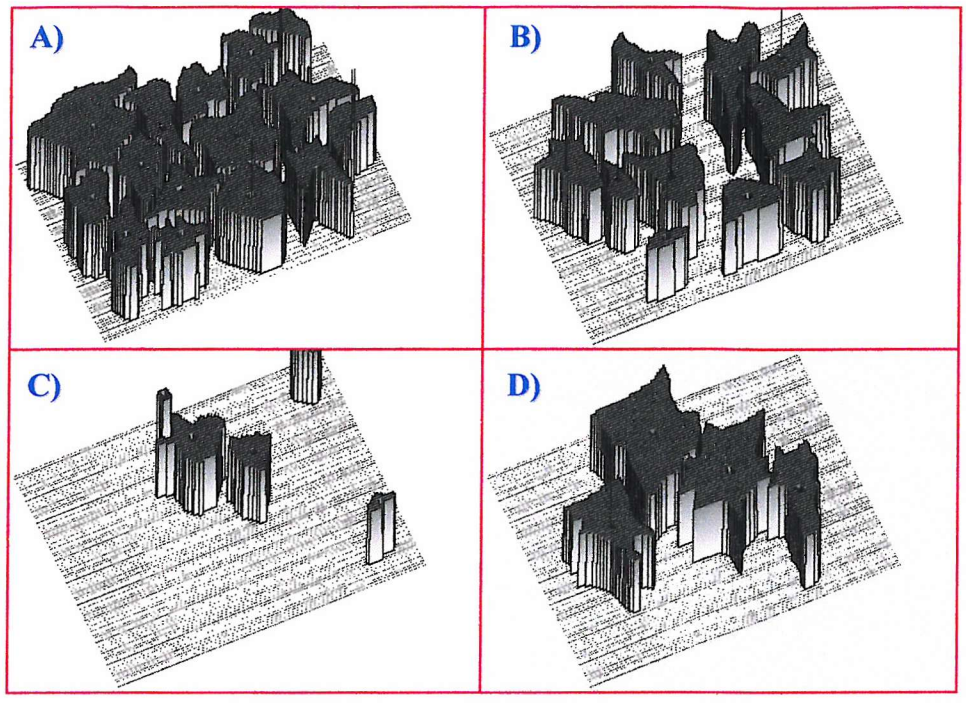


Figure 3-2 Surface plot images of human bone marrow cell attachment and spreading on PLA films (×200): A) Enhanced cell attachment and spreading were observed in serum free conditions on PLA films coated with FN, or B) coupled with PLL-GRGDS, or C) PLA film alone and, D) tissue culture plastic coated with serum after 24 hours as detected by fluorescence microscopy.

3.3.2. Quantitation of cell attachment and spreading on PLA films

Human bone marrow cell adhesion and growth on FN adsorbed and PLL-GRGDS coupled PLA films was concentration dependent (20pM – 0.2μM FN and 30nM – 30μM PLL-GRGDS) and significant at concentration as low as 2nM fibronectin (Fig. 3-3A and 3-3B; Table 3-1 and Table 3-2) and 30nM PLL-GRGDS (Fig. 3-3B and Table 3-2). Quantitation of cell area by image analysis confirmed the marked variation in cell size distribution between the different surfaces. Figure 3-3 and Table 3-1,2 show the distribution of mean and total cell area on each material after 5 hours and 24 hours of culture. As expected from the mean cell area data, total area was dramatically reduced in cells cultured on PLA alone compared to cells grown on FN adsorbed and PLL-GRGDS coupled PLA films. The cell spreading observed was comparable to TCPS conditions (positive control). Interestingly, high concentrations of PLL-GRGDS (30μM and 3μM groups) inhibited the attachment and spreading of human bone marrow cells on the PLA films (Fig. 3-3B). This was attributed to either an excess of adhesion motif peptide (RGD), which is known to reduce cell migration, or excess PLL, which has recently been shown to have an inhibitory effect on cell spreading (Quirk et al, 2001).

Table 3-1 HBM Cell attachment and spreading on FN adsorbed PLA films

	Groups	FN adsorbed PLA films					PLA	TCPS
		200nM	20nM	2nM	0.2nM	0.02nM		
5 hours	Cell Number	101	75	58	65	66	61	67
	Total Area μm^2	408841	306015	200987	154513	117751	46666	234461
24 hours	Cell Number	92	69	59	65	59	55	59
	Total Area μm^2	474219	367245	228303	172976	168349	65474	349228

Table 3-2 HBM Cell attachment and spreading on FN-PLA & PLL-GRGDS-PLA films

	Groups	GRGDS-PLL-PLA				FN-PLA		PLA	TCPS
		30μM	3μM	0.3μM	0.03μM	200nM	2nM		
5 Hour	Cell Number	109	153	113	104	83	71	82	75
	Total Area μm^2	44379	92263	171344	264792	237434	166334	53526	202167
24 Hour	Cell Number	99	125	113	100	94	116	89	62
	Total Area μm^2	30972	46091	155676	137641	238237	108270	42387	162289

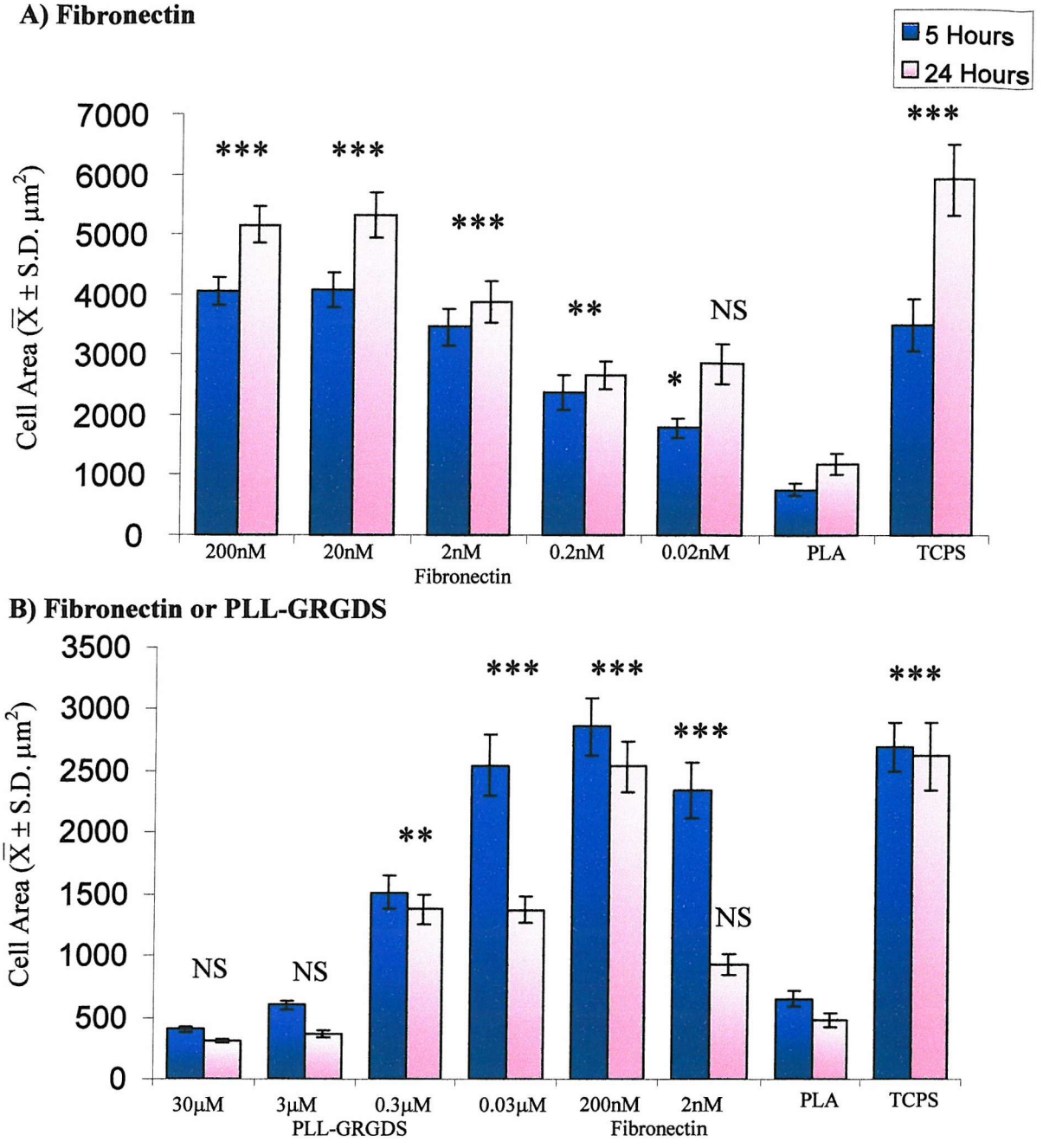


Figure 3-3 Quantitation of cell attachment and spreading showing the distribution of cell areas following culture of HBM cells on a) fibronectin coated films (20 pM – 0.2 μM) and b) PLA films coated with the adhesion peptide GRGDS using PLL (30 nM – 30 μM). TCPS was used as a positive control and unmodified PLA alone was run in all studies. Data represents measurements from nine random areas (area 1.77×10^5 μm^2). NS: not significant, *: $P<0.05$, **: $P<0.01$, ***: $P< 0.001$ compared to PLA alone.

3.3.3. Osteoblast phenotype on surface-modified PLA films

Immunohistochemical reactivity to type I collagen (Fig. 3-4A-D) and histochemical staining of alkaline phosphatase (Fig. 3-4E-H) confirmed maintenance of the osteoblast phenotype following culture of human bone marrow cells on PLA films coupled with -RGD- or adsorbed with FN. Expression of type I collagen and alkaline phosphatase on cells grown on FN or GRGDS adsorbed films was maintained in culture compared to PLA alone (Fig. 3.4C,G).

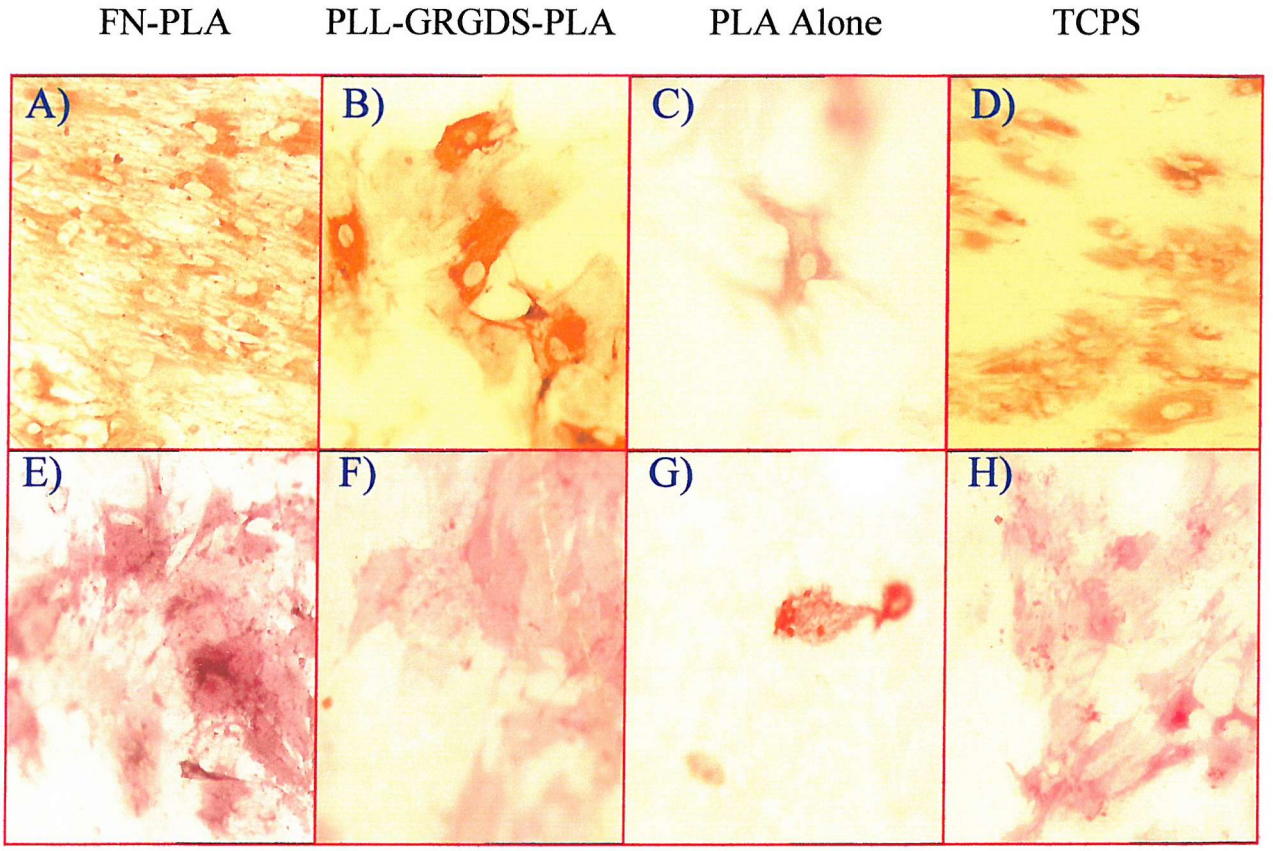


Figure 3-4 Expression of Type I collagen (A-D) and alkaline phosphatase activity (E-H): Type I collagen immunohistochemistry using LF 67 on PLA films and TCPS. Note enhanced adhesion and expression of type I collagen on modified PLA films (A-D). Comparable alkaline phosphatase activity detected histochemically on PLA films and TCPS (E-H). Negligible cell adhesion was observed on PLA alone (C, G). Original magnification: ×200.

3.3.4. Cell attachment and growth on three-dimensional polymer scaffolds

Human bone marrow cell adhesion and growth was observed on porous PLGA scaffolds coated with 0.2μM FN in PBS (Fig.3-5A-D,F). Confocal and fluorescence microscopy confirmed extensive growth of human bone marrow cells on PLGA scaffolds, initially coated with FN or fetal calf serum for 24 hours in αMEM alone, followed by growth in osteogenic conditions for up to 28 days (Fig. 3-5A-D,F). Negligible cell adhesion and growth was observed on unmodified PLGA scaffolds (Fig. 3-5E). The growth of the cells was evidenced by intense fluorescence staining following incorporation of the Cell Tracker^{TM} green probe into viable cells and negligible EH-1 staining (Fig. 3-5C,D,F).

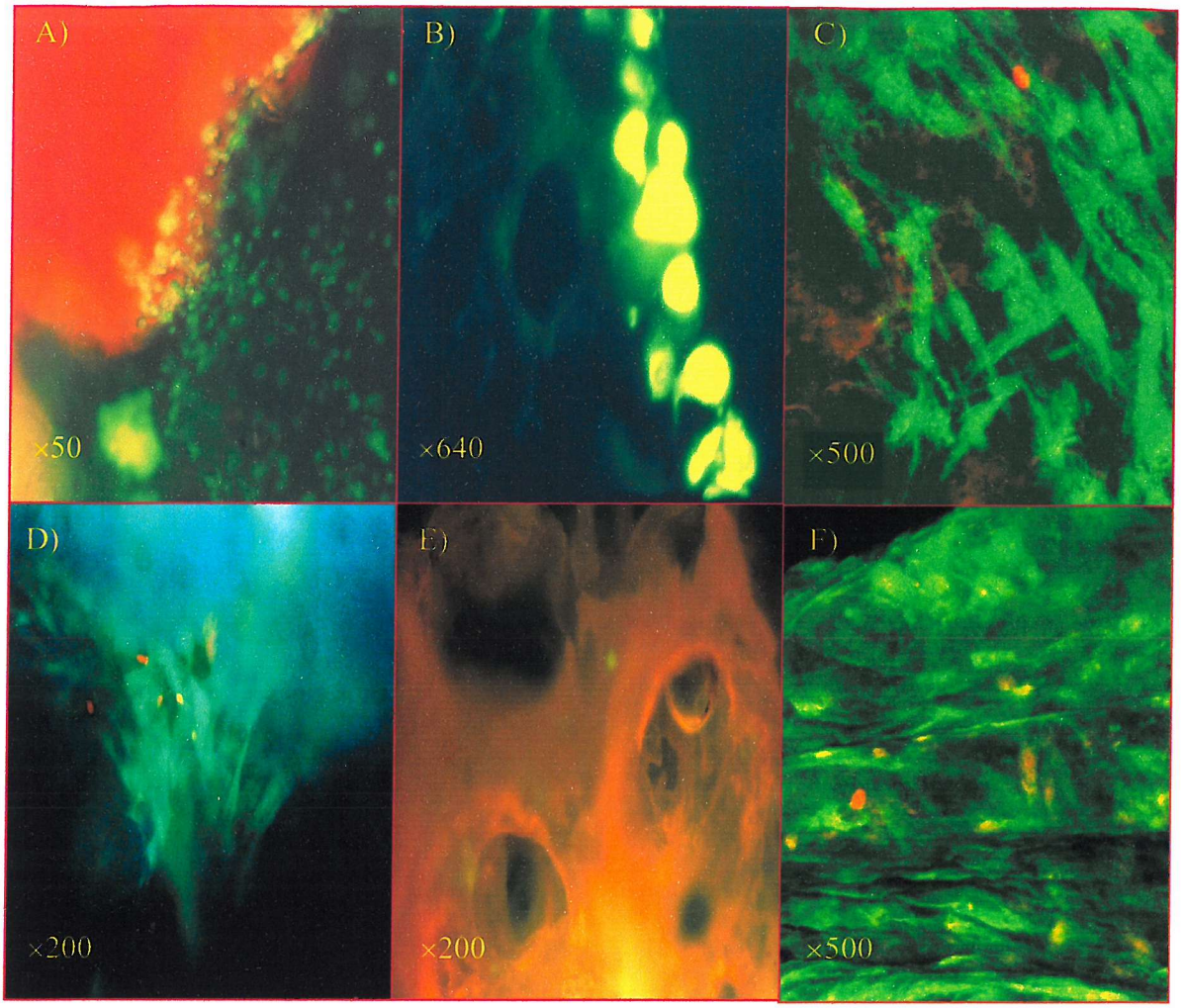


Figure 3-5 Fluorescence photomicrographs of human bone marrow cells after 5 hours (A-B), 6 weeks of culture (C-E) and 12 weeks (F) on PLGA scaffolds coated with FN. Viable cells (green) detected using Cell Tracker^{TM} green and necrotic cells (red) using EH-1. Negligible cell adhesion was observed on unmodified PLGA (E). Original magnification: A) ×50, B) ×640, C) ×500, D) ×100, E) ×100, F) ×500.

3.3.5. Cell differentiation on three-dimensional polymer scaffolds

To confirm the potential of using biomimetic scaffolds as tissue engineering constructs, human bone marrow cells were cultured for extended periods of time on FN adsorbed PLGA scaffolds.

Extensive ingrowth of human bone marrow cells into the porous PLGA scaffold was observed after culture for 6 weeks in osteogenic media (Fig. 3-6A-D). Penetration of human bone marrow cells into and throughout the porous scaffold could be demonstrated on paraffin sections (Fig. 3-6E-H). The mature osteoblastic phenotype of the cells and cell growth was confirmed by staining for alkaline phosphatase as well as for type I collagen, *cbfa-1* and osteocalcin immunocytochemistry (Fig. 3-6E-H). Osteocalcin expression was observed in cultures pre-treated with (10nM) $1,25(OH)_2D_3$ for 48 hours using the monoclonal antibody OS 35 (Fig. 3-6D). No immunoreactivity was observed on scaffolds not pre-treated with (10nM) $1,25(OH)_2D_3$.

Following characterisation of the osteoblast phenotype by immunocytochemistry, cultures were examined for mineral formation. Cell ingrowth and initiation of mineralised matrix formation was observed after four weeks in human bone marrow cells on PLGA scaffolds adsorbed with FN. After 6 weeks numerous foci of mineralisation could be observed (Fig. 3-6E, F, H and Fig. 3-7). The formation of mineralised structures within the scaffolds was confirmed by von Kossa staining (Fig. 3-6H and Fig. 3-7A,B). Mineralisation was not observed in bone marrow cells cultured on tissue culture plastic alone or nomodified PLA (Fig. 3-7C).

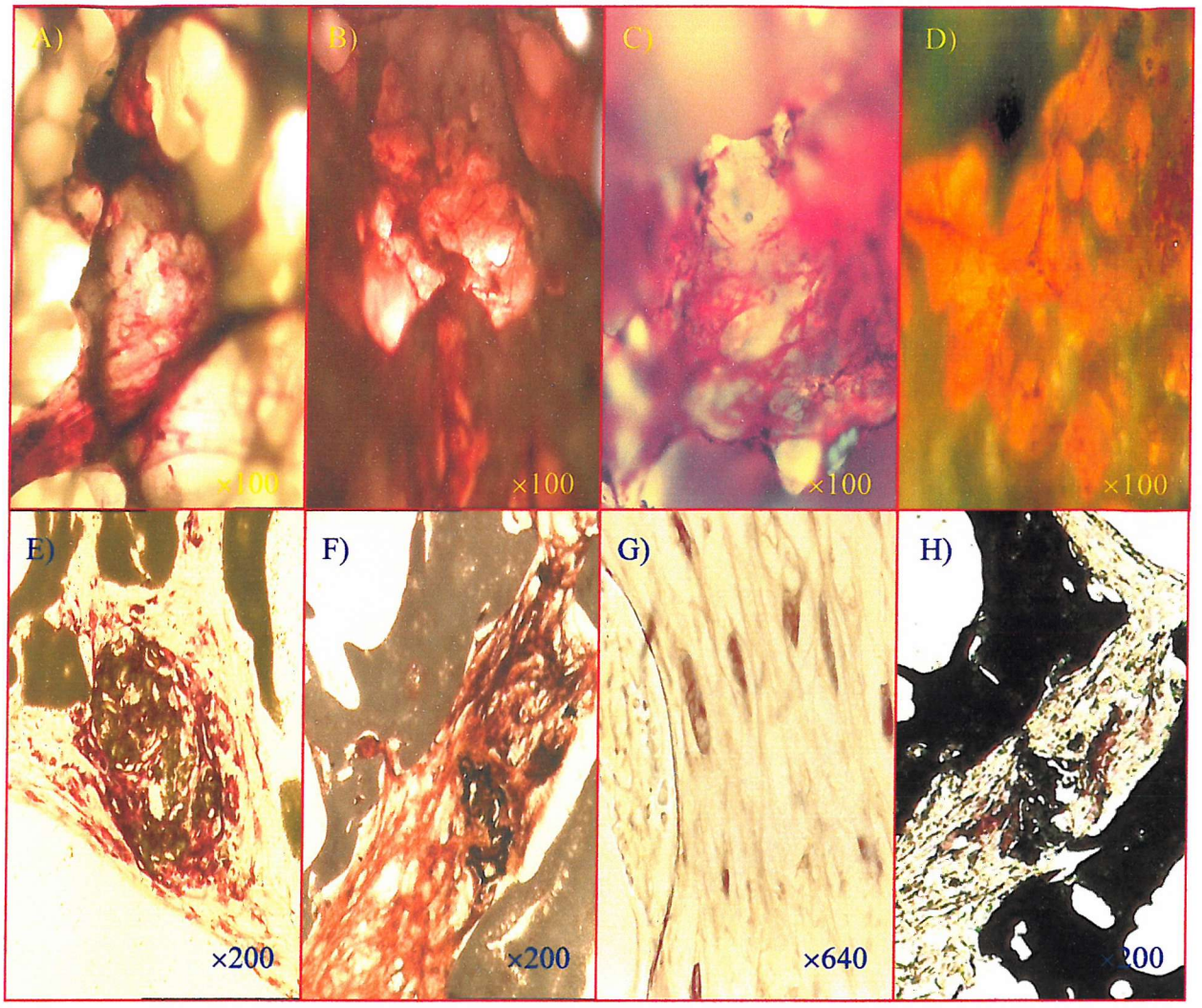


Figure 3-6 Expression of bone markers by human bone marrow cells, in *situ* (A-D) and on paraffin sections (E-H), as detected by immunocytochemistry, on PLGA scaffolds coated with FN following 6 weeks culture. A and E) Alkaline phosphatase activity detected by histochemistry. B and F) Type I collagen. C and G) *cbfa-1* expression (PEBP2αA). D) Osteocalcin expression. H) Cell ingrowth and mineralisation as detected by von Kossa and alkaline phosphatase histochemistry. Mineralisation can also be observed in sections E and F. Original magnification: A-D) ×100, E,F,H) ×200, G) ×640.

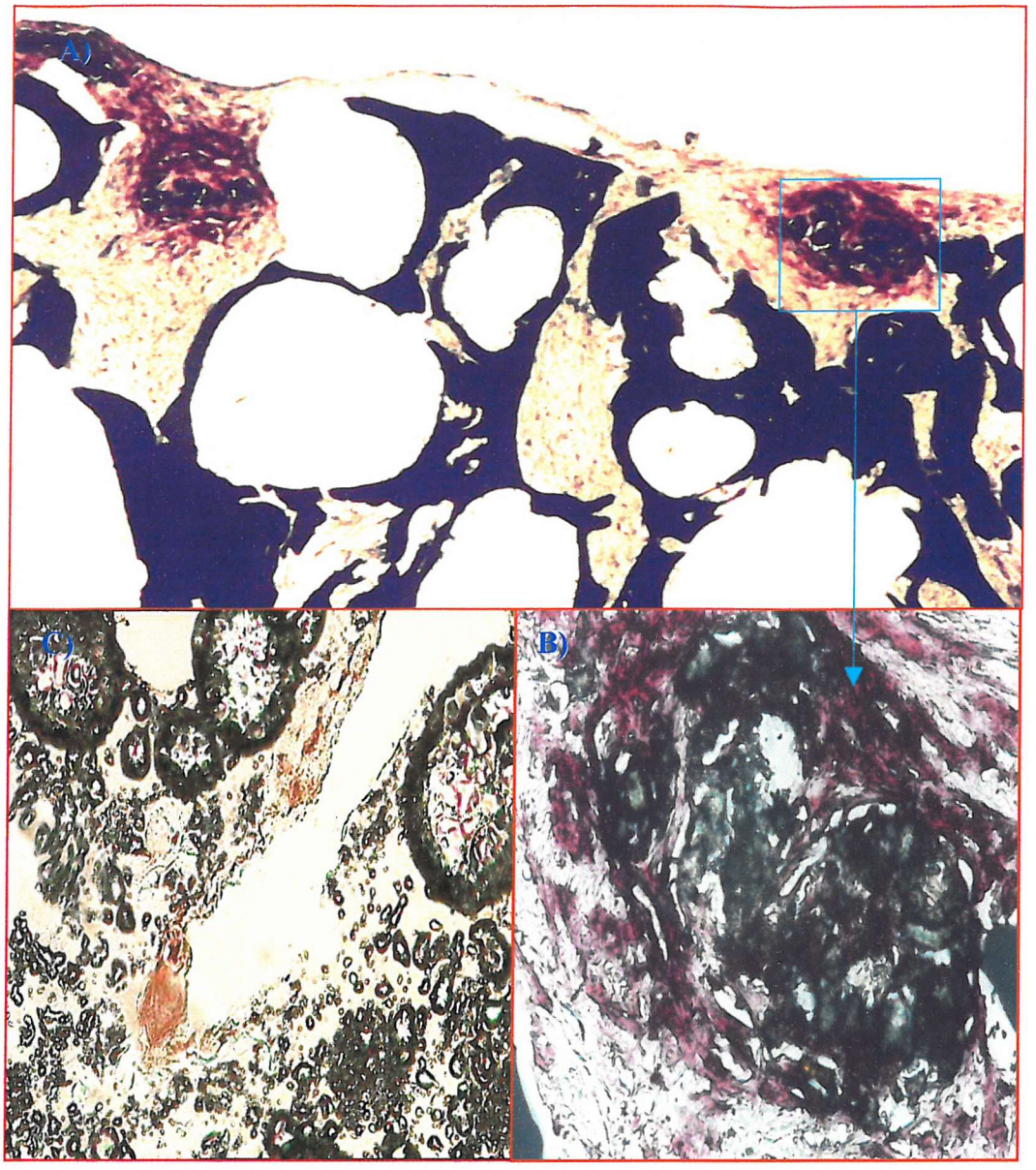


Figure 3-7 Human bone marrow cell ingrowth and mineralisation on/in PLGA scaffolds coated with FN following 6 weeks culture (A,B: Alkaline phosphatase and von-Kossa staining). No mineralised matrix deposition was observed on PLGA scaffold alone (C: Type I collagen and von-kossa staining). Original magnification: A) ×100, B) ×450, C) ×200.

3.4. Discussion

This study has examined the attachment, proliferation and differentiation of human bone marrow cells on biomimetic scaffolds generated by modification of PLA films and PLGA porous degradable scaffolds. Negligible adhesion, growth and differentiation was observed on the hydrophobic PLA film alone, whereas cell attachment was enhanced on PLA films adsorbed with FN or coupled with the integrin adhesion motif RGD. Extension of these studies to porous PLGA scaffolds suggests that biomaterial surfaces, which on their own do not support cell growth, can be modified to enhance human bone marrow cell growth, differentiation and mineralisation. The use of PLGA scaffolds adsorbed with FN, resulted in extensive cell growth after 4–6 weeks of culture in osteogenic media. Expression of the osteoblast phenotype was maintained as examined by alkaline phosphatase, type I collagen, *cbfa-1* and osteocalcin expression. In addition, initiation of mineralisation was confirmed by von Kossa staining on the porous scaffolds. These findings indicate the capacity to generate biomimetic surfaces within a porous template for human bone marrow cells that will allow the differentiation and ultimately mineralisation of bone marrow cells. In this study, the use of fluorescence markers (Cell Tracker^{TM} green and EH-1) have confirmed human bone marrow cell spreading and adhesion on FN-PLA, PLL-GRGDS-PLA films compared to attachment on PLA polymer alone. The osteoblast response was found to be dose dependent (20pM – 0.2μM FN and 30nM – 30μM PLL-GRGDS) and significant at concentrations as low as 2nM FN and 30nM PLL-GRGDS. In addition, cell adhesion and spreading on FN-PLA films was comparable to tissue culture plastic serum controls.

Due to the hydrophobic nature of the PLA polymer, PLA alone resulted in negligible adhesion and cell spreading. These studies confirmed as expected that a principle

binding domain of adhesion proteins is -RGD-. It has been shown the effect on adhesion of bone cells could be inhibited partially (50-60%) with -RGD-containing peptides and antibodies (Puleo and Bizios, 1991; Dalton et al, 1995). Over-presentation of the surface RGD has been shown to result in sub-optimal cell migration (Johnson, 1998; Isogai et al, 1999). It cannot be discounted in this case, as previously shown by Quirk *et al* (2001), that the surface concentration of PLL in the PLL-GRGDS system may also interfere with integrin-mediated cell response.

A previous report by Ishaug and coworkers (1994) showed rat osteoblasts can be cultured on films of biodegradable poly(L-lactic acid) (PLLA), 75:25 PLGA, 50:50 PLGA and poly(glycolic acid) in the presence of serum. Extensive studies from the same group using three-dimensional poly(DL-lactic-co-÷glycolic acid) foams and rat calvarial osteoblasts (Ishaug et al, 1997; Ishaug-Riley et al, 1998; Wake et al, 1998) in the presence of serum, showed PLGA foams supported osteoblast differentiation and mineralisation in long-term cultures (56 days) indicating poly(alpha-hydroxyacids) are suitable substrates for osteoblast growth. Further support for the suitability of poly(alpha-hydroxy esters) for osteoblast growth is provided by Matsuzaka and co-workers (Matsuzaka et al, 1999) in their study on the effects of micro grooves and surface texture on osteoblast growth on PLA and polystyrene surfaces. Surface texture was shown to increase differentiation and mineralisation on PLA micro textured surfaces. However, all these studies were performed in the presence of serum, rich in adhesion proteins, thus the precise adhesion and spreading interaction between osteoblastic cells and the biomaterial are unclear (Cannizzaro et al, 1998).

The current work, as presented, confirms the use of a simple approach developed by Quirk and coworkers (2001) to couple adhesion peptide sequences to PLL, which immobilizes the peptide through adsorption at the PLA surface. The ability to modify

hydrophobic PLA, shows the cell surface interaction of any biomaterial is critical in the provision of close appositional bone differentiation and growth. In the current study, the use of PLL to immobilize -RGD- or the adsorption of FN provides new approaches to generating biomimetic environments. Other groups have examined the ability to modulate osteoblast activity on biomimetic structures. Rezania and Healy (Rezania and Healy, 1999) using rat calvarial osteoblast populations, showed that peptide sequences incorporating both cell (-RGD-) and consensus heparin binding domains (-Phe-His-Arg-Arg-Ile-Lys-Ala) (-FHRRIKA-) immobilized on quartz surfaces could enhance the cell surface interaction and promote cell spreading and mineralisation. Most cell types, except erythrocytes, will adhere to fibronectin immobilized to certain surfaces by interacting specifically with the glycoprotein. A number of oligopeptide cell adhesion domains have been recognized on fibronectin and other matrix proteins including, for example, -Lys-Gln-Ala-Gly-Asp-Val- (-KQAGDV-) on fibrinogen for platelets and -Glu-Ile-Leu-Asp-Val- (-EILDV-) on fibronectin for melanoma cells. Bhatnagar and co-workers (Bhatnagar et al, 1999) have shown the potential to construct biomimetic environments by immobilizing a collagen-derived high-affinity cell-binding peptide P-15, in three-dimensional templates to promote attachment of human dermal fibroblasts to inorganic bovine bone mineral phase. In these studies, increased cell coupling to the collagen coated bovine bone mineral phase was associated with increased mineralisation.

This study demonstrated the ability to modulate surface structures to promote human osteoprogenitor adhesion, spreading, growth and differentiation on FN or PLL-GRGDS peptide coated PLA films and the possibility of human bone marrow cell adhesion and spreading on PLGA porous scaffolds. The use of peptides/proteins and 3-D structures to provide positional and environmental information, as in these studies, indicate the

potential for biomimetic structures coupled with appropriate osteoinductive factors in the development of environments for conductive and inductive bone formation.

CHAPTER 4

THE EFFECT OF AN ADMIX OF BMP'S ('RETENTATE') ON HUMAN OSTEOPROGENITOR ACTIVITY

4.1. Introduction

Biodegradable polymers such as PLA and PLGA have been used as structural supportive scaffolds for tissue regeneration. However, these biomaterials are not osteoinductive and are not capable of supporting cell attachment and growth. Thus, the combination of these biodegradable biomaterials with selected osteogenic growth factors may provide biomimetic scaffolds with an appropriate microenvironment to stimulate cell adhesion, proliferation, differentiation and bone matrix formation (Freed et al, 1994; Ishaug et al, 1997; Patel et al, 1998; Peter et al, 1998a; Quirk et al, 2000). Osteoinductive factors, many present within bone matrix, have the potential to stimulate bone growth. As stated in Chapter 1.3.2.1, BMP's are secreted signaling molecules, which are cell growth and differentiation factors involved in the development of new bone and cartilage tissues (Urist, 1965; Wozney and Rosen, 1998). Previous studies indicate the potential of BMP-2, BMP-4 and BMP-7 in the treatment of fracture repair, segmental bone defects and in the fixation of prosthetic implants (Yasko et al, 1992; Yamashita et al, 1996; Wozney and Rosen, 1998; Oreffo and Triffitt, 1999), while recent studies indicate BMP-3, or osteogenin, may also be a negative regulator of bone density (Daluiski et al, 2001). This osteoinductive capacity has led to extensive research in the present use of bone morphogenetic proteins as therapeutic agents for the creation of new bone for clinical applications in a variety of orthopaedic situations.

However, the ideal combination of bone morphogenetic proteins for bone formation remains unclear. Anderson and coworkers (1992) discovered that cell conditioned media from Saos-2 human osteosarcoma cells contains an admix of BMP's (specially BMP-1, -2, -3, -4, -5, -6, -7) (Table 4-1) and the non-collagenous matrix proteins including bone sialoprotein, osteonectin, osteopontin and osteocalcin. Cell conditioned

media from Saos-2 cells was shown to induce new endochondral bone formation when implanted *in vivo* in Nu/Nu mice (Hsu et al, 1999; Anderson et al, 2002). Therefore, the use of these cell extracts may offer a possible approach to targeting the appropriate factors for osteoinduction in bone tissue engineering.

Table 4-1 Relative abundance of BMP's in 'retentate' and Saos-2 cells

	Saos-2-conditioned media 'retentate'	Saos-2 cells
BMP-1	+++	++
BMP-2	+	+
BMP-3	+++	++
BMP-4	+++	+
BMP-5	+	++
BMP-6	+	++
BMP-7	+	+

+++: Maximal; ++: Moderate; +: Trace (Adapted from Anderson et al, 2002)

The aim of this study was to test the efficacy of osteoinductive extracts of Saos-2 cells adsorbed onto porous biodegradable scaffolds, generated from PLGA, on the proliferation, differentiation and induction of mineralisation in cultures of primary human osteoprogenitor cells.

4.2. Materials and Methods

For general materials and methods see Chapter 2.1 and Chapter 2.2. Saos-2 cell extract ('retentate') was a gift from Prof. Anderson (Kansas city, USA).

4.2.1. Cell culture

Bone marrow samples (12 samples: 5 male and 7 female, 63-80 years of age, mean age 69.8 years) were obtained from patients undergoing routine total hip replacement surgery. Primary cultures of bone marrow cells were established as detailed in Chapter 2.2.1.

4.2.2. Colony forming unit-fibroblastic culture

Primary human bone marrow cells were cultured in 6 well tissue culture plates (5×10^5 cell/well) in the presence and absence of Saos-2 'retentate' (0.5ng/ml – 500ng/ml) for 12 days in basal or 9 days in osteogenic media. Cells grown in the presence of dexamethasone/ascorbic acid-2-phosphate were stopped on day 9 to prevent merging of the colonies (Cannizzaro et al, 1998; Oreffo et al, 1999). After fixation and staining, total and alkaline phosphatase-positive colonies were counted by eye using an Anderman colony counter. Mean values for each group were derived from 3 samples (See Chapter 2.2.5.).

4.2.3. Soas-2 'retentate' production

Serum-free Saos-2 cell-conditioned medium was passed through 0.45-μm pore-size filters after precentrifugation at 1000 RPM for 20min to remove and discard large cell fragments. Microsome-sized cell fragments retained by 0.45-μm filters ("retentate") were extracted into 6M urea for 7 days at 4°C. The extract was dialyzed against H2O to remove urea, and lyophilized prior to storage at -20°C (Anderson et al, 2002).

4.3. Results

4.3.1. Effects of Saos-2 'retentate' on CFU-F formation

Addition of Saos-2 'retentate' to αMEM supplemented with osteogenic media significantly stimulated human bone marrow stromal cells growth and total colony formation by 40% compared to control cultures at concentrations of 'retentate' as low as 5ng/ml (Fig. 4-1).

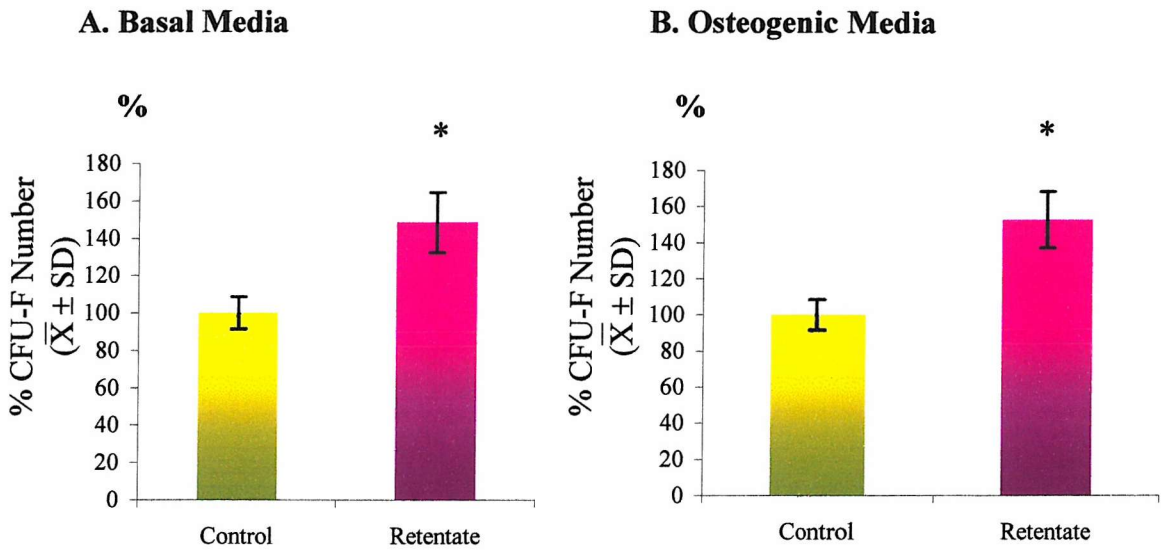


Figure 4-1 Effect of Saos-2 'retentate' on total CFU-F number. A) Cells cultured in the presence and absence of Saos-2 'retentate' (50ng/ml) in basal media for 12 days and, B) osteogenic media for 9 days. Each point represents mean ± SD, n=3.
* $P < 0.05$ compared to control.

4.3.2. Effects of Saos-2 'retentate' on alkaline phosphatase specific activity

Saos-2 'retentate' significantly stimulated alkaline phosphatase specific activity in basal (Fig.4-2A) or osteogenic media (Fig.4-2B) at a concentration as low as 5ng/ml. Alkaline phosphatase specific activity was increased by around 30% compared to control cultures. DNA content was not significantly (3%) compared to control cultures.

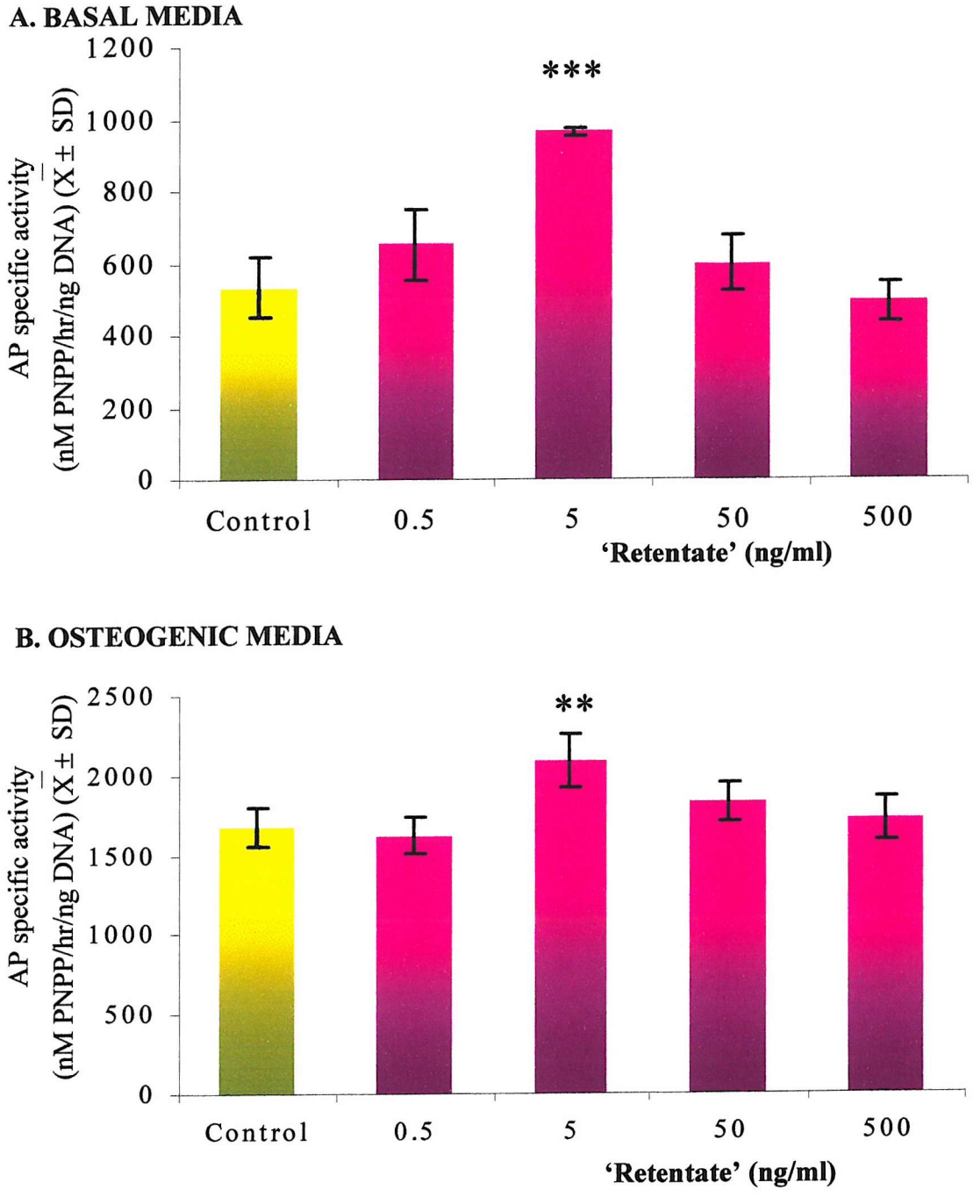


Figure 4-2 Effect of Saos-2 'retentate' on alkaline phosphatase specific activity. Cells cultured in the presence and absence of Saos-2 'retentate' in basal (A) and osteogenic media (B) for 21 days. Each point represents mean ± SD, n=3.
** $P \leq 0.01$; *** $P \leq 0.001$ compared to control.

4.3.3. Cell attachment and growth on three-dimensional polymer scaffolds

Human bone marrow cell adhesion and growth was observed on porous PLGA scaffolds adsorbed with 50ng/ml Saos-2 'retentate' in αMEM (Fig.4-3A-F). The growth of the cells was evidenced by intense fluorescence expression following CFMDA and EH-1 labeling. Fluorescence (Fig.4-3A) and confocal microscopy (Fig.4-3B) confirmed extensive growth of human bone marrow cells on 'retentate' adsorbed PLGA scaffolds followed by growth in osteogenic conditions for up to 6 weeks. Negligible cell adhesion and growth was observed on unmodified PLGA scaffolds (Fig. 4-3C). In *situ* (Fig. 4-3D) and scanning electron microscopy (Fig. 4-3E,F) confirmed the proliferation, differentiation and mineralisation of HBMCs on biomimetic PLGA scaffolds.

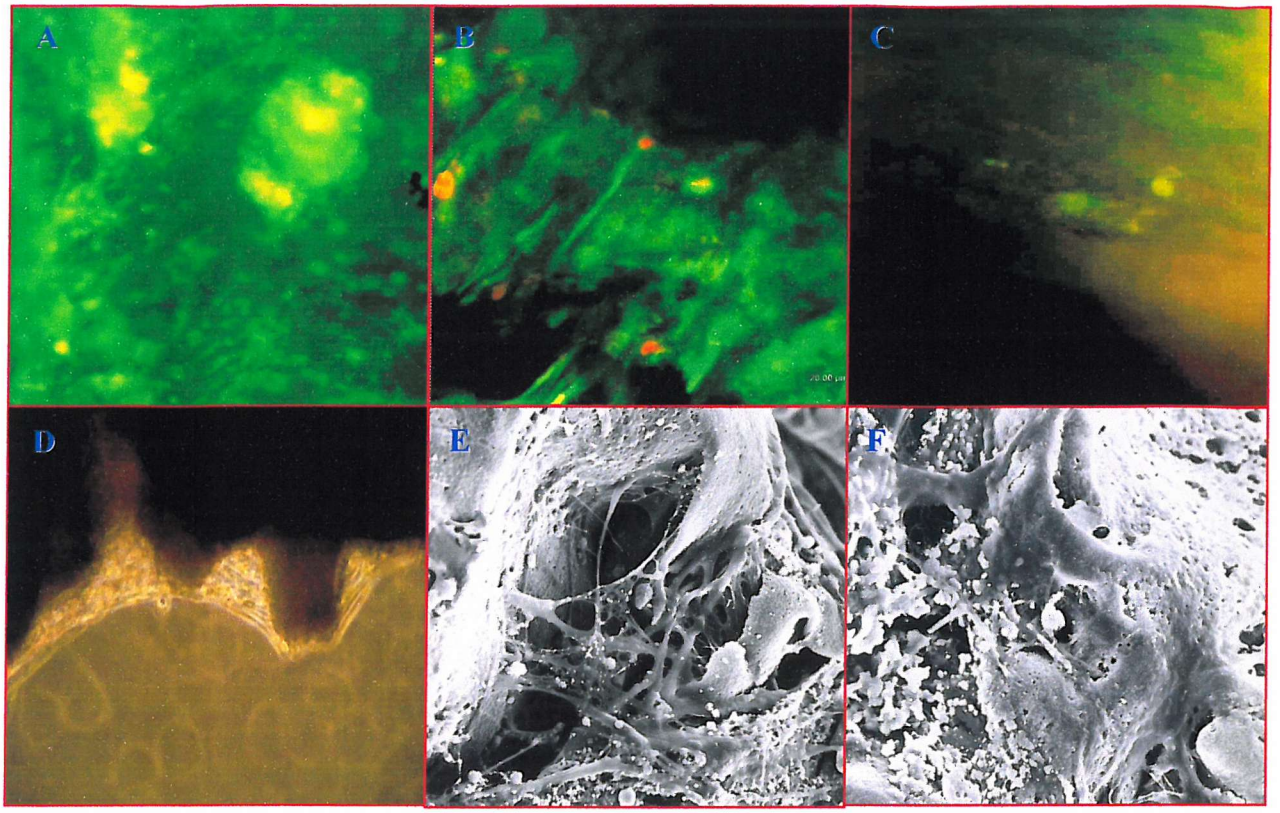


Figure 4-3 Human bone marrow cell attachment and growth on Saos-2 'retentate' adsorbed PLGA scaffold after 6 weeks of culture. Fluorescence photomicrographs (A,B) showed viable cells (green) and necrotic cells (red). Poor cell adhesion and spreading was observed on PLGA alone (C). Cell proliferation was observed on PLGA-'retentate' scaffold *in situ* (D) and by SEM (E,F). Original magnification: A,C) ×100, B) ×500, D) ×50, E,F) ×1000.

4.3.4. Human bone marrow cell differentiation on 'retentate'-adsorbed scaffolds

Following demonstration of the potential of Saos-2 'retentate' to stimulate differentiation of human osteoprogenitor cells, this study examined the potential of 'retentate' adsorbed on PLGA scaffolds to induce differentiation and mineralisation in 3-D scaffold templates. 10 fold excess of 'retentate' was used to allow for loss of 'retentate' on dilution in media. Cell growth and differentiation was observed on PLGA scaffolds adsorbed with 'retentate' as examined by staining with Alcian blue/Sirius red (Fig. 4-4A), immunocytochemistry for type I collagen expression (Fig. 4-4C,D). Increased cell proliferation and mineralisation compared to control cultures was observed on PLGA scaffolds coated with 'retentate' 50ng/ml as observed by von Kossa staining (Fig. 4-4B).

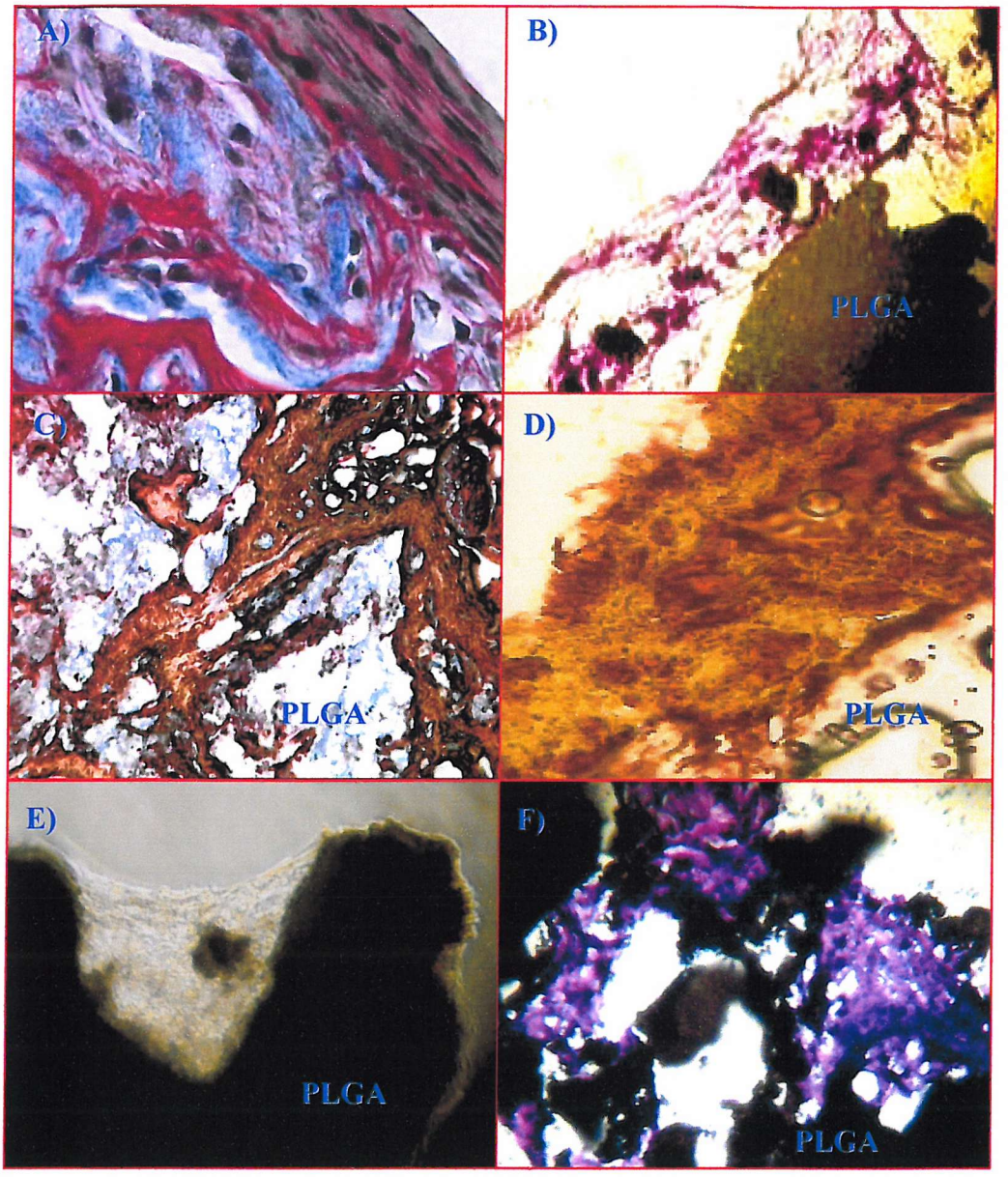


Figure 4-4 Cell growth and expression of bone markers by human bone marrow stromal cells on paraffin sections stained for Alcian blue and Sirius red (A), toluidine blue (B,F), type I collagen (C,D), mineralisation by von-Kossa (B) and *in situ* (E). Original magnification: A,D) ×250, B,C,F) ×100, E) ×50. * PLGA: poly(lactic-c-÷glycolic acid) scaffold.

4.4. Discussion

The present studies demonstrate that 'retentate' of Saos-2 cells promoted cell adhesion, growth and differentiation of human osteoblasts and osteoblast precursors on PLGA scaffolds resulting in mineralised structures *in vitro*. Expression of the markers alkaline phosphatase, type I collagen and subsequent mineralisation, after 6 weeks, confirmed differentiation of the osteoprogenitor cells along the osteogenic lineage.

The capacity of bone for growth, regeneration and remodeling has been attributed to a variety of signals including members of the TGF-β superfamily, pivotal in the recruitment and differentiation of bone progenitors. The BMP family of proteins are important osteoinductive factors in the recruitment, commitment and differentiation of bone progenitors (Urist, 1965; Wozney and Rosen, 1998). However, despite the availability of recombinant BMP's, it remains unclear which BMP, or which combination of BMP's, will be most efficacious and cost-effective for bone induction and regeneration in clinical practice. Native BMP's are 100-1000 fold more effective in inducing bone than individual recombinant BMP's (De Groot, 1998). In the current study, an admix of BMP's (specifically BMP 1-7) produced by Saos-2 cells and termed 'retentate' has been used. 'Retentate' induced colony formation, increased alkaline phosphatase specific activity and differentiation by 30-40% in both basal and osteogenic conditions. The concentrations of 'retentate' required to induce differentiation and mineralisation were extremely low, approximately 100-fold lower than concentrations of rhBMP's required to achieve similar effects. It remains unclear, at this stage, which of the BMP's in this admix are optimal for bone differentiation and induction although, by comparing the BMP expression profile of Saos-2 cells with that of the non-osteoinductive U2-OS cells. Anderson et al (2002) have shown that BMP-1, 3, 4 and bone sialoprotein appear to be the most important for the osteoinductive action

of Saos-2 cells. The same may apply to current study, but it cannot be discounted that there remain other as yet unidentified BMP's or other osteogenic factors and cofactors within the 'retentate' complex for osteoprogenitor differentiation and bone formation.

In conclusion, osteoinductive factors isolated from Saos-2 cells have the ability to promote adhesion, expansion and differentiation of human osteoprogenitor cells resulting in bone matrix deposition on/in porous biodegradable scaffold. These results indicate the capacity to generate osteoinductive surfaces within a porous template for de novo bone formation.

CHAPTER 5

PLEIOTROPHIN STIMULATES BONE AND CARTILAGE FORMATION *IN VITRO* AND *IN VIVO*

5.1. Introduction

As described in Chapter 1.3.2, a variety of osteoinductive factors, many present within bone matrix, have the potential to stimulate bone growth. A 136 amino acid bone growth factor rich in lysine and cysteine residues, pleiotrophin, also named OSF-1 or HB-GAM, has been suggested to play a role in bone formation (Imai et al, 1998). PTN is highly conserved across species with more than 90% homology between chick, rat, bovine and human sequences (Merenmies and Rauvala, 1990; Hampton et al, 1992; Deuel et al, 2002). PTN shares 50% homology with the mouse midkine (MK) protein and MK-type chicken protein, retinoic acid induced heparin binding protein (RHIB) (Zhang and Deuel, 1999). Expression postnatally of PTN is predominantly in bone and brain, from where it was first isolated. Transgenic mice over-expressing the human PTN gene have been shown to achieve a higher peak bone mass, which compensated for bone loss due to estrogen-deficiency (Masuda et al, 1997) and to contain 10% higher bone mineral content (Tare et al, 2001). PTN is highly expressed in embryonic and early post-natal fibre pathways of the nervous system (Rauvala et al, 1994). In developing and regenerating bone, PTN was identified as a matrix-bound chemotactic signaling molecule for migration of osteoblasts/osteoblast precursors (Imai et al, 1998). Zhou and coworkers (1992) have shown that PTN can enhance the attachment and subsequent differentiation of MC3T3-E1 cells. Tare et al (2001) and Masuda et al (1997) have shown that PTN over-expressing transgenic mice had an increased bone mineral content and, furthermore, that the metaphysis function of these mice remained active for longer.

The aim of this study was to investigate the ability of the osteotropic factor, PTN, to modulate human bone marrow stromal cell adhesion, chemotaxis, proliferation, differentiation and CFU-F formation. Furthermore, scaffolds adsorbed with PTN to

modulate bone formation were examined *in vivo* using the subcutaneous and the diffusion chamber assays.

5.2. Methods and Materials

For general materials and methods see Chapter 2.1 and Chapter 2.2.

5.2.1. Cell culture

Bone marrow samples (17 patients: 8 females and 9 males, 50-83 years of age, with a mean age of 66.7 years) were obtained from patients undergoing routine total hip replacement surgery. Primary cultures of bone marrow cells were established as previously described (See Chapter 2.2.1).

5.2.2. Colony forming unit-fibroblastic (CFU-F) culture

Primary human bone marrow cells were cultured in 6 well tissue culture plates (5×10^5 cell/well) in the presence and absence of PTN (5-50pg/ml) for 12 days in basal or 9 days in osteogenic media. After fixation and staining, total and alkaline phosphatase-positive colonies were counted by eye using an Anderman colony counter. Mean values for each group were derived from 3 samples (See Chapter 2.2.5). For time course studies, 10pg/ml PTN was added to cultures either at day 0-6 and day 6-9 (osteogenic media) or at day 0-7 and day 7-12 (basal media).

5.2.3. Chemotaxis studies

The chemotactic potential of PTN was examined on patterned surfaces generated using EM grids (Mesh: 400μm; Bar: 80μm from Agar Scientific) on tissue culture plastic coated with and without PTN (50μg/ml) and irradiated with UV light (254nm) to denature the exposed PTN as previously described (Imai et al, 1998). After removal of the grids, the chambers were washed thoroughly with sterile distilled water. Human bone marrow cells (passage 1) were plated at 1.25×10^5 cell/cm^2 and cultured in serum

free medium for up to 24 hours. Chamber slides coated with αMEM supplemented with 20% FCS or 10mg/ml BSA was used as control substrates.

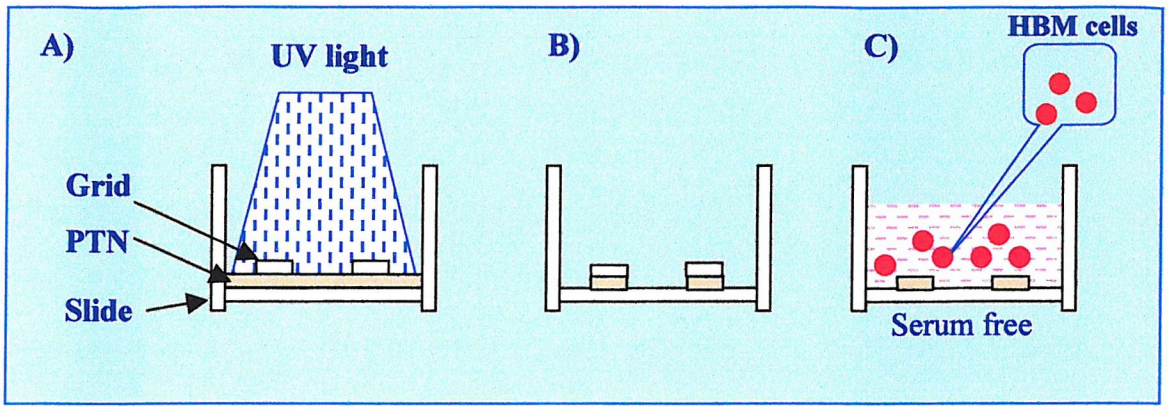


Figure 5-1 Illustration of the chemotactic assay: A) and B) denaturation of exposed PTN, C) examination of human bone marrow stromal cell chemotaxis.

5.3. Results

5.3.1. Effects of PTN on human osteoprogenitor chemotaxis *in vitro*

The chemotactic ability of PTN was examined on patterned surfaces generated using EM grids (Fig. 5-2D) on tissue culture plastic coated with PTN (50μg/ml) and irradiated with UV light (254nm) (Fig. 5-2). Human bone marrow stromal cells, which were randomly distributed after 30 minutes (Fig. 5-2A), were clearly recruited to areas of intact PTN after 24 hours (Fig. 5-2B-D) of culture. No migration of human bone marrow cells was observed on cell culture plastic in the absence of PTN.

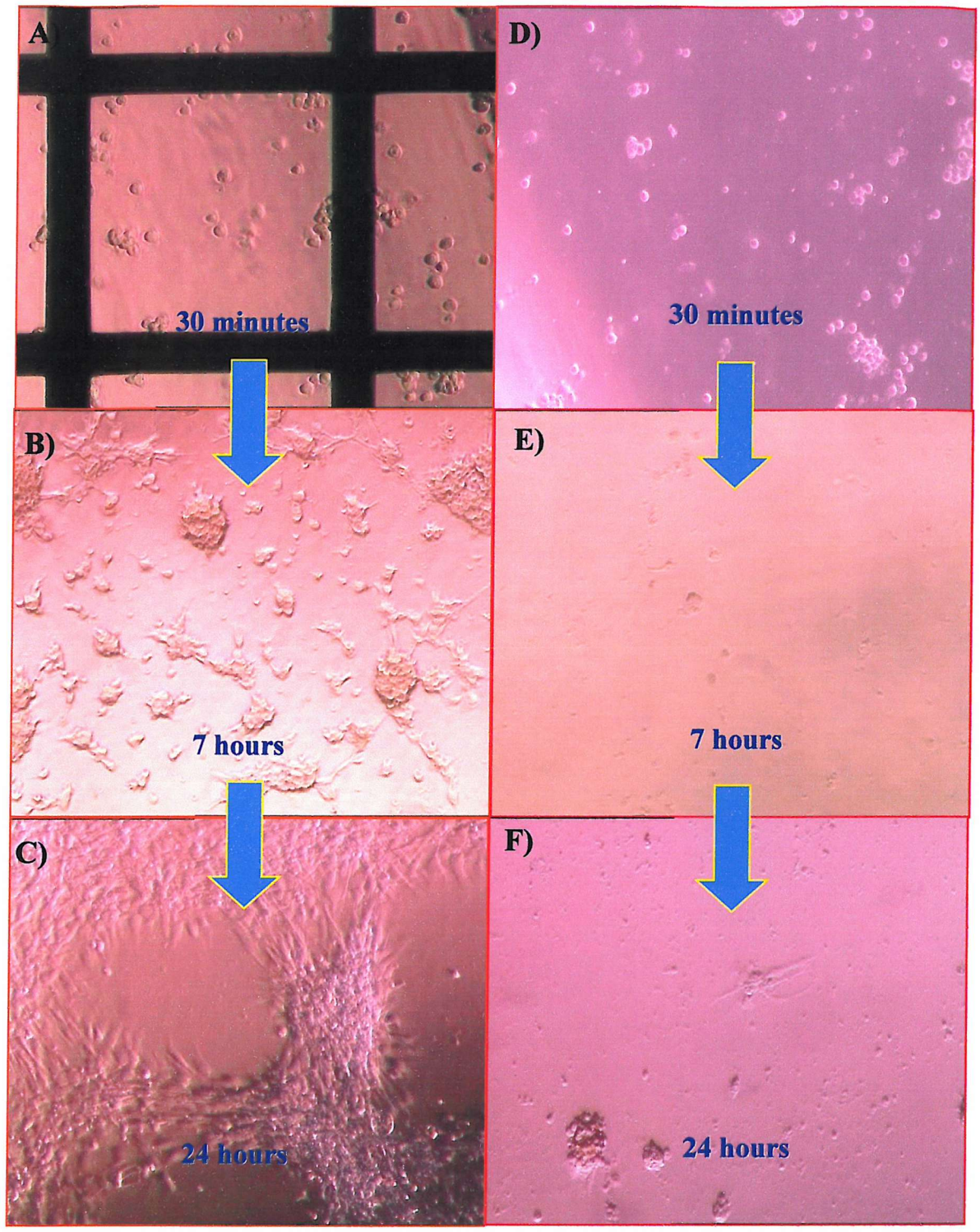


Figure 5-2 Effect of PTN on human osteoprogenitor migration, growth and differentiation *in vitro*: Attachment of human bone marrow cells on PTN patterned culture dishes (A-C). (A) 30 minutes after initial seeding, cells migration to areas of intact PTN after 7 hours (C) and 24 hours (D). No migration was observed in BSA negative control (D-F). Original magnification: ×100.

5.3.2. Effects of PTN on CFU-F formation

In these studies, PTN (10pg/ml) addition resulted in a significant stimulation of total CFU-F colony formation as well as alkaline phosphatase positive colony formation (Fig. 5-3) compared with control cultures. In osteogenic media, colony number and alkaline phosphatase-positive CFU-F colonies (Fig. 5-3B) were significantly stimulated above levels observed in basal culture. Addition of PTN in basal conditions enhanced CFU-F number and alkaline phosphatase-positive CFU-F colony number (Fig. 5-3A). In osteogenic media supplemented with PTN, a stimulation of total CFU-F colony formation by approximately 30% and alkaline phosphatase positive colony formation by approximately 80% was observed at 10pg/ml (Fig. 5-3B).

The effects of PTN on modulation of total colony formation in basal media and osteogenic media were dependent on time of addition, with increased colony formation during the late phase of culture (Fig. 5-4A and 5-4B). Thus in basal cultures, addition of PTN from day 7-12 significantly enhanced total colony formation as well as alkaline phosphatase positive CFU-F formation (Fig. 5-4A) and similarly, addition of PTN in osteogenic conditions (between day 6-9) significantly enhanced total colony formation (Fig. 5-4B) and alkaline phosphatase CFU-F formation, suggesting an effect on late osteoprogenitors.

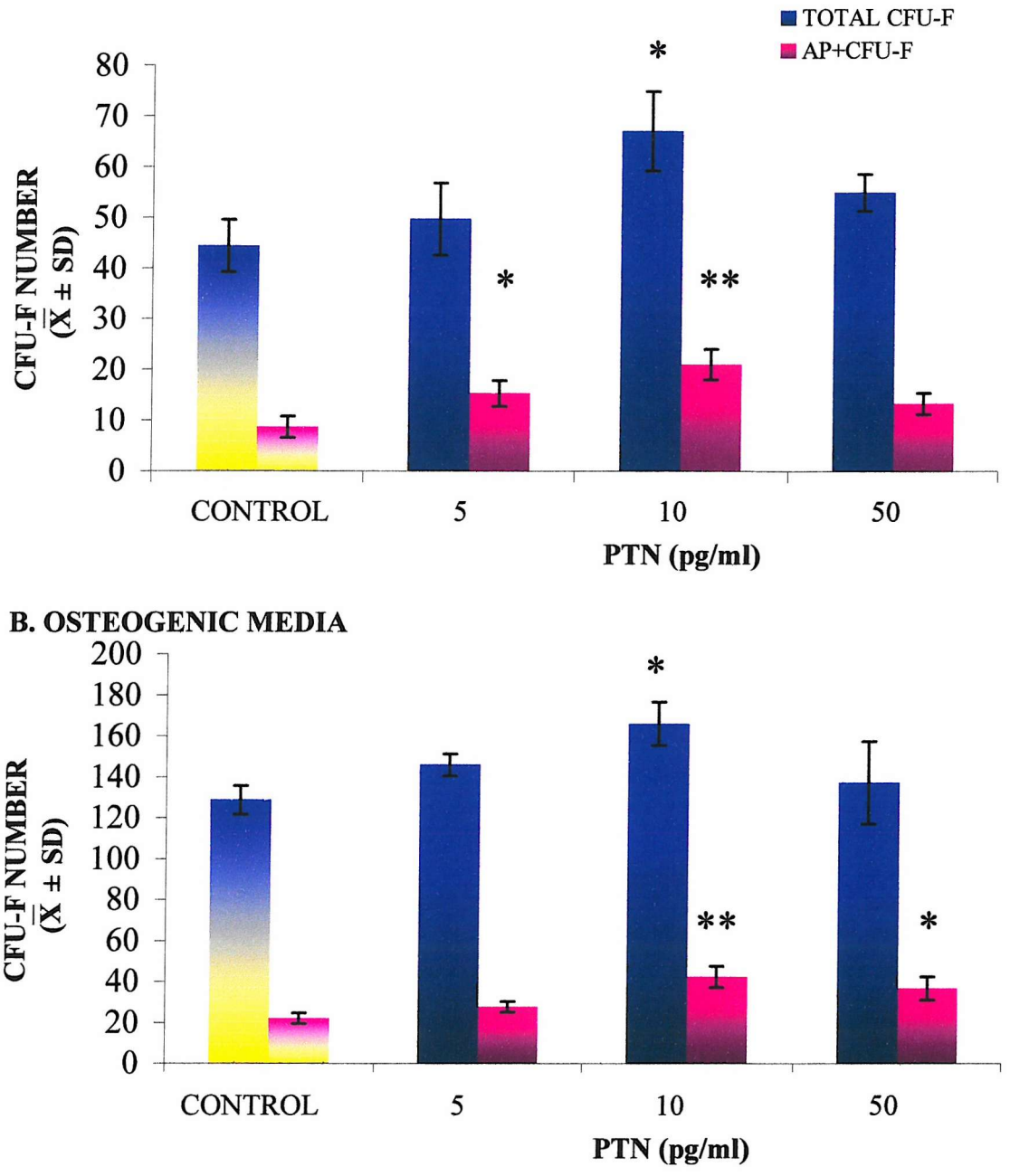


Figure 5-3 Effect of PTN on total and alkaline phosphatase-positive CFU-F number: Cells were cultured in the absence (αMEM/10%FCS) and presence of PTN for A) 12 days in basal conditions or, B) 9 days in osteogenic conditions as described in the materials and methods. Each point represents mean ± SD, n=3. *P≤0.05, **P≤0.01 compared to control.

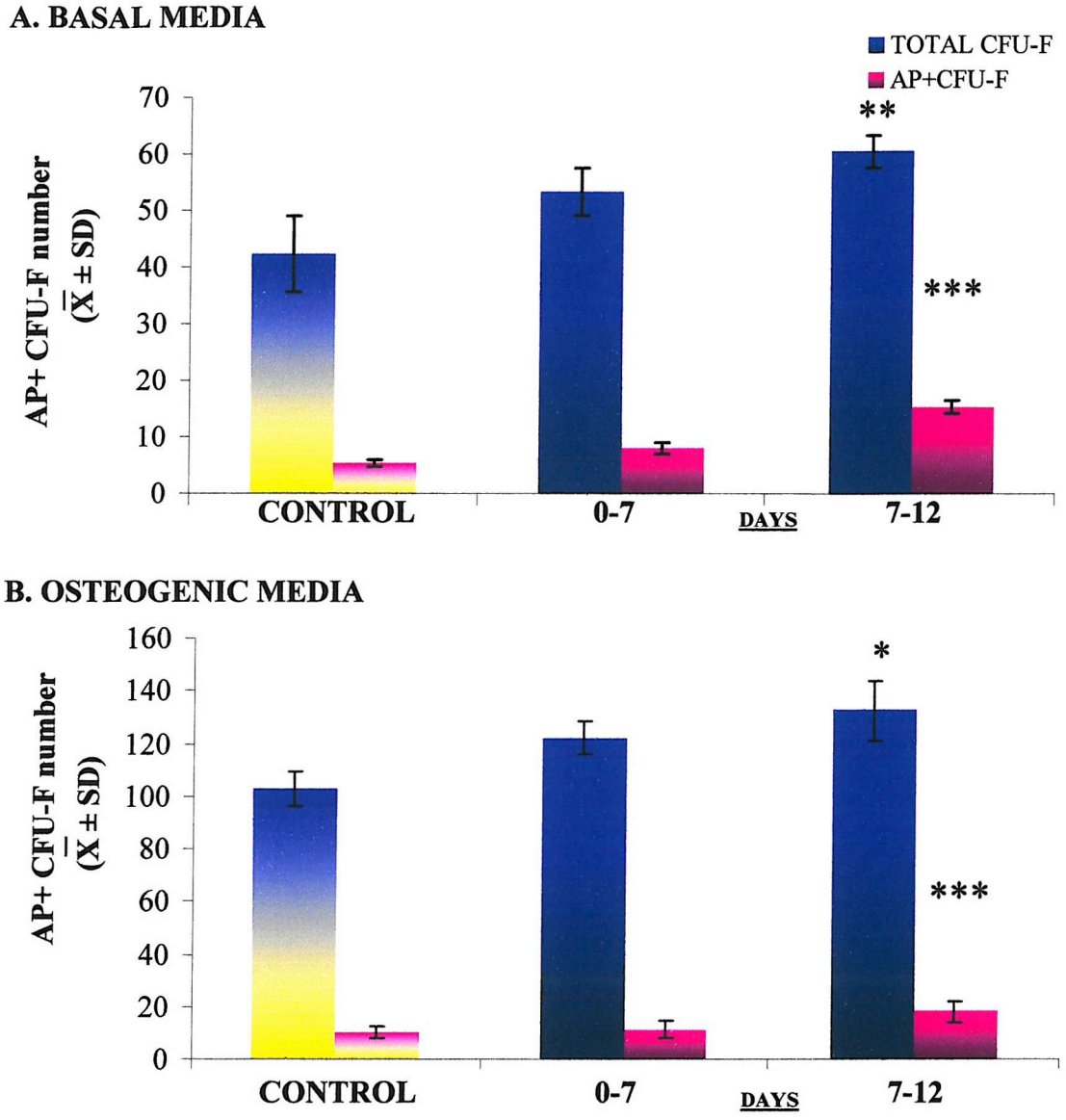
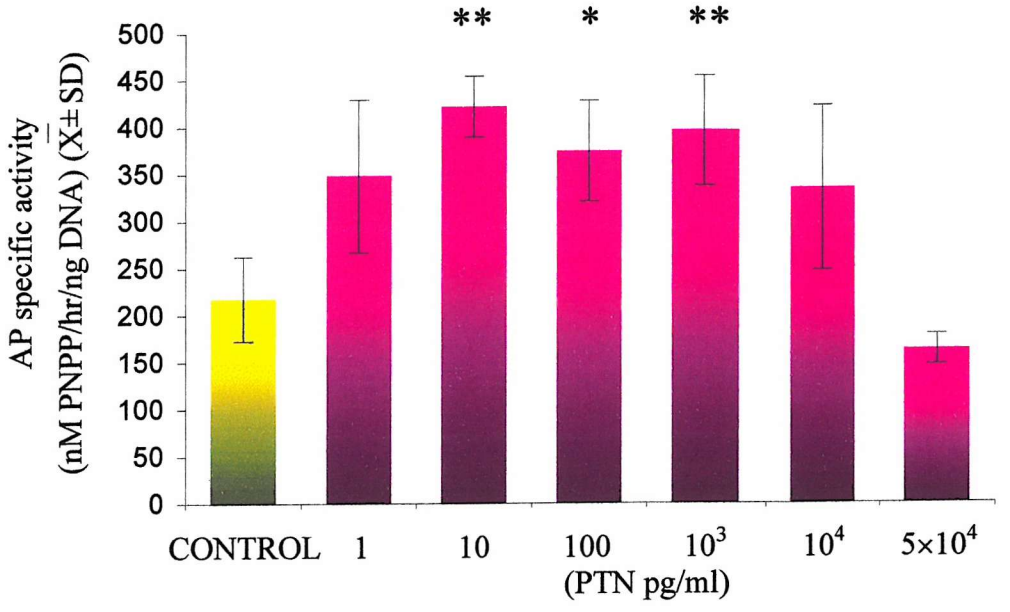


Figure 5-4 Variation in total and alkaline phosphatase-positive CFU-F number in human bone marrow cells following exposure to PTN. Cells cultured in the presence and absence of PTN in A) basal media for either 0-7 or 7-12 days or, B) in osteogenic media for 0-6 and 6-9 days as described in Materials and Methods. Each point represents mean ± SD, n=3. *P≤0.05, **P≤0.01, ***P≤0.001 compared to control.

5.3.3. Effects of PTN on alkaline phosphatase activity

PTN produced a dose-dependent stimulation of alkaline phosphatase specific activity with a significant increase in activity compared to cultures in basal media or in osteogenic media at concentrations as low as 10pg/ml (Fig 5-5A). In osteogenic media, the basal level of enzyme activity was increased approximately 10-fold compared with that in basal media and addition of PTN resulted in further enhancement by 55% of alkaline phosphatase activity (Fig 5-5B). As observed in the colony formation studies, high concentrations of PTN (>1ng/ml) had no effect on alkaline phosphatase activity in basal or osteogenic conditions. DNA content was not significantly (5%) compared to control cultures.

A. BASAL MEDIA



B. OSTEOGENIC MEDIA

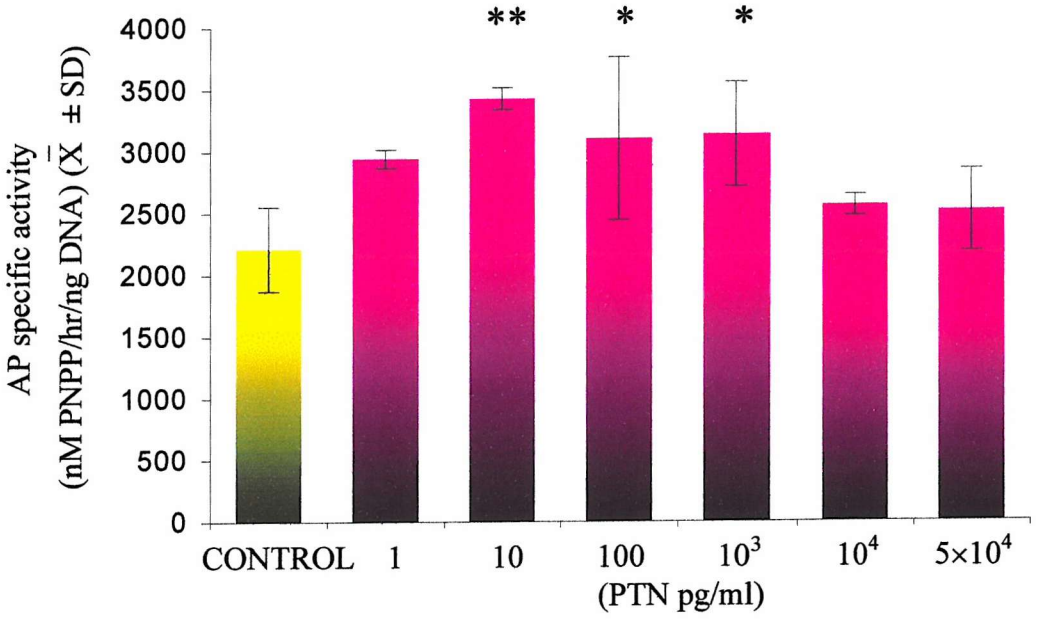


Figure 5-5 Effect of PTN on alkaline phosphatase specific activity in human bone marrow stromal cells cultured in A) basal media or B) osteogenic media. Cells plated at 5×10^5 cell/well in 6 well plates and incubated for 21 days with the indicated groups (mean ± SD, n=3). *P≤0.05, **P≤0.01, ***P≤0.001 compared to control.

5.3.4. Cell growth on PTN adsorbed scaffolds *in vitro*

Following demonstration of the potential of PTN to stimulate chemotaxis, adhesion and differentiation of human osteoprogenitor cells, the potential of PTN adsorbed on PLGA scaffolds to induce adhesion, differentiation and mineralisation in 3-D scaffold templates was examined.

Cell adhesion, spreading and proliferation on PLGA scaffold

Cell viability after 4-6 weeks of static culture was examined using Cell Tracker^{TM} green and EH-1 expression to detect live and necrotic cells respectively (Fig. 5-6A,B). Cell adhesion, spreading and growth was observed on PLGA scaffolds adsorbed with PTN as examined by fluorescence and confocal microscopy (Fig. 5-6A,B) as well as SEM (Fig. 5-6C). Increased cell attachment and proliferation was observed on PTN (50ng/ml) adsorbed scaffolds with few necrotic cells as evidenced by the scarcity of EH-1 positive cells (Fig. 5-6A-C). In contrast, poor cell attachment, migration, growth were observed on PLGA scaffolds alone (Fig. 5-6D).

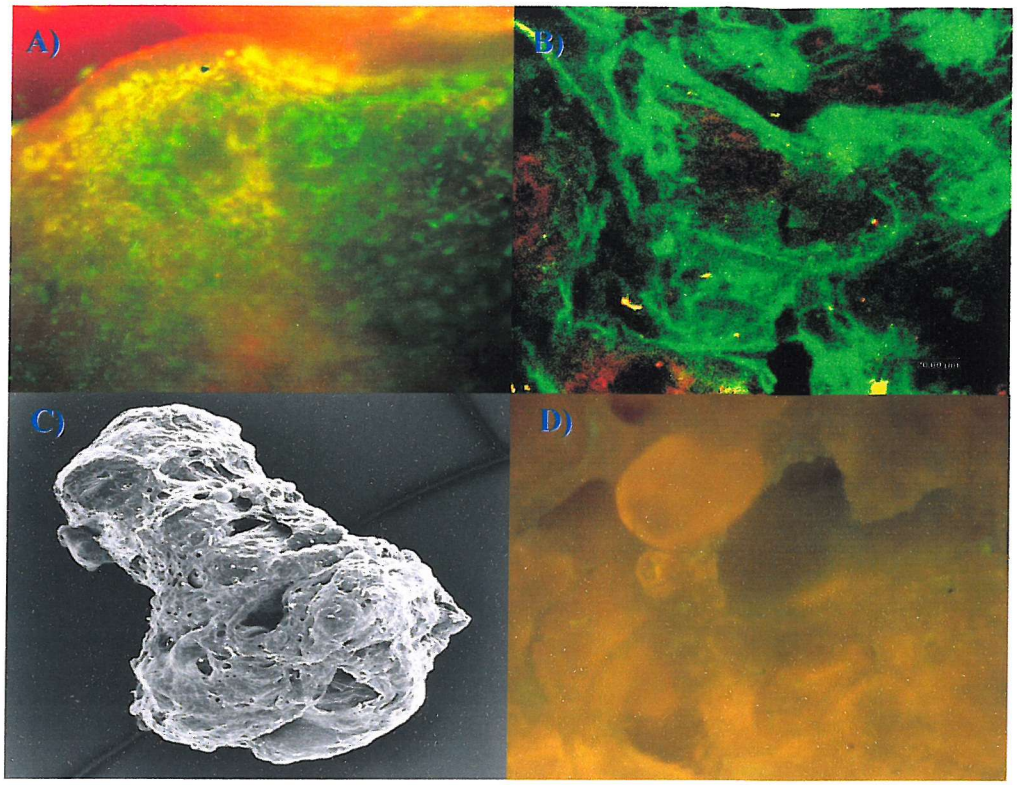


Figure 5-6 Examination after 6 weeks of human bone marrow cell viability and growth using Cell Tracker Green and ethidium homodimer-1 (A, B, D), confocal (B) and scanning electron microscopy (C) on PLGA scaffolds. Viable cells detected using Cell Tracker green and necrotic cells (red) using EH-1. Note the extensive cell adhesion and spreading observed following culture on PTN adsorbed PLGA scaffolds. Negligible cell adhesion and cell growth was observed on PLGA scaffold alone (D). Original magnification: A) ×50, B) ×500, C) ×50, D) ×100

Effect of PTN on the differentiation of HBMCs and mineralisation on PLGA scaffold

PTN stimulated differentiation and bone matrix synthesis by human bone marrow cells on porous PLGA scaffolds cultured in static culture over 4-6 weeks, as visualized by extensive expression of alkaline phosphatase, PTN, *cbfa-1*, osteocalcin and type I collagen in the matrix between the scaffold material (Fig 5-7 and Fig. 5-8). Further evidence that PTN stimulated bone matrix synthesis was provided following visualisation by Sirius red staining and Alcian blue for bone matrix and cartilaginous proteins respectively (Fig 5-8A and 5-8B). Collagen deposition and new matrix synthesis was confirmed by birefringence microscopy (Fig 5-8C). Von Kossa staining, after culture in osteogenic conditions, identified areas of mineral deposits indicative of expression of the mature osteogenic phenotype (Fig 5-8C and Fig 5-8D). Negligible mineralisation was observed on PLGA scaffolds alone.

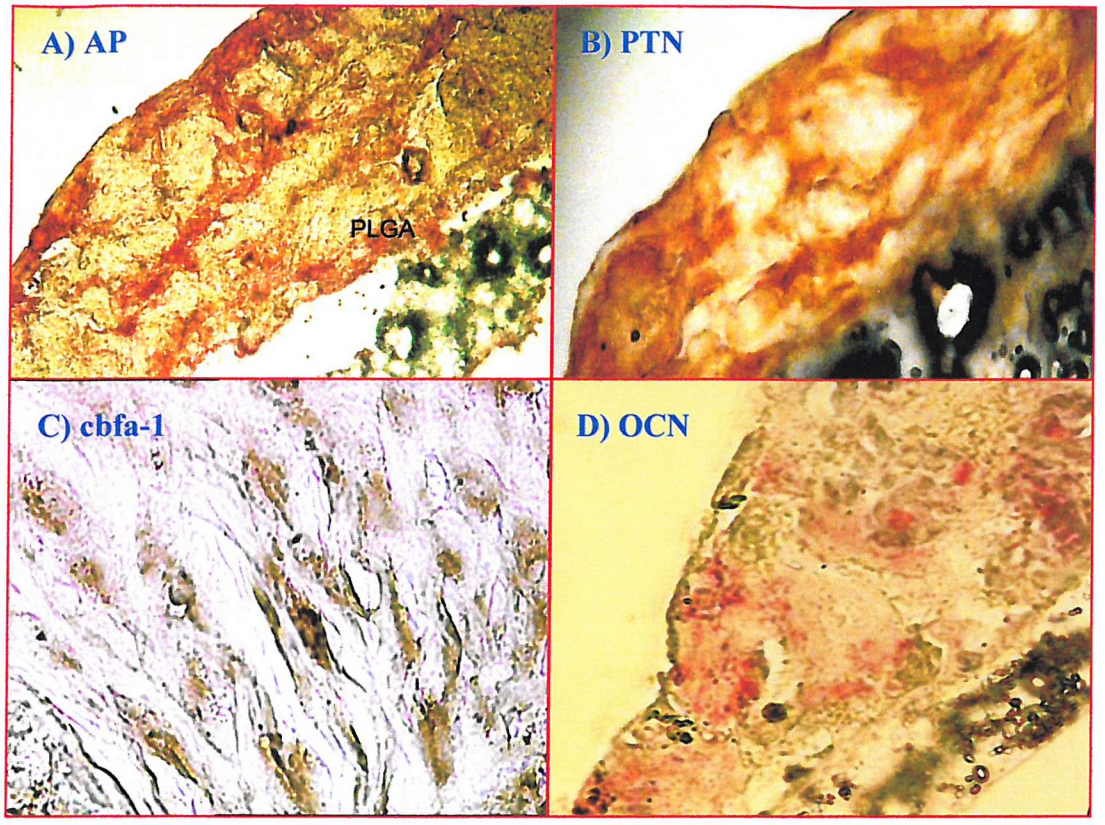


Figure 5-7 Expression of bone markers following 6 weeks of culture in osteogenic conditions by human bone marrow cells on PLGA scaffolds adsorbed with PTN alone as detected by histochemistry and immunocytochemistry on paraffin sections (A-D). A) Alkaline phosphatase activity detected by histochemistry, B) PTN, C) *cbfa-1* and, D) osteocalcin detected by immunocytochemistry. Original magnification: A,B,D) ×100, C) ×250. * PLGA: poly(lactic-c-÷glycolic acid) scaffold.

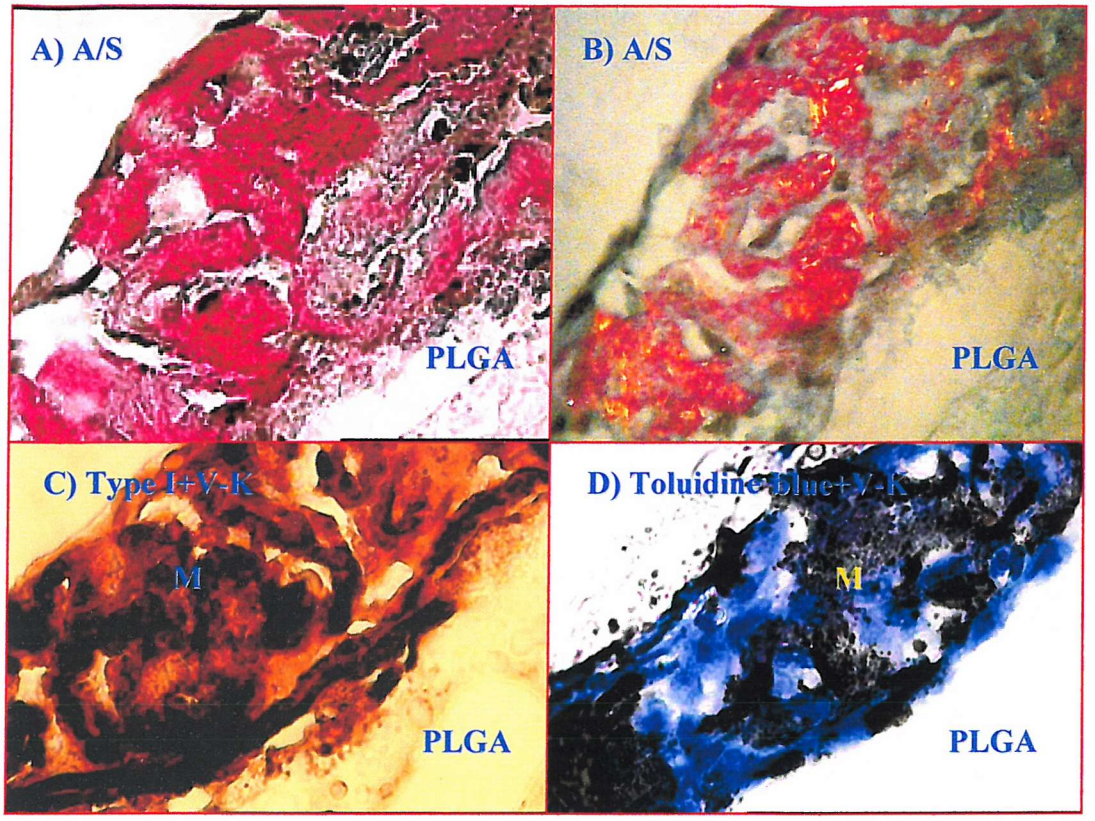


Figure 5-8 Expression of bone markers following 6 weeks of culture by human bone marrow cells on PLGA scaffolds adsorbed with PTN alone, prior to culture in osteogenic conditions as detected by histochemistry and immunocytochemistry on paraffin sections (A-D). Mineralisation and bone matrix synthesis was examined using Sirius red/birefringence (A,B) as well as type I collagen (C) and von-Kossa (D) staining. Original magnification: ×100. * PLGA: poly(lactic-c-÷glycolic acid) scaffold, M: mineral.

5.3.5. *In vivo* studies

Subcutaneous implant model

Primary human bone marrow cells were impregnated onto PLGA porous scaffolds adsorbed with or without recombinant human PTN (50ng/ml) as described in the materials and methods. Cell/growth factor constructs were placed subcutaneously into 6 athymic mice. After 4 to 6 weeks, subcutaneous implants were removed and analyzed for new bone formation (Fig 5-9). Human bone marrow cell differentiation and bone matrix synthesis on/within PTN absorbed PLGA scaffolds showed morphological evidence of new bone matrix synthesis as detected by Sirius red staining (Fig 5-9A,D), expression of type I collagen (Fig 5-9C) and expression of osteocalcin (Fig 5-9D). Evidence of organized new woven bone was confirmed by birefringence of collagen using polarized microscopy (Fig 5-9B). As observed in the *in vitro* studies, negligible cell attachment was observed on PLGA scaffolds alone in the absence of PTN and no evidence of cell differentiation, mineralisation and bone formation but fibrous tissue was observed in these constructs after subcutaneous implantation (Fig. 5-9E).

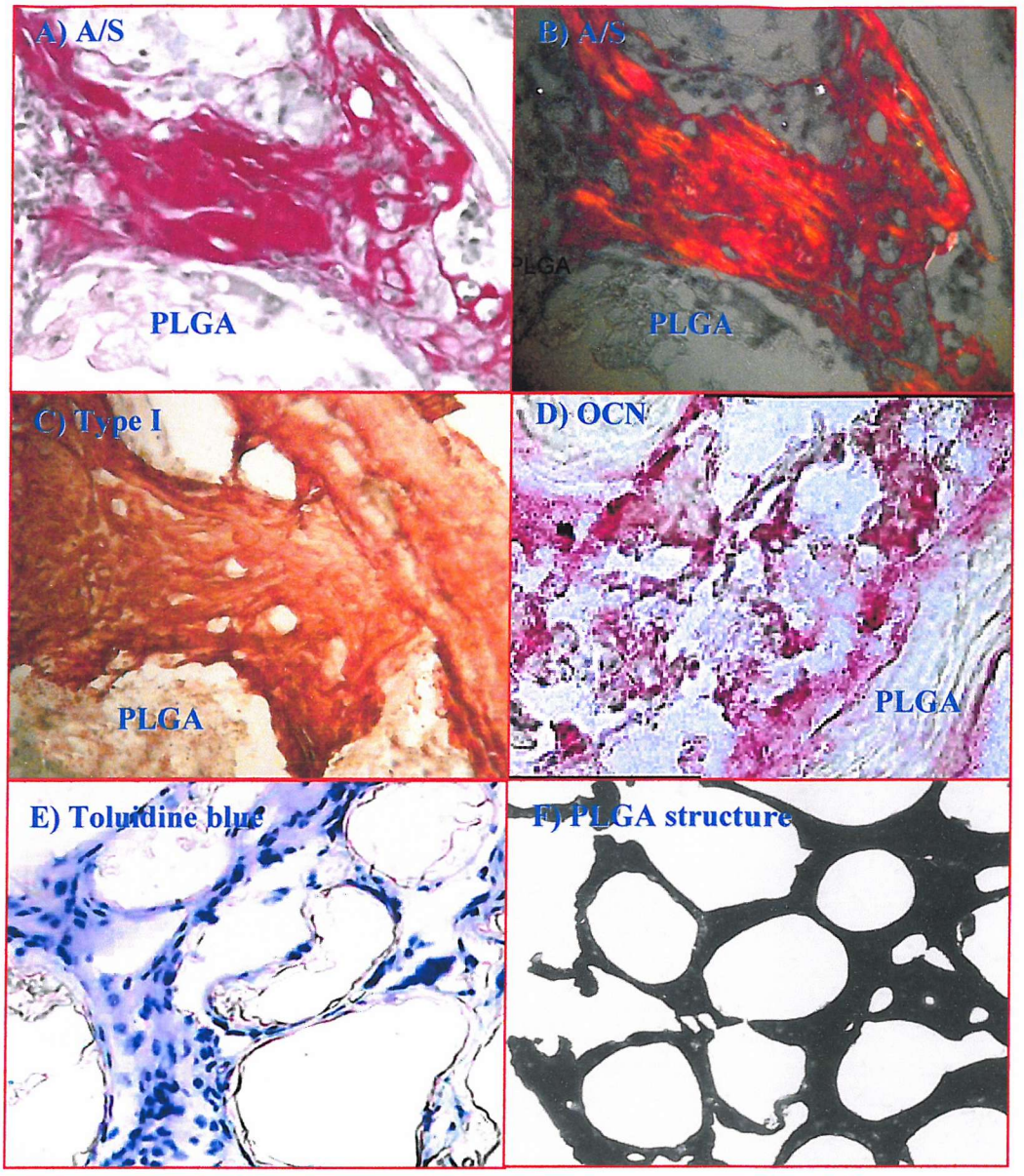


Figure 5-9 Effect of PTN on human bone marrow cell growth, differentiation and bone matrix formation *in vivo* using the subcutaneous implant model. The subcutaneous implant model was run for 6 weeks as detailed in materials and methods. A) New woven bone/ formation by human bone marrow cells within PLGA scaffolds adsorbed with PTN as detected by Sirius red and birefringence of collagen (A,B). Expression of bone markers, type I collagen (C) and osteocalcin (D), within PLGA/PTN constructs as detected by immunocytochemistry on paraffin sections. No bone matrix but fibrous tissue was observed in PLGA alone (E). F) PLGA scaffold in situ. Original magnification: A-D) ×250; E,F) ×100. * PLGA: poly(lactic-c-÷glycolic acid) scaffold.

Diffusion chamber model

Scaffold/cell/PTN (50ng/ml) constructs were sealed in diffusion chambers and placed into 9 athymic mice. PTN adsorbed constructs showed morphologic evidence of new bone matrix and cartilage formation by human bone marrow cells within diffusion chambers as evidenced by X-ray analysis (Fig 5-10A), staining for bone and cartilage matrix using Alcian blue and Sirius red (Fig 5-11A-D and Fig. 5-12A-C) after 10 weeks. Cartilage formation was observed within PLGA scaffolds confirming penetration of human osteoprogenitors through the scaffold constructs (Fig. 5-11A-D). In addition, formation of organized new woven bone was confirmed by birefringence of collagen (Fig. 5-11B and Fig. 5-12B, C) and extensive alkaline phosphatase expression and mineralisation (Fig. 5-10B and Fig. 5-12D) in 4 of 9 chambers. No bone formation was observed in human bone marrow/scaffold constructs alone (Fig. 5-10C,D). Metachromatic staining was observed using toluidine blue as well as expression of type I collagen (Fig. 5-12E,F). Quantitation of mineral formation from x-ray analysis showed 4.89 ± 3.84% (range 1.32-8.95%) of the diffusion chambers were filled with mineral whereas no mineral was observed in human bone marrow/ scaffolds constructs alone.

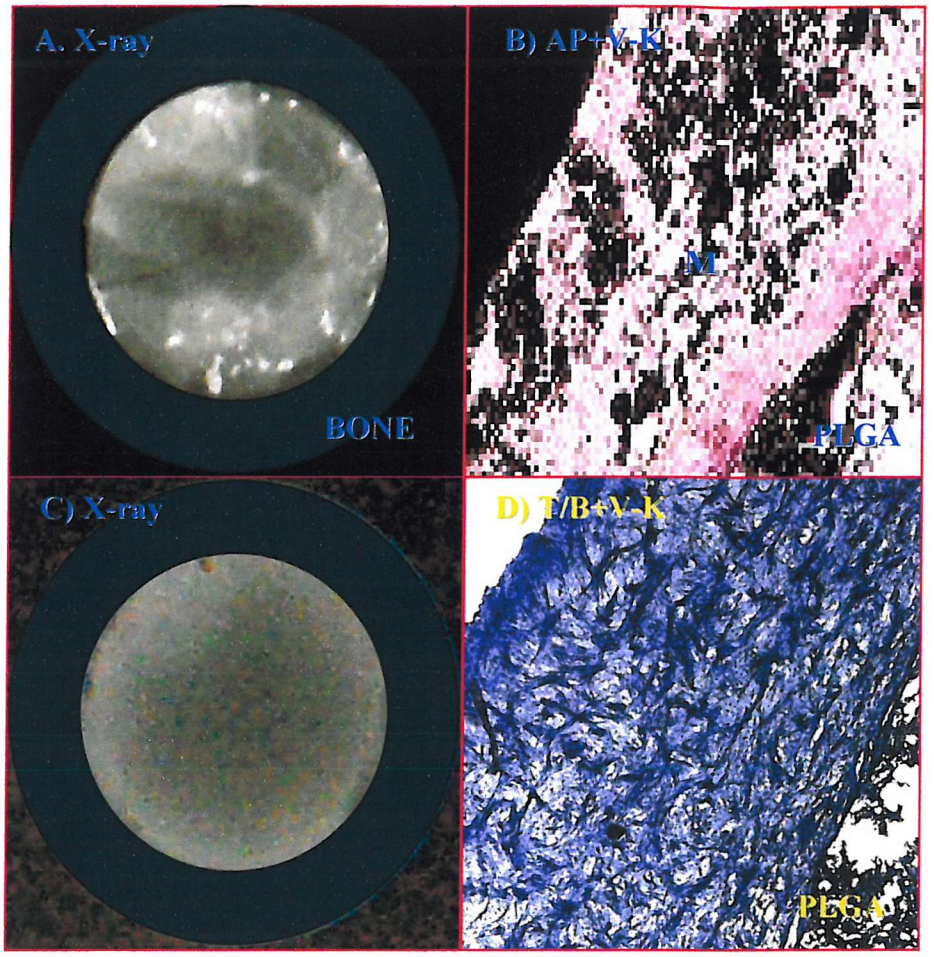


Figure 5-10 Assessment of X-ray (A,C) and paraffin sections (B,D): Bone formation by human bone marrow cells on PTN adsorbed PLGA scaffolds within diffusion chambers after 10 weeks (A,B). No bone matrix formation was observed in PLGA alone (C,D). Original magnification: A,C) ×5, B,D) ×100. * PLGA: poly(lactic-c-÷glycolic acid) scaffold, M: mineral.

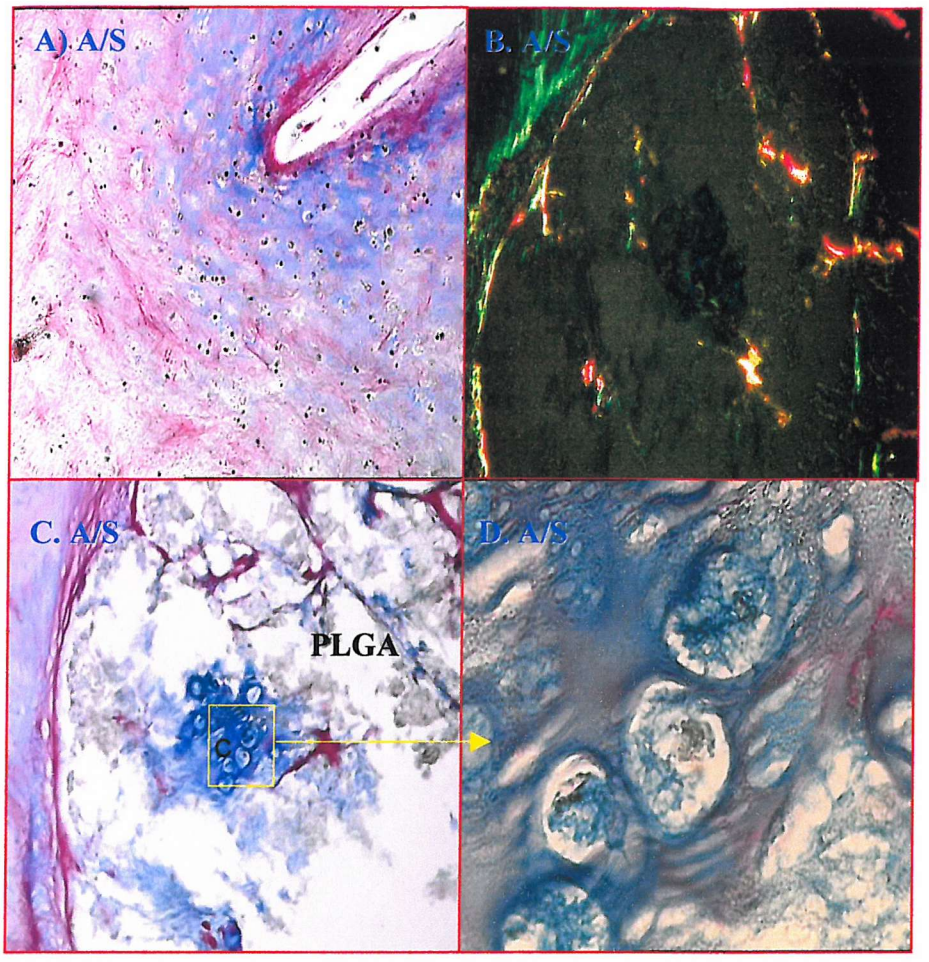


Figure 5-11 Cartilage and bone formation by human bone marrow cells on PTN adsorbed PLGA scaffolds within diffusion chambers after 10 weeks as analyzed on paraffin sections. A) Alcian blue and Sirius red staining showed new bone and cartilage matrix formation within chambers;
B) Demonstration of collagen birefringence within the PLGA constructs by polarized microscopy from a parallel section. C,D) new cartilage tissue within PLGA/PTN scaffolds as detected by Alcian blue staining. Original magnification: A-C) ×100, D) ×250. * PLGA: poly(lactic-c-÷glycolic acid) scaffold, C: cartilage.

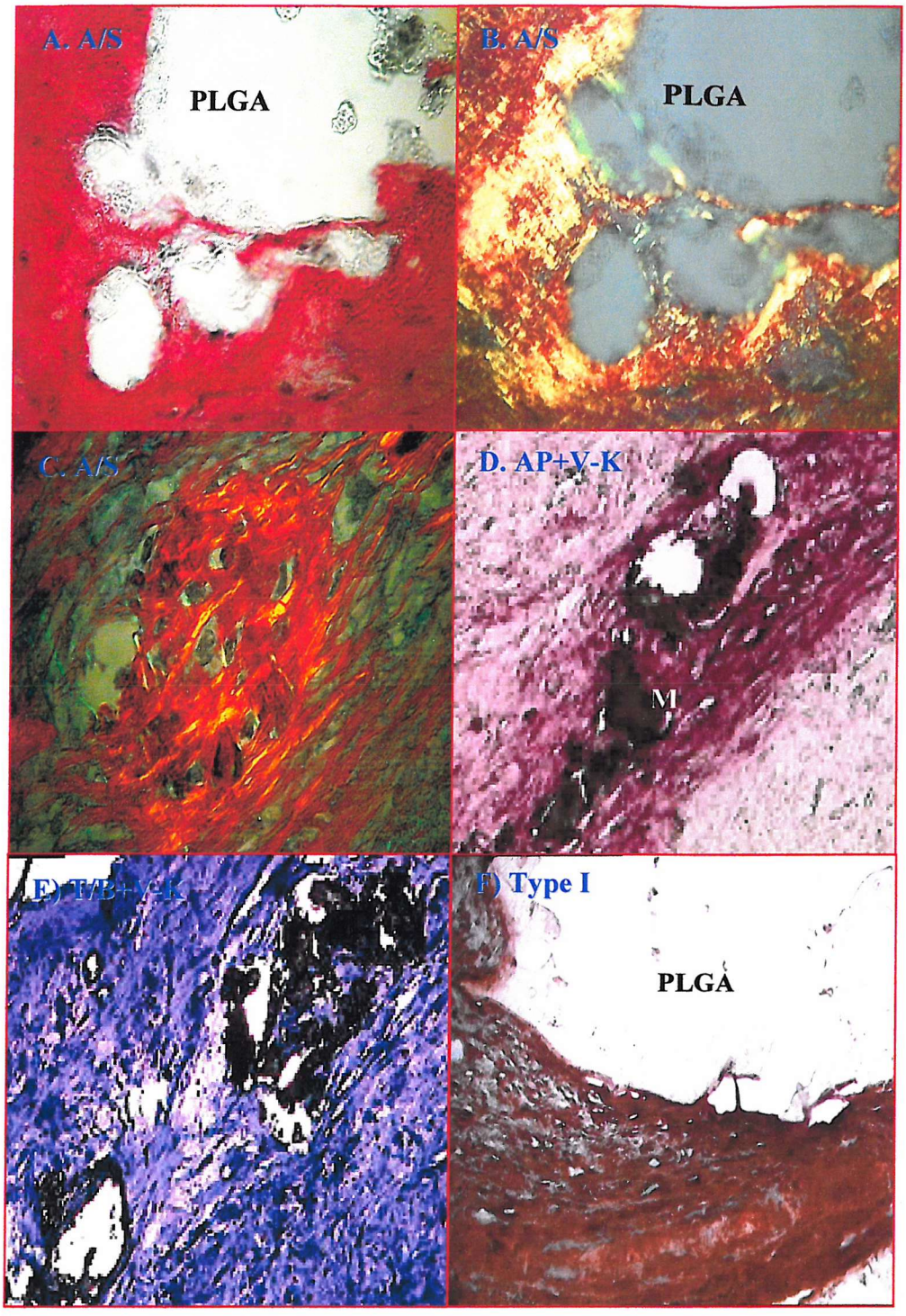


Figure 5-12 New bone matrix and evidence of woven bone formation on and within PTN adsorbed PLGA scaffolds by Sirius red staining (A-C) and birefringence of collagen using polarized microscopy from a parallel section (B, C). Alkaline phosphatase (D) and mineral (D,E) as detected by von Kossa histochemistry. Type I collagen expression (F). Original magnification: ×250.

* PLGA: poly(lactic-c-÷glycolic acid) scaffold, M: mineral.

5.4. Discussion

These studies demonstrate the use of human osteoprogenitor cells in combination with PTN adsorbed PLGA scaffolds to provide an appropriate microenvironment to promote cell-matrix interaction facilitating human bone precursor cell attraction, adhesion, growth and differentiation. These results indicate that PTN recruited human osteoblasts and osteoblast precursors and promoted cell adhesion, growth and differentiation on the PLGA scaffolds resulting in mineralised structures ex vivo. PTN was chemotactic to human osteoprogenitors and significantly stimulated total and alkaline phosphatase-positive colony formation, as well as significantly stimulating alkaline phosphatase specific activity in basal conditions by 93% and by 55% in osteogenic conditions compared to controls. Expression of the bone markers *cbfa-1*, alkaline phosphatase, Type I collagen, osteocalcin and subsequent mineralisation, after 3 weeks, confirmed differentiation of the osteoprogenitor cells along the osteogenic lineage. In vivo studies confirmed the ability of PTN to drive primed human osteoprogenitor cells, following culture in osteogenic conditions, to form bone following subcutaneous implantation or within the enclosed environment of the diffusion chamber. The diffusion chamber provides unequivocal demonstration of the new bone formation by the implanted cells as opposed to host cells. The inability to demonstrate cartilage and bone formation on PLGA scaffolds in the absence of PTN, indicate the potential of coupling osteogenic matrix proteins, such as PTN, to porous biodegradable three-dimensional scaffolds to provide an appropriate biomimetic structure for bone growth differentiation, development and ultimately the augmentation of de *noval* bone formation.

The demonstration using PTN, of human osteoblast migration followed by attachment, as shown by the chemotactic assay, confirmed and extended the findings of Imai et al (1998) in their studies using a variety of mammalian cell lines. Thus PTN may act on

these cells in a paracrine fashion. In the current studies, PTN was found to stimulate colony formation, including alkaline phosphatase positive CFU-F number. Specifically the effects of PTN, on CFU-F formation, were observed in the late phase of cell culture irrespective of whether preparations were grown in basal or osteogenic conditions. Thus, the effects of PTN on colony formation indicate an effect on late osteoprogenitor populations. In support of this, PTN promoted mineralisation and bone formation in primed osteoprogenitor preparations as assessed by *in vitro* static culture, subcutaneous and diffusion chamber in vivo assays. Gundle and co-workers (Gundle et al, 1995) demonstrated bone formation in human bone marrow cell preparations derived from young donors (14-27 years of age) following extended culture, for over 6 weeks, in osteogenic conditions prior to implantation in combination with a hydroxyapatite carrier. In the present study, primary human osteoprogenitors derived from individuals primed have been used for 21 days in osteogenic conditions prior to implantation, demonstrating the ability of PTN to drive primed mesenchymal populations along the osteogenic lineage.

However, the cultures of human primary bone marrow stromal cells used in this study include non-adherent haemopoietic cells, which are present until their extensive removal, by vigorous washing, on day 6. It cannot be excluded that the effects of PTN on stromal progenitors may be mediated, at least in small part, indirectly, by effects on hematopoietic cells and hematopoietic progenitors. To date, the effects of PTN on colony formation of hematopoietic progenitor cells, including granulocyte, erythroid, macrophage and megakaryocyte are unknown.

Bone formation involves the directed differentiation of mesenchymal cells into osteogenic cells, a process subject to regulation by a variety of hormones and factors (Bianco et al, 2001; Bianco and Robey, 2001). BMP's, originally identified as proteins

which could induce new cartilage and bone formation in non-bony tissues, are major osteoinductive factors central in the recruitment, commitment and differentiation of bone progenitors (Wozney and Rosen, 1998). Native BMP's are 100-1000 fold more effective in inducing bone than individual recombinant BMP's (De Groot, 1998). However, the identification of an anabolic agent for skeletal repair remains, to date, elusive. With this background, the concentrations of PTN required to induce differentiation and mineralisation were extremely low, approximately 100-fold lower than concentrations of rhBMP's required to achieve similar effects. In the current study, the effects of PTN on human osteoblast differentiation and mineralisation on 3-D PLGA scaffolds, were observed using concentrations of PTN as low as 10pg/ml. Given the relatively high levels of PTN, as much as 3.5mg/kg of PTN, present in bone matrix (Zhou et al, 1992), the current findings add further support to the proposed role of PTN in maintaining osteoblastic activity of recruited populations whether at damaged sites or at areas of altered mechanical loading.

There have been reports for and against a potential mitogenic role for pleiotrophin (Li et al, 1990; Milner et al, 1992; Chauhan et al, 1993). Pleiotrophin was originally purified as a weak mitogen from bovine uterus by Deuel's group (Milner et al, 1989) and the same group went on to show that pleiotrophin was mitogenic for, and promoted, neurite outgrowth (Li et al, 1990). Chauhan et al (1993) showed over expression of bovine pleiotrophin cDNA in NIH 3T3 cells resulted in enhanced cell number, anchorage dependent growth and tumour formation in nude mice indicating a profound influence on cell growth. In contrast, a number of other groups failed to find a mitogenic effect on neurite outgrowth for PTN (Kuo et al, 1990; Raulo et al, 1992). Hampton and coworkers (1992) showed PTN was not a mitogen for Balb/3T3 cells or human umbilical vein endothelial cells although when presented as a substrate to chick

embryo cerebral cortical derived neurons, neurite extension activity was observed. Studies using Swiss mouse 3T3 and NIH 3T3 cells by Kuo and coworkers (1990) showed little, if any, mitogenic activity. Furthermore, Rauvala and Peng (1997) have suggested PTN may even be a proliferation arrest gene and suggest that PTN has a role in cell contact-dependent proliferation arrest. The current studies indicate PTN promotes adhesion, migration, growth and differentiation of human mesenchymal cell populations, however only a modest mitogenic effect was observed, as assessed by total colony formation. Further *in vitro* and in vivo studies using PTN and mesenchymal populations are required to determine if PTN is mitogenic to stem cells.

In the current studies, using adsorbed growth factors, the exact concentration of PTN adsorbed on to the PLGA scaffolds is unknown. However, these studies indicate that sufficient PTN is present to induce attachment and migration into the scaffold compared to PLGA scaffolds lacking either PTN or serum in which negligible cell attachment was observed. A number of tissue engineering strategies are in progress to incorporate growth factors, typically BMP's, into delivery systems and tissue engineered scaffolds for skeletal repair, including the use of collagen or hyaluronic acid with PLGA or PLA and encapsulation of BMP-2 in poly(DL-lactide-co-glycolide) (Brekke and Toth, 1998; Winn et al, 1999; Mori et al, 2000). Clearly, the ability to generate scaffolds containing encapsulated PTN and other guest agents will provide a natural developmental step to explore, which may provide unique delivery vehicles for skeletal regeneration.

In conclusion, these studies indicate PTN has the ability to promote adhesion, migration, expansion and differentiation of human osteoprogenitor cells. Furthermore, PTN appears to act specifically on late human osteoprogenitor populations rather than early osteoprogenitor sub-populations as evidenced by the time-course studies on PTN

action. These findings indicate the capacity to generate osteoinductive surfaces within a porous template for human bone marrow cell growth, differentiation and, ultimately, mineralisation.

CHAPTER 6

GENETIC MANIPULATION OF HUMAN OSTEO-PROGENITORS - *IN VITRO* AND *IN VIVO* BONE FORMATION ON POLYMER SCAFFOLDS

6.1. Introduction

Over the last few years, research on bone formation has focused on using recombinant growth factors to stimulate osteoprogenitor and osteoblast proliferation and differentiation to enhance bone formation. To date, BMP's have been identified as the main osteoinductive factors capable of inducing differentiation of multipotential mesenchymal stem cells as well as in endochondral ossification (Urist, 1965; Ahrens et al, 1993; Reddi, 1997; Wozney and Rosen, 1998). However, the effect of endogenous biologically active BMP-2 is about 100-1000 fold higher than recombinant BMP-2 *in vivo* (De Groot, 1998). Although BMP's can induce bone formation, the inability to identify a suitable delivery system and associated costs for patient administration has, to date, limited their clinical use. Thus, the use of a cell delivery vehicle for gene therapy offers a simple solution to this problem and has been pursued by many workers in a numbers of studies (Musgrave et al, 1999; Lieberman et al, 1999; Baltzer et al, 1999; Cheng et al, 2001). A numbers of studies have demonstrated the possibility of using adenoviral gene transfer to manipulate endogenous bone stem cells with gene-encoding specific osteoinductive growth factors. Typically, growth factor overexpression is maintained for several weeks (Oakes and Lieberman, 2000; Lind and Bunger, 2001).

As described in Chapter 1, synthetic materials, such as PLA, PLGA and PGA have emerged as potential scaffolds for cell transplantation and tissue growth. Thus, an alternative attractive approach for skeletal repair is the genetic modulation of primary human osteoprogenitor cells in combination with biodegradable polymer scaffolds, which interact and promote osteoblast differentiation and osteogenesis for cartilage and bone tissue regeneration. In these studies, Dr Kris Partridge performed the transfer of BMP-2 using an adenoviral vector provided by Dr Yasunori Okubo and Professor

Kazuhisa Bessho (Sakyo-ku, Japan). All *in vitro* and *in vivo* studies were performed by myself.

6.2. Methods and Materials

For general materials and methods see Chapter 2.1 and Chapter 2.2.

6.2.1. Cell culture

Bone marrows (7 patients: 4 females and 3 males, 61-80 years of age, with a mean age of 70 years) were obtained from patients undergoing routine total hip replacement surgery. Primary cultures of bone marrow cells were established as details in Chapter 2.2.1.

6.2.2. Infection of cells with adenovirus expressing BMP-2 (Dr Kris Partridge)

Cell lines were transduced with AxCAOBMP-2, a replication-deficient adenoviral vector carrying the human BMP-2 gene or AxCALacZ (hereafter termed AdBMP-2 or AdLacZ, respectively) (Gifts of Dr Yasunori Okubo and Professor Bessho, Japan), a control vector carrying the *Escherichia coli (E. coli) LacZ* gene. Cells were infected with the adenovirus once confluence had been reached and maintained for several days. Virus was added to the cells at multiplicities of infection (MOI) of 20 in media containing 5% FCS. Flasks were rotated every 30 min for 1.5 hours before addition of the same volume of fresh 5% FCS αMEM. An MOI of 20 was chosen after dose ranging studies (6.25-100) by Dr. Kris Partridge.

6.2.3. *In vivo* studies

Adenovirally labeled human bone marrow cells were cultured in αMEM medium containing 5% FCS prior to intra-peritoneal implantation (2×10^6 cells/chamber) using diffusion chambers. Diffusion chambers were implanted intraperitoneally into 8 athymic MF1-nu/nu mice. Control chambers contained human bone marrow samples

on PLGA scaffolds alone. After 10 weeks the mice were killed, chambers removed and examined by X-ray analysis and histology (See Chapter 2.2.13).

6.3. Results

6.3.1. Growth of adenovirally transduced HBM cells on PLGA scaffolds *in vitro*

Human bone marrow cells transduced with AdBMP-2 were seeded onto biodegradable porous PLGA scaffolds in basal media (media supplemented with 10%foetal calf serum alone). Cell adhesion and extensive cell in-growth were observed following culture for 4 weeks as observed by scanning electron microscopy (Fig. 6-1A). Cell viability was examined using Cell Tracker^{TM} green and EH-1 (Fig. 6-1B). The presence of intense fluorescence staining of the human osteoprogenitor cells as a consequence of label incorporation confirmed cell viability of transduced cells after 4 weeks on the porous scaffolds. The lack of cell necrosis was evidenced by the absence of ethidium homodimer-1 staining. These results indicate that adenovirally transduced cells are capable of adhering and proliferating on PLGA scaffold *in vitro*.

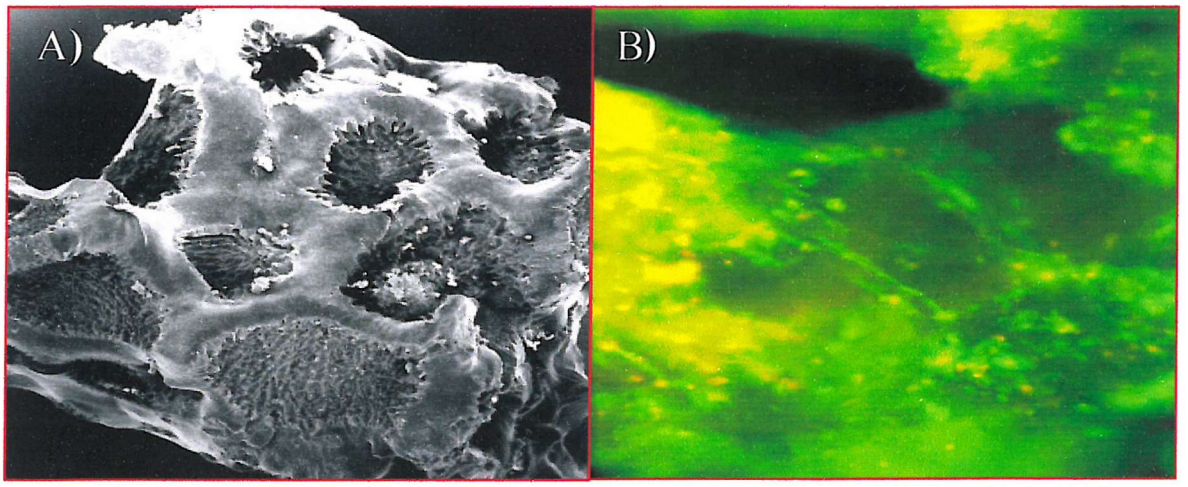


Figure 6-1 Primary human bone marrow cells in culture transduced with AdBMP-2, seeded onto PLGA scaffolds and maintained for 4 weeks. A) SEM image of scaffold alone. B) Confocal image of cells stained with Cell Tracker^{TM} green and EH-1 showing adhesion of the cells onto the scaffold. Original magnification: A) ×50, B) ×100.

6.3.2. Differentiation of adenovirally transduced human bone marrow cells on PLGA scaffolds *in vitro*

Differentiation of cells to the osteoblast phenotype was confirmed by histochemical staining for type I collagen, Alcian blue/Sirius red and evidence of extensive mineralisation (Fig. 6-2). Immunostaining for Type I collagen was observed after extended *in vitro* culture for 4 weeks (Fig. 6-2A) and extracellular matrix formation was observed by Sirius red and Alcian blue staining (Fig. 6-2B). Further evidence of matrix formation and mineralisation on the scaffolds was confirmed by von Kossa staining (Fig. 6-2C). Mineralisation was not observed on control samples (Fig. 6-2D).

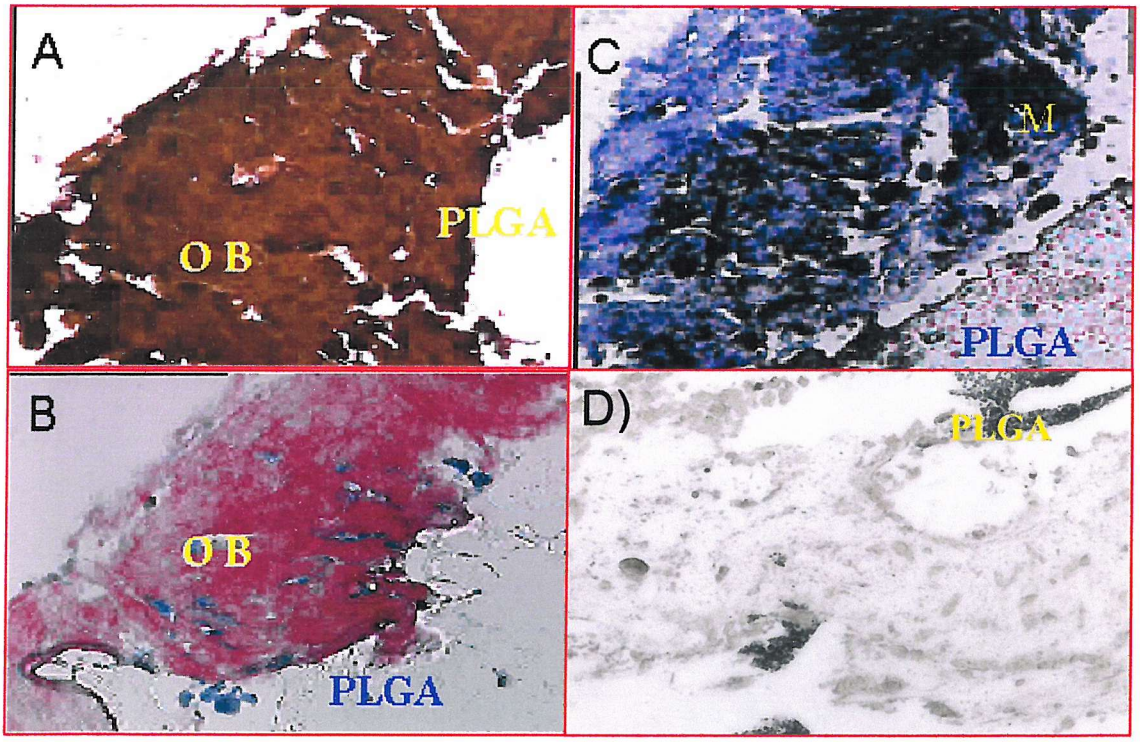


Figure 6-2 Cultured primary human bone marrow cells transduced with AdBMP-2, seeded on PLGA scaffold and maintained in basal medium for 4 weeks. Samples were embedded in paraffin and sectioned. A) Type I collagen; B) Staining with Alcian blue and Sirius red demonstrating extensive matrix formation; C) Toluidine Blue and von Kossa; D) von Kossa alone (Control). Original magnification: ×100. OB: osteoblast, M: mineral, PLGA: poly(lactic-c-÷glycolic acid) scaffold.

6.3.3. Differentiation of adenovirally-transduced human bone marrow cells *in vivo*

Adenovirally transduced human bone marrow cells expressing BMP-2 cultured in basal media only (in the absence of ascorbate and dexamethasone) were injected into diffusion chambers containing PLGA scaffolds and implanted intraperitoneally in 5 nude mice. The chambers were removed after 10 weeks and examined for bone formation by X-ray analysis and histochemical analysis. X-ray analysis indicated the presence of bone tissue (Fig. 6-3A) after only 10 weeks in 3 of 5 mice. On histological analysis, extensive cell growth on the PLGA scaffolds was observed and morphological evidence for the formation of cartilage and bone tissue by adenovirally transduced human bone marrow cells expressing BMP-2 (Fig. 6-3B-E and Fig. 6-4A-D). Immunolocalisation and histochemical analysis showed expression of alkaline phosphatase (Fig. 6-3E), type I collagen and extensive matrix formation (Fig. 6-3C). Metachromatic staining with toluidine blue in combination with von Kossa staining indicated the presence of bone and cartilage respectively (Fig. 6-3B, E). New bone formation was confirmed using polarizing light microscopy to demonstrate, by birefringence, new collagen formation (Fig. 6-3C, D and Fig. 6-4A, B). In diffusion chambers containing human bone marrow cells alone, in the absence of BMP-2, no evidence of bone and cartilage formation was observed (Fig. 6-3F). These results indicate osteoblastic differentiation and bone formation in cells transduced with an adenoviral vector expressing human BMP-2 *in vivo*.

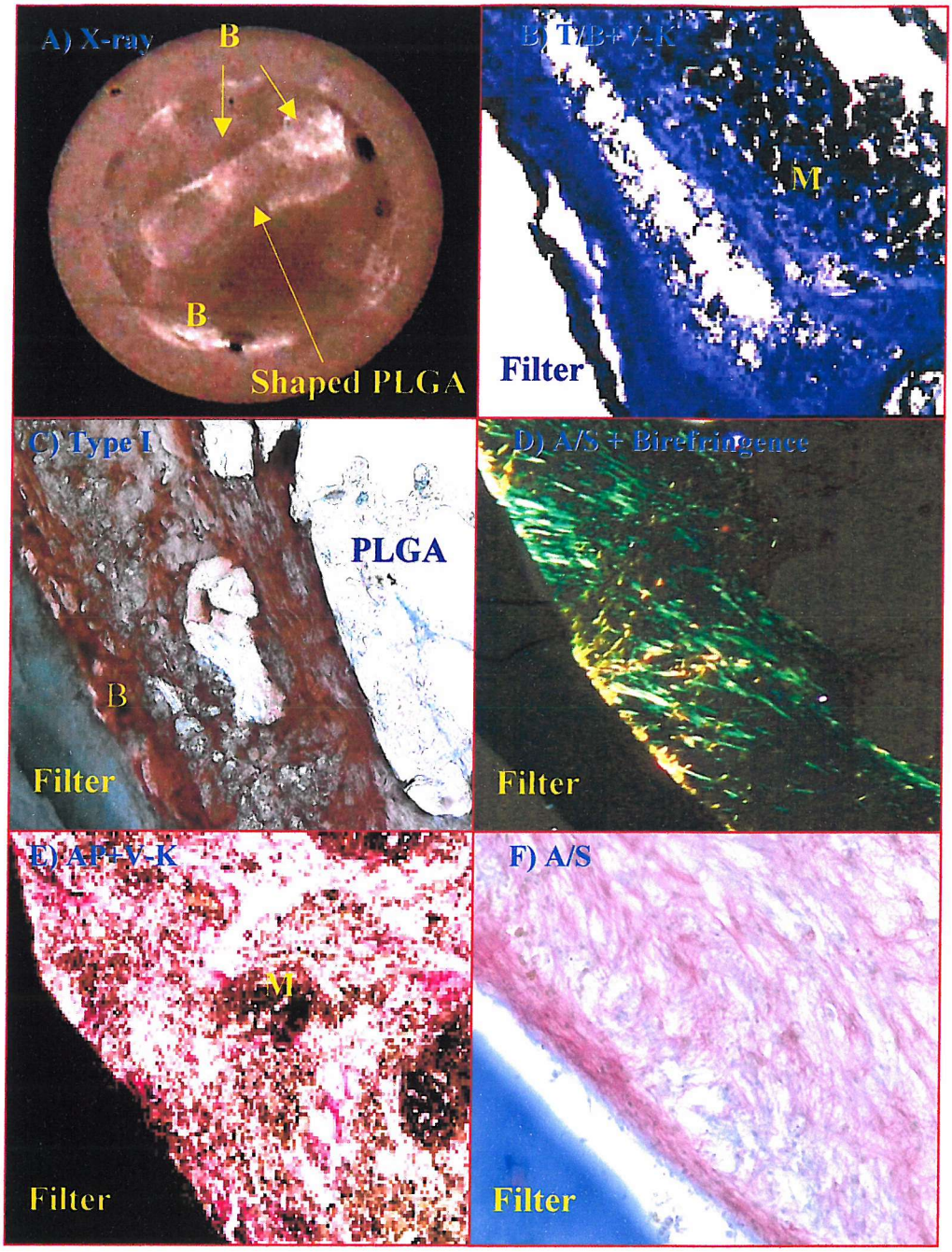


Figure 6-3 Bone formation by X-ray analysis on PLGA scaffolds within diffusion chambers after 10 weeks by cultured primary human bone marrow cells transduced with AdBMP-2 (A). Mineralisation observed by von Kossa staining (B,E). Alkaline phosphatase expression (E). Type I collagen immunocytochemistry (C) and parallel sections viewed using polarized light birefringence (D). Only fibrous tissues was seen in diffusion chambers with human bone marrow cells alone (F). Original magnification: A) ×5, B-F) ×100. *B: bone, M: mineral, PLGA: poly(lactic-co-÷glycolic acid) scaffold.

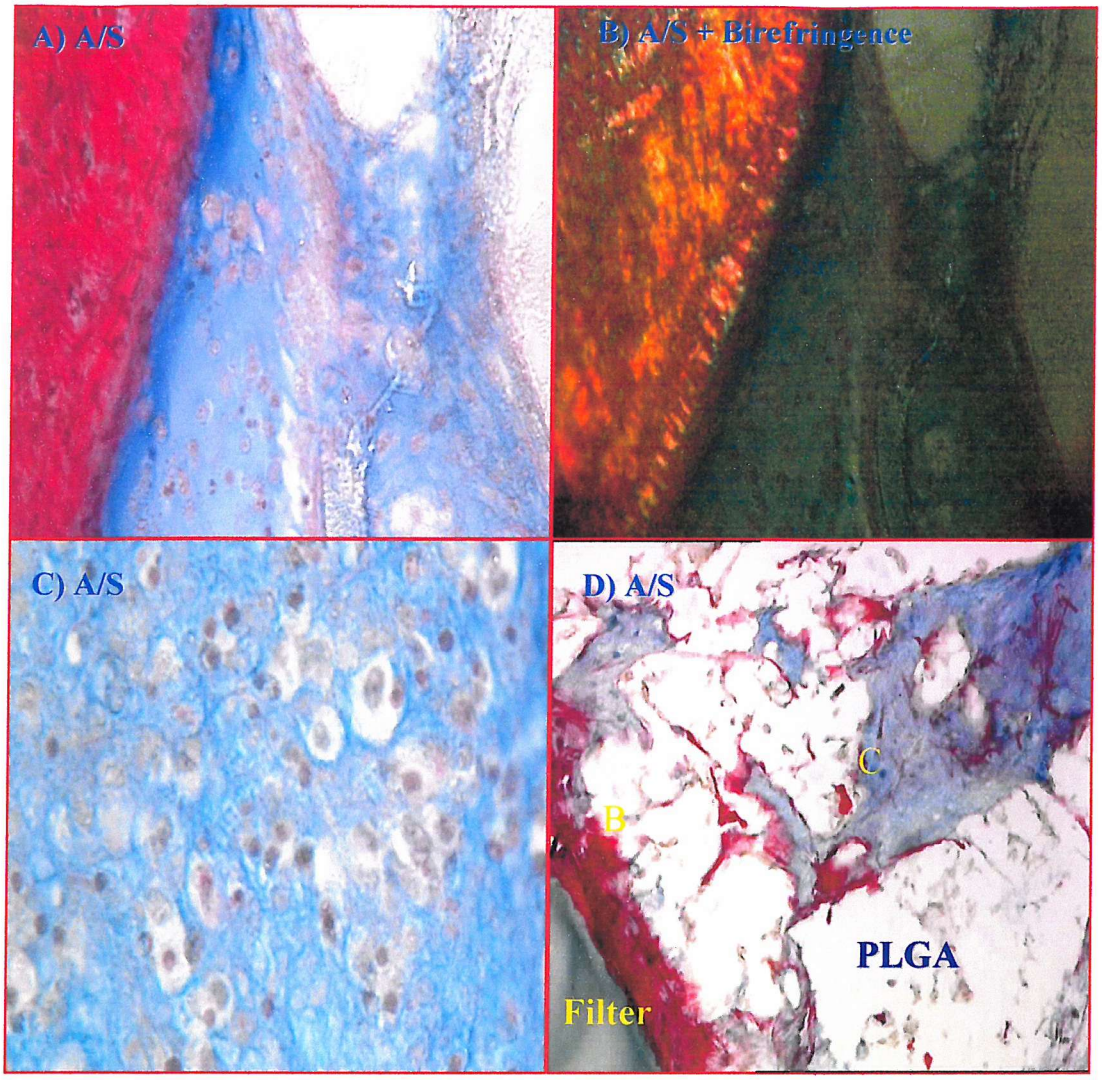


Figure 6-4 Cartilage and bone formation within diffusion chambers after 10 weeks by cultured primary human bone marrow cells in basal media transduced with AdBMP-2, seeded on PLGA scaffold and implanted intraperitoneally into MF1nu/nu mice. A,B,D) islands of bone within the PLGA scaffold as viewed by Alcian blue and Sirius red staining respectively, B) parallel section viewed using polarized light and, C,D) cartilage formation as demonstrated by Alcian blue staining. Original magnification: A,B) ×100, C) ×200, D) ×50. * PLGA: poly(lactic-co-÷glycolic acid) scaffold, C) cartilage.

6.4. Discussion

The current study demonstrates the expression and maintenance of the osteoblast phenotype on porous PLGA scaffolds following culture *in vitro* for 4 weeks using human bone marrow cells transduced with an adenoviral vector expressing BMP-2. All studies were performed using basal media (media supplemented with 10% fetal calf serum alone) in the absence of dexamethasone or ascorbate. Evidence for the transduced constructs to generate bone tissue was confirmed using a diffusion chamber assay, which allows unequivocal demonstration of new bone tissue formation by the implanted cells within a closed environment. New bone formation in primary human bone marrow cells expressing human BMP-2 on a porous PLGA scaffold was observed.

The ability to infect a wide variety of cell types including dividing and non-dividing cells combined with ease of manipulation, and high efficiency of gene transfer in the absence of integration into the host genome, makes recombinant adenoviruses an attractive choice for gene therapy. In this study the high level of infection of the heterogeneous target cell population containing osteoprogenitor cells at different stages of differentiation confirms the selective advantage of adenoviral vectors for gene therapy. However adenoviral infection has several key limitations 1) immunogenicity *in vivo* is a significant issue, 2) long infection times are deleterious, 3) delivery of cells requires expansion in culture prior to viral infection and reimplantation and, 4) fate of adenoviral cells is unclear. A number of these issues have been circumvented by using a simple and reproducible technique to generate high titre of virus enabling infections of target cell populations at low MOI.

Previous studies indicate a dose of 10^7 particle units or an MOI of over 50 will provoke an immune response (Chirmule et al, 1999; Harvey et al, 1999). In this study, a low

MOI in comparison to the work of others has been used (Lieberman et al, 1998; Cheng et al, 2001; Turgeman et al, 2001; Viggeswarapu et al, 2001), which may help to reduce these complications. The observation of over 98% efficiency of transduction with AdLacZ with low MOI differs to literature reports (Conget and Minguell, 2000). However, Baltzer et al (1999) reported 100% AdLacZ transduction in an osteoblast cell line. The importance of the relationship between efficiency of transduction and levels of protein secretion remains to be determined, although, in this system sufficient protein was expressed to induce differentiation whilst using a low MOI.

Another factor that may contribute to a reduction in immunogenicity is highlighted by Cheng et al (2001) who found that increasing the time to 7 days between transduction and implantation increased bone formation. In the current study, cells were implanted 3-7 days after transduction with 3 out of 5 positive chambers for bone formation by X-ray and 5 of 5 positive for bone markers histologically. This delay may allow for differentiation to begin before implantation when the immune response may clear the virus. To eliminate the problems associated with delivery of BMP-2 to the required site Musgrove and co-workers (Musgrave et al, 1999) reported on the use of direct adenoviral mediated gene therapy to deliver active BMP-2 and produce bone in skeletal muscle. Direct adenoviral mediated gene therapy, although attractive, produced little bone probably due to rapid immune clearance of the virus and raised issues of pathogenecity and safety. In contrast to Turgeman (Turgeman et al, 2001) who allowed the virus to remain in the media, this study demonstrates that an exposure of the virus for 24 hours followed by washing and provision of fresh medium was sufficient for transduction. The use of a low MOI, delay in implantation and removal of the virus, may reduces issues of immunogenicity.

Interestingly, Olmsted and co-workers (2001) were unable to demonstrate BMP-2 secretion from primary bone marrow stromal cells that had reached confluence and were transduced with an adenoviral vector expressing BMP-2 (Olmsted et al, 2001). In the current system, cells were allowed to become confluent before transduction and subsequently expressed BMP-2. This discrepancy may be explained by the different manner in which the cells were handled prior to confluence. Cells were cultured in the absence of antibiotics and antifungals and cells were used at primary and first passage only. Clearly differences in experimental approaches have the potential to subtly alter the phenotype of primary cells, potentially either enabling or prohibiting adenoviral transduction.

The exact levels of BMP-2 present *in vivo* in this study are not clear. Active BMP-2 was measured by Dr Kris Partridge at 10-165 nM in comparison to rhBMP-2 standards and was secreted for up to 8 days. These levels were sufficient to induce osteoblastic differentiation without any evidence of toxicity to the cells. The time scale of secretion is in agreement with others measurements of adenoviral BMP-2 production in cell lines (Lieberman et al, 1998; Olmsted et al, 2001) with a peak at approximately 6 days followed by a rapid decrease.

This study expands on the tissue-engineering theme by seeding transduced cells on a biodegradable scaffold, which should avoid the potential for unwanted migration of cells. The generation of 3-D porous biodegradable constructs *in vitro* is attractive in the development of skeletal repair strategies providing a scaffold/filler for bone regeneration. The development of supercritical fluid mixing technology to generate porous polymer scaffolds of defined porosity and degradation characteristics by Howdle and colleagues (2001) have provided new platform technologies for osteoprogenitor differentiation and mineralisation. These scaffolds resorb by hydrolysis

and have found relevance for use as tissue engineering scaffolds as their resorption results in a natural replacement tissue without the long-term complications associated with foreign implants (Howdle et al, 2001).

In summary, these studies demonstrate the successful delivery of active BMP-2 using bone osteoprogenitors on porous biodegradable scaffolds. The generation of mineralised 3-D structures *in vitro* and the subsequent demonstration of bone formation *in vivo* using the diffusion chamber assay, with such constructs indicates the potential to tissue-engineer bone. However, the balance between osteoblastic differentiation and induction of an immune response will need to be determined.

CHAPTER 7

BONE FORMATION BY HUMAN OSTEOPROGENITORS USING ENCAPSULATED BONE MORPHOGENETIC PROTEIN-2 ON BIODEGRADABLE POLYMER SCAFFOLDS

7.1. Introduction

As previously described in Chapter 1.1.6.1 and Chapter 1.3.1, bone marrow contains multipotential stromal stem cells, which can be used to directly differentiate into osteogenic cells to form bone (Owen, 1988; Friedenstein, 1995; Triffitt, 1996; Bruder et al, 1998). This process of bone formation is subject to regulation by a variety of osteotropic factors(Triffitt, 1996). To date, much is known of the osteogenic factors implicated in enhancing bone formation in particular proteins of the TGF-β superfamily. The various individual BMP's, of which there are over 30 members, have been shown to induce cartilage and bone formation when implanted or injected subcutaneous in rats and to induce complete union in a rat segmental bone defect model (Yasko et al, 1992; Lee et al, 1994; Rodgers et al, 1998; Ripamonti et al, 2001). However, key in the formation of new bone by these osteoinductive agents, in a tissue engineering context, is an appropriate delivery system or carrier material required to prevent the rapid diffusion of these molecules (Zellin and Linde, 1997; Murphy and Mooney, 1999; Uludag et al, 2001; Saito et al, 2001). Delivery systems, to date, have included natural or synthetic materials, type I collagen (Ripamonti et al, 2001; Suh et al, 2002), gelatin (Tabata and Ikada, 1999; Meinel et al, 2001), biodegradable polyorthoester (Busch et al, 1996), poly(L-Lactic acid) (Whitaker et al, 2001), and a copolymer of polylactic and polyglycolide, polypropylene fumarate and hydroxyapatite ceramics (Ishaug-Riley et al, 1998; Tjia et al, 1999; Lu et al, 2000; Tamai et al, 2002). Recent developments by Howdle and coworkers (2001) have led to the generation of porous PLA scaffolds using novel supercritical fluid technology. These scaffolds have a number of advantages including the ability to generate structures of defined porosity, degradation characteristics and, significantly, the potential for the incorporation of guest particles/factors/cells. The absence of solvents and thermal processing in the

generation of these scaffolds makes this an attractive approach to growth factor incorporation. Howdle and coworkers (2001) have shown high protein (ribonuclease) loading into foamed PLA scaffolds will retain full activity on subsequent release from the PLA over 3 months (Whitaker et al, 2001). Furthermore, PLA and PGA have FDA approval, are biodegradable and can be chemically modified (see Chapter 1.3.4). Thus the possibility of incorporating guest agents such as osteoinductive factors into poly lactic acid scaffolds to generate controlled release scaffolds is a realistic and clinically desirable objective.

BMP-2 has been shown to induce the differentiation of mesenchymal stem cells into cells of osteoblast lineage and is currently under clinical evaluate for fracture repair and for use in a variety of orthopaedic applications. However, the protocols for the use of BMP-2 have been limited by issues of availability (Baylink et al, 1993; Yamaguchi et al, 2000) (See Chapter 1.3.2.1). The aim of this study was to generate porous biodegradable PLA scaffolds encapsulating an osteogenic protein, recombinant human bone morphogenetic protein-2 and to examine the ability of these scaffolds to promote human osteoprogenitor differentiation and bone formation *in vitro* and *in vivo*. BMP-2 encapsulated PLA scaffolds were generated using supercritical fluid process technology by Professor Howdle's group (University of Nottingham, UK). The ability of BMP-2 released from encapsulated constructs to promote adhesion, migration, expansion and differentiation of human osteoprogenitor cells on 3-D scaffolds was examined by *in vitro* cell culture and within a novel *ex vivo* chick chorioallantoic membrane bone formation model and by *in vivo* bone formation assays using the subcutaneous and diffusion chamber assays.

7.2. Materials and Methods

For general materials and methods see Chapter 2.1 and Chapter 2.2.

Recmbinant human BMP-2 adsorbed PLA scaffold were used for examination of the effect of rhBMP-2 on human bone marrow stromal cell growth and differentiation *in vitro* and *in vivo* followed by using the rhBMP-2 encapsulated PLA scaffolds *in vivo*.

7.2.1. Cell culture

Bone marrow samples (16 patients in total: 11 females and 5 males aged 14–83, with a mean age of 63.81 years) were obtained from haematological normal patients undergoing routine total hip replacement surgery. Primary cultures of bone marrow cells were established as details in Chapter 2.2.1.

7.2.2. Chorioallantoic membrane assay

Fertilized eggs were incubated for 10-18 days using a Multihatch automatic incubator at 37°C in a humidified atmosphere. Chick femurs were excised from day 18 chick embryos and a wedge-shaped segmental defect created in the middle of the femur, into which the scaffold construct was placed to fill the defect site. Chick bone and scaffolds (29 samples) were placed directly onto the CAM of 10-day-old eggs and incubation continued for a further 7 days. After completion of culture, explants were harvested for histochemical analysis as previously described (See Chapter 2.2.10 and Chapter 2.2.12)

7.2.3. Growth factor encapsulation

Recombinant human BMP-2 was encapsulated in PLA (100ng/mg PLA) by Professor Howdle in University of Nottingham using a supercritical carbon dioxide method as described in Chapter 2.2.7.

7.2.4. C2C12 alkaline phosphatase assay

BMP-2 has the ability to induce C2C12 promyoblast differentiation into the osteoblast lineage (Katagiri et al, 1994; Okubo et al, 1999; Kirsch et al, 2000; Partridge et al,

2002). In this study, C2C12 cells were used to assay the activity of rhBMP-2 following encapsulation within PLA scaffold. Briefly, human bone marrow stromal cells were cultured in the presence or absence of rhBMP-2 encapsulated PLA scaffold, or passaged onto rhBMP-2 encapsulated PLA scaffold or PLA scaffold alone in 10% FCS DMEM at 37°C and 5% CO_2 for three days. Samples were fixed in ethanol and stained for alkaline phosphatase.

7.3. Results

7.3.1. Human bone marrow stromal cell growth on rhBMP-2 adsorbed PLA scaffolds

Initial studies examined the ability using rhBMP-2 adsorbed PLA to modulate human bone marrow stromal cell growth.

In vitro study

Human bone marrow stromal cells were cultured on rhBMP-2 (50ng/ml) adsorbed PLA scaffold for 6 weeks. Recombinant human BMP-2 promoted human bone marrow stromal cell adhesion, spreading, proliferation, and differentiation on PLA porous scaffold *in vitro* as observed by SEM, confocal microscopy and expression of type I collagen histochemistry (Fig. 7-1).

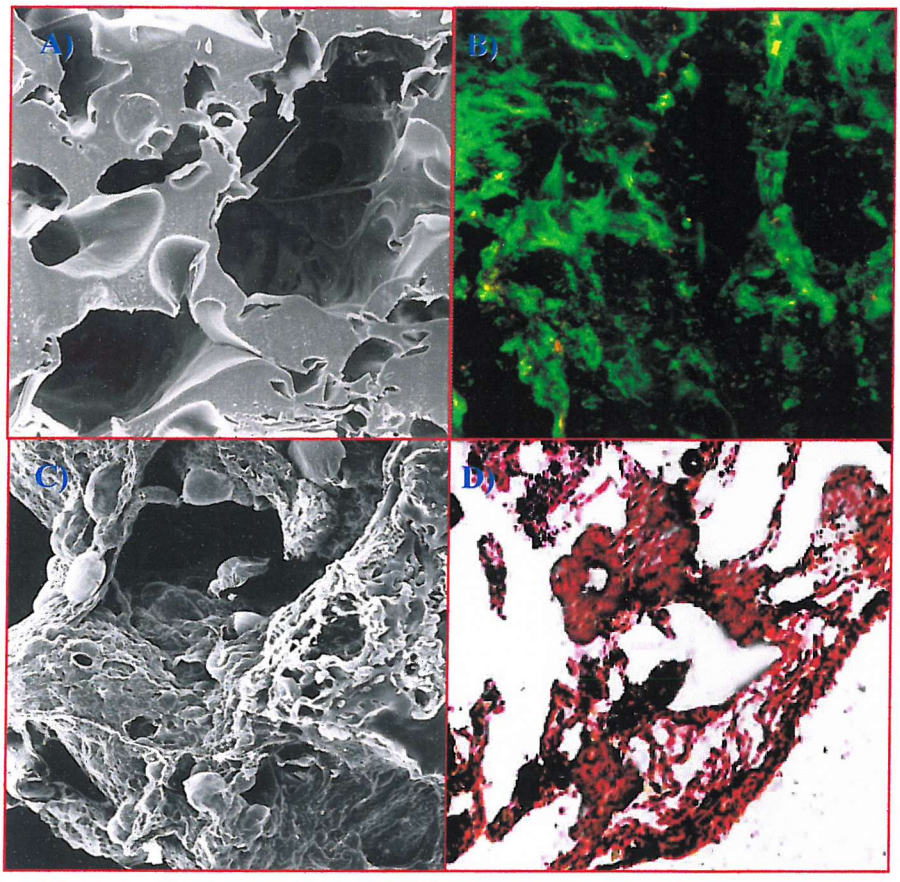


Figure 7-1 Human bone marrow stromal cell growth on rhBMP-2 adsorbed PLA scaffolds. A) Original PLA structure without cells (SEM). B) Cell adhesion and proliferation on rhBMP-2 adsorbed PLA scaffold as observed by confocal microscopy (viable cells-green and necrotic cells-red). C) SEM. D) Expression of Type I collagen by immunohistochemistry confirmed the maintenance of the osteoblast phenotype. Original magnification: A,C) ×100, B) ×500, D) ×100.

In vivo study

The ability of rhBMP-2 to stimulate bone formation by human bone marrow cell *in vivo* was examined using the subcutaneous implant model. Following implantation (6 samples) for 6 weeks, rhBMP-2 (50ng/ml) stimulated bone matrix formation not only on the surface but through out the scaffold with evidence of ingrowth into the porous PLA scaffold as observed in paraffin sections stained with Alcian blue/Sirius red (Fig. 7-2A), Type I collagen (Fig. 7-2B), toluidine blue (Fig. 7-2C) and birefringence (Fig. 7-2D).

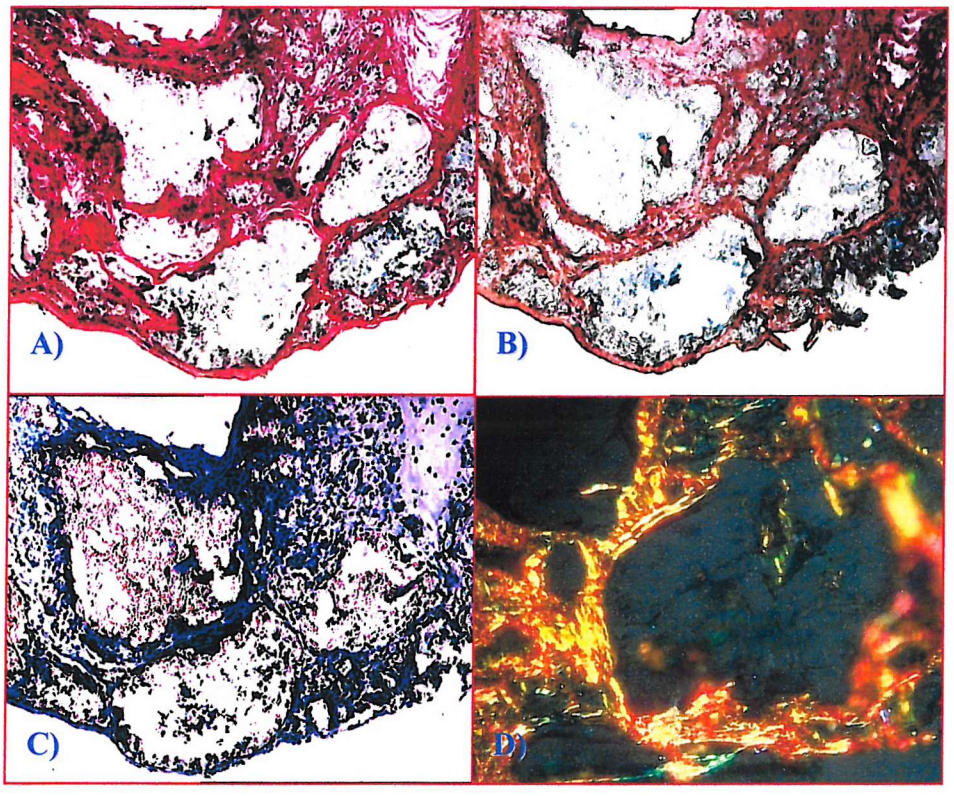


Figure 7-2 *In vivo* subcutaneous implant: HBM cell in-growth into rhBMP-2 adsorbed PLA scaffolds. New bone matrix formation was observed on/in rhBMP-2 adsorbed PLA scaffolds as detected by Alcian blue and Sirius red staining (A), Type I collagen staining (B), Toluidine blue staining (C) and birefringence (D). Original magnification: ×100.

7.3.2. Bioactivity of rhBMP-2 encapsulated PLA scaffolds

Following demonstration of new bone matrix formation by human bone marrow stromal cells on rhBMP-2 adsorbed scaffolds, rhBMP-2 was encapsulated within PLA scaffold (100ng/mg PLA) by Professor Howdle. The bioactivity of rhBMP-2 encapsulated PLA scaffolds were determined using induction of the C2C12 promyoblast cell line into the osteogenic lineage as detected by alkaline phosphatase expression. Alkaline phosphatase-positive cells were observed following culture of C2C12 cells in presence of or on rhBMP-2 encapsulated PLA scaffolds (Fig. 7-3A, C). No induction of alkaline phosphatase-positive cells was observed using blank scaffolds (Fig. 7-3B, D).

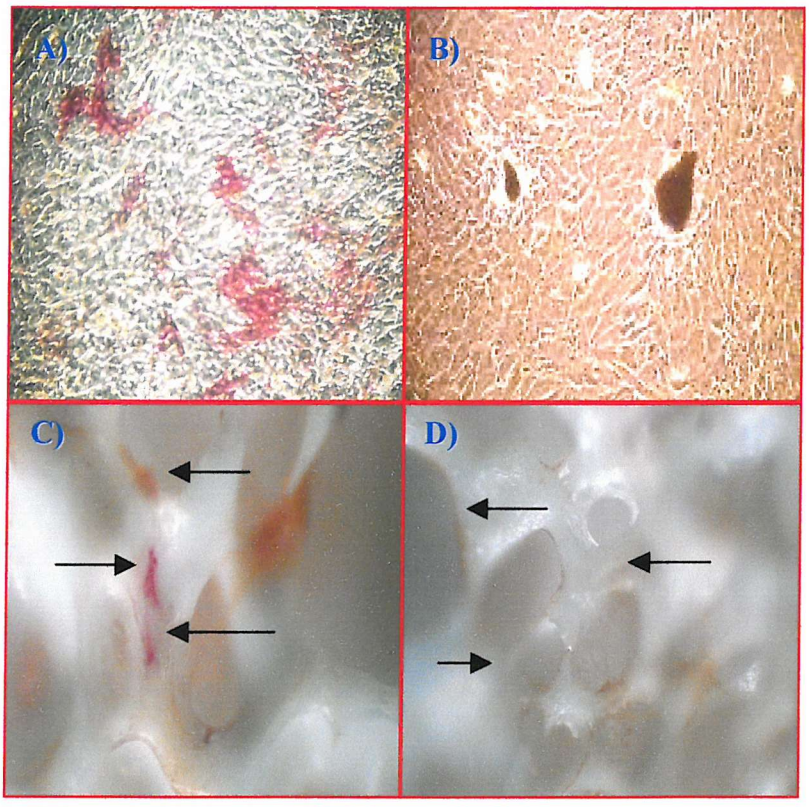


Figure 7-3 Induction of C2C12 cells: C2C12 cells were cultured in presence of rhBMP-2 encapsulated PLA scaffold for 3 days and cell differentiation detected by alkaline phosphatase positive staining (A, C). No induction of alkaline phosphatase positive cells was observed using PLA blank scaffolds (B, D). Original magnification: ×200.

7.3.3. Human osteoprogenitor growth on rhBMP-2 encapsulated scaffolds

Following demonstration of the ability of using rhBMP-2 encapsulated PLA scaffold to stimulate differentiation of C2C12 promyoblast towards the osteoblast lineage, this study has examined the potential of this scaffold to induce differentiation and mineralisation of human bone marrow stromal cell.

CAM culture

Culture of human osteoprogenitors on rhBMP-2 encapsulated PLA scaffolds on the chick chorioallantoic membrane model showed that encapsulated rhBMP-2 stimulated human bone marrow stromal cell growth and differentiation in the PLA scaffold. Extensive angiogenesis was observed on the scaffold/cell constructs. New cartilage and bone were observed within the chick bone defect (Fig. 7-4).

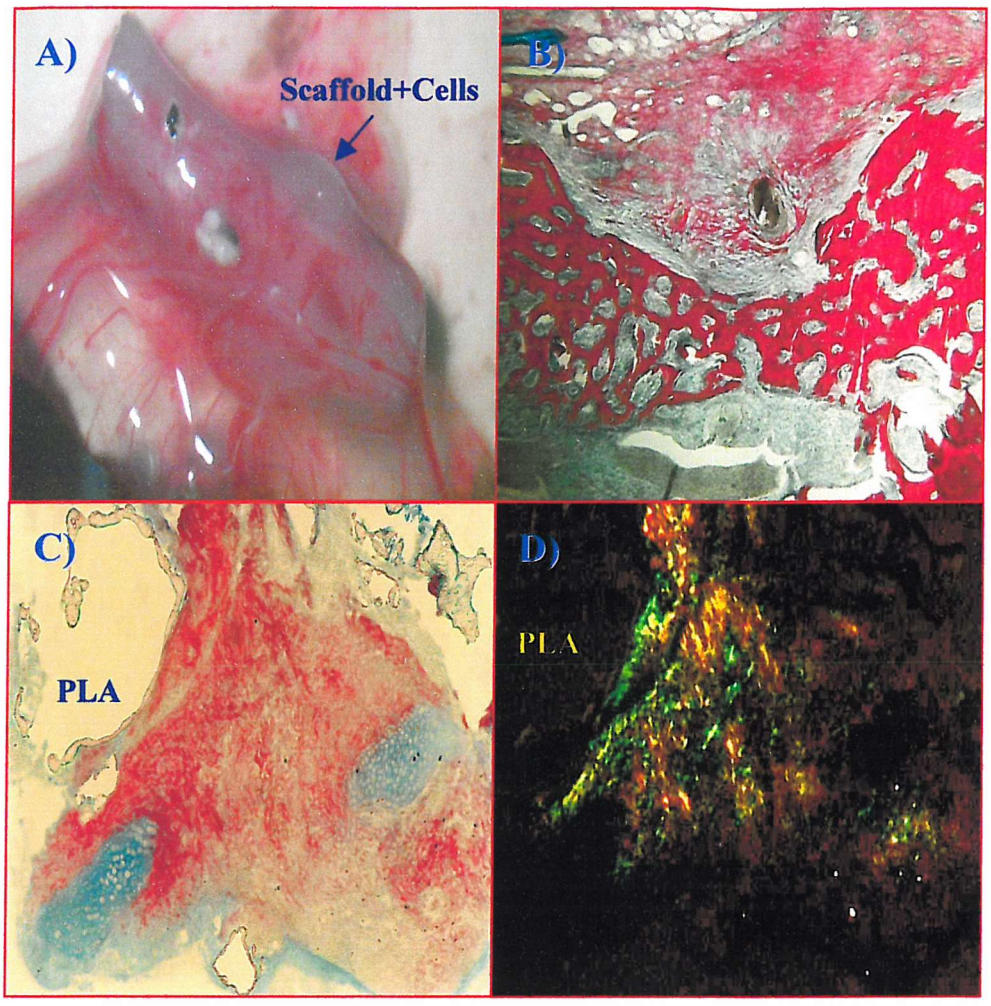


Figure 7-4 CAM culture model: Vascular supply to the chick bone-PLA-rhBMP-2-HBMCs was observed (A). New bone matrix detected within the chick bone defect (B). Cartilage and bone matrix formation in rhBMP-2 encapsulated PLA scaffolds as stained by Alcian blue and Sirius red (C). Original magnification: A) ×50, B) ×100, C,D) ×150. *PLA: poly(lactic acid) scaffold.

Subcutaneous implant model

Primary human bone marrow cells were impregnated onto PLA scaffolds encapsulated with rhBMP-2 and subcutaneous implanted (8 samples) in nude mice for 6 weeks (PLA alone as a negative control). The rhBMP-2 encapsulated scaffolds promoted human bone marrow stromal cell adhesion, proliferation, differentiation with evidence of new bone matrix deposition as detected by Alcian blue/Sirius red staining (Fig. 7-5A-C) and birefringence (Fig. 7-5B and C). In contrast, poor cell growth and negligible bone matrix synthesis was observed on PLA scaffolds alone in the absence of rhBMP-2 (Fig. 7-5D).

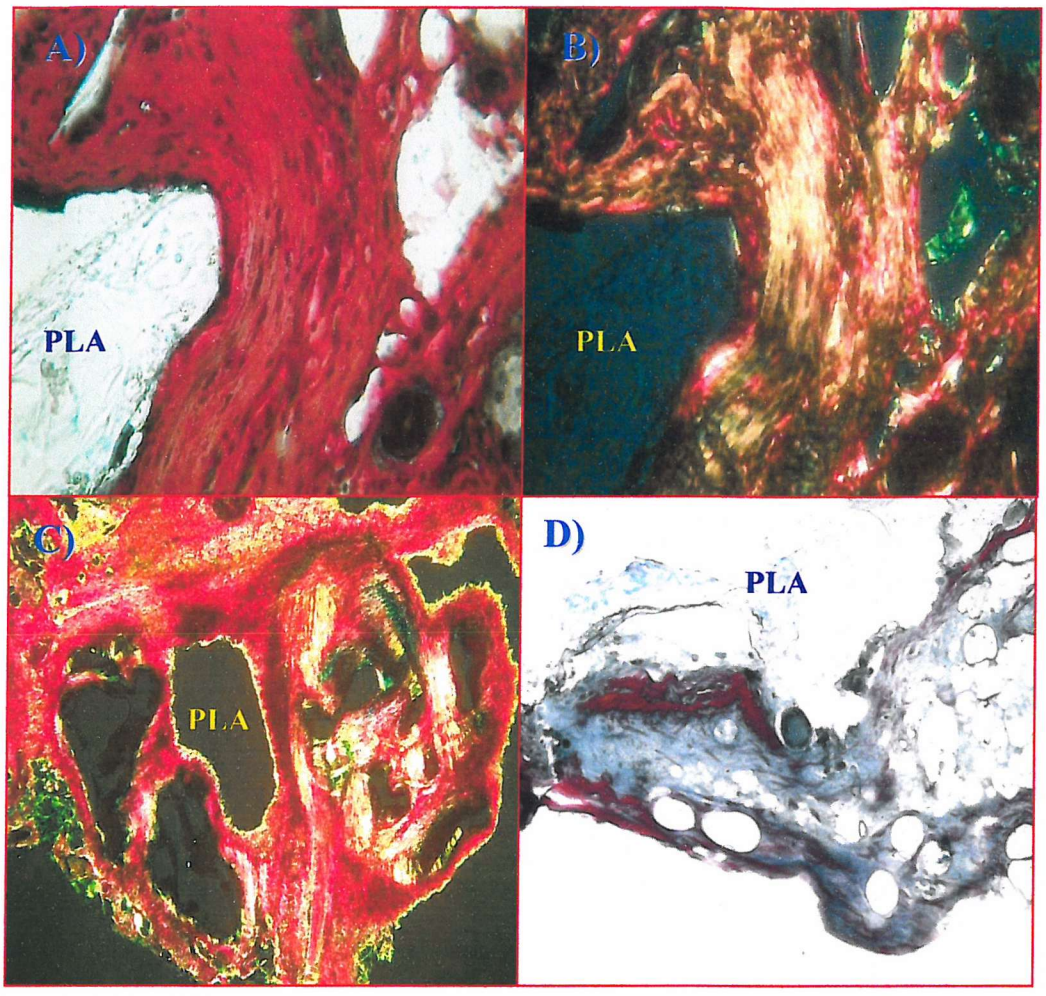


Figure 7-5 Subcutaneous implant: Bone matrix formation in rhBMP-2 encapsulated PLA porous scaffolds were observed in paraffin sections stained with Alcian blue and Sirius red (A) as well as birefringence (B, C). Only fibrous tissue and fat tissue were observed in PLA alone (D). Original magnification: A,B) ×200, C,D) ×100. *PLA: poly(lactic acid) scaffold.

Diffusion chamber model

Recombinant human BMP-2 encapsulated PLA scaffolds seeded with human osteoprogenitor cells, showed morphologic evidence of new bone and cartilage matrix formation as visualisation by Alcian blue and Sirius red staining (Fig. 7-6A, B, D, E) and by X-ray analysis (Fig. 7-6C) after 10 weeks implantation within diffusion chambers. Collagen deposition and new matrix synthesis was confirmed by birefringence microscopy (Fig. 7-6B). Cartilage formation was observed within rhBMP-2 encapsulated PLA scaffolds confirming penetration of human osteoprogenitors through the scaffold constructs (Fig. 7-6D, E). No bone formation was observed on cell/PLA scaffold constructs alone (Fig. 7-6F).

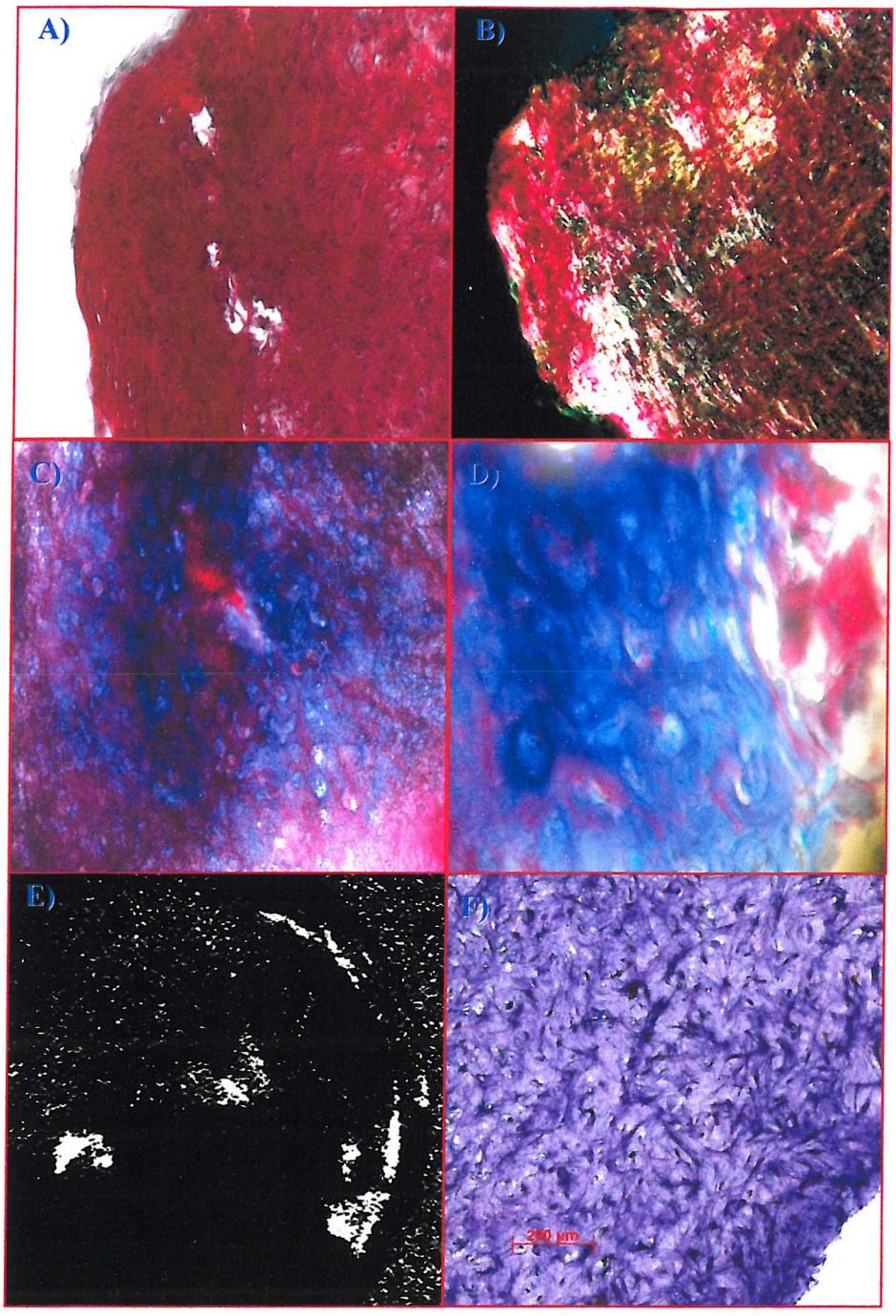


Figure 7-6 Diffusion chamber assay: Bone matrix formation within rhBMP-2 encapsulated PLA scaffold as detected by Alcian blue/Sirius red staining (A-D), birefringence (B) as well as X-ray assay (E). Cartilage tissue formation was confirmed by Alcian blue and Sirius red staining (C, D). No bone matrix deposition was observed in PLA alone in nude mice (F). Original magnification: A,B,C) ×100, D,F) ×200, E) ×10.

7.4. Discussion

Successful tissue growth is often dependent on the ability to deliver specific growth factors to cells within regenerating tissues and a requirement to promote vascularisation. Thus, strategies in tissue engineering have emerged for the development of sophisticated growth factor delivery mechanisms to mimic the endogenous profiles of growth factor production during natural tissue morphogenesis or regeneration. Furthermore, with the current advancement of material science and biotechnology in targeting drug release and release patterns, it is possible to increase the efficacy of these proteins through controlled release. To achieve this target, biomimetic tissue engineering scaffolds need to meet the following conditions: 1) an ability to maintain the biological function of the growth factors during the fabrication of the scaffolds, 2) an understanding of the precise kinetics of growth factor release, 3) development of scaffolds that can control the release of two or more molecule types and, 4) potentially targeted delivery to specific cell populations (Langer and Vacanti, 1993; Whang et al, 1998; Whitaker et al, 2001). In this study, rhBMP-2 released from PLA scaffolds was shown to promote C2C12 osteogenic differentiation and stimulate bone formation by human bone marrow cell populations.

The BMP family of proteins are known osteoinductive factors pivotal in the recruitment, commitment and differentiation of bone progenitors (Wozney and Rosen, 1998). BMP-2 has been shown to induce bone formation in monkey muscle, rat segmental non-union defects, and human femoral non-unions (Johnson et al, 1988; Yamaguchi et al, 1991; Miyamoto et al, 1993) However, issues of bioactivity and sustained release *in vivo* have limited their use clinically. Thus, a scaffold system that could deliver rhBMP-2 in a controlled manner with precise release kinetics and retain

function within a 3-D supportive matrix would have tremendous therapeutic application (Whang et al, 1998).

This study used bioactive polymer composites generated by Professor Howdle using supercritical fluid mixing (Whitaker et al, 2001; Howdle et al, 2001). In this procedure, the efficient agitation of the scCO2 swollen polymer suspension at near ambient temperatures results in a homogeneous distribution of the bioactive factor throughout the polymer matrix. The absence of solvents and thermal processing in the generation of these scaffolds allows retention of rhBMP-2 activity resulting in biomimetic osteoinductive structures for bone tissue engineering.

At present, the efficacy of these innovative constructs to generate bone can only be evaluated in animal models. The use of these model is always the subject of moral, ethical as well as financial considerations. However, the chorioallantoic membrane of growing chick embryos has been widely used as an assay system for angiogenesis and as an explanted tissue culture system (Roach et al, 1998). This is due to the potential of the blood vessels of the CAM to invade any tissue explants placed on it. These studies have extended the standard CAM culture model to generate an easy, reproducible and cost-effective *ex vivo* assay model for the evaluation of innovative bone formation regimes. The application as a pre-screening assay prior to *in vivo* evaluation remains an area for further exploration.

These studies have examined the ability to generate new bone and cartilage tissues using rhBMP-2 encapsulated PLA porous scaffolds combined with human bone marrow stromal cells *ex vivo* and *in vivo.* The results have established the proof of such approach by development of innovative biomimetic scaffolds incorporating osteoinductive factors (rhBMP-2) released over time from biodegradable scaffolds containing human osteoprogenitor cells.

In conclusion, these studies have developed a simple *ex vivo* model for the assay of bone formation regime and used this model together with an *in vivo* model to evaluate the possibility to generate human bone and cartilage tissue using innovative tissue constructs that allow the release of osteoinductive factors on biodegradable scaffolds. Future work will examine the ability to release multiple growth factors to promote human osteoprogenitor differentiation and to enhance bone formation. Inaddition, the present results suggest the chorioallantoic membrane culture may be a useful model as an *ex vivo* assay system for tissue engineering.

CHAPTER 8

DISCUSSION

The ability to generate new bone for skeletal use is a major clinical need. Biomimetic scaffolds that interact and promote osteoblast differentiation and osteogenesis from human bone marrow stromal cells have important therapeutic implications and offer a promising approach to the generation of skeletal tissue to resolve this major healthcare issue (Langer and Vacanti, 1993; Bruder et al, 1997; Oreffo and Triffitt, 1999; Rose and Oreffo, 2002). Towards this goal, a central strategy has involved the use of 1) the isolation or characterisation of a source of progenitor cells, 2) the generation of a scaffold with an appropriate surface texture and 3-D structure support tissue regeneration, 3) the exploration of appropriate signaling molecules or growth factors and, 4) the investigation of cells, matrix and growth factors interaction.

Previous studies have shown the ability of rat osteoblasts to differentiate on 2-D films or 3-D foams of biodegradable PLA, PLGA and PGA in the presence of serum indicating poly(alpha-hydroxyacids) are suitable substrates for osteoblast growth (Ishaug et al, 1994; Ishaug-Riley et al, 1998; Matsuzaka et al, 1999). However, all these studies were performed in the presence of serum, which is rich in adhesion proteins, so the precise adhesion and spreading interaction between osteoblastic cells and the biomaterial are still unclear. A number of studies have shown that polymer materials themselves lack a chemical reactive pendent chain and are not suitable for cell attachment and growth (Quirk et al, 2001; Yang et al, 2001). To date, many procedures of surface modification have been developed to address this issue including the use of surface hydrolysis, plasma deposition and the generation of polymer peptide hybrids (Cannizzaro et al, 1998; Shakesheff et al, 1998). Thus, the ability to modify hydrophobic polymer biomaterials, shows the cell surface interaction, is critical in bone tissue engineering.

Current studies have investigated a tissue engineering approach combining a source of bone cells with selective osteoinductive growth factors, biodegradable polymer scaffolds and gene therapy for the development of a living tissue substitute for bone.

In initial studies, the matrix protein FN and the integrin adhesion motif RGD have been used to modify the surface chemistry of two dimensional (2-D) PLA films. FN and PLL-GRGDS were found to enhance the adhesion, proliferation and differentiation of human bone marrow stromal cells on surface-modified biodegradable PLA compared to the hydrophobic PLA films alone, indicating the potential to use PLA to generate three dimensional porous supportive scaffolds for bone tissue regeneration. With optimal concentrations of FN or RGD, cell adhesion and spreading was found to be comparable to tissue culture plastic serum controls. Extension of these studies to use porous PLGA scaffolds adsorbed with FN showed extensive human osteoprogenitor cell growth. Expression of the osteoblast phenotype was maintained and the initiation of mineralisation of the collagenous matrix was observed. As expected, the use of hydrophobic PLA alone resulted in negligible cell adhesion and spreading. These findings suggests that PLA surfaces, which on their own do not support cell growth, can be modified to enhance human bone marrow cell growth, differentiation and mineralisation, which indicate the capacity to generate biomimetic surfaces within a porous template and to allow the adhesion, proliferation, differentiation and mineralisation of human bone marrow cells. Furthermore, the results with FN and GRGDS agree with reports that a principle binding domain of adhesion proteins was RGD and that adhesion of bone cells could be inhibited with RGD containing peptides (Puleo and Bizios, 1991; Dalton et al, 1995; Johnson, 1998; Isogai et al, 1999). However, it cannot be discounted in these studies, as previously shown by Quirk et al

(2001), that the surface concentration of PLL in the PLL-GRGDS system may also have interfered with the integrin-mediated cell response.

Most cell types, except erythrocytes, will adhere to fibronectin immobilized to certain surfaces by interacting the specifically with the glycoprotein. Following the demonstration of the effect of cell adhesion motif and extracellular matrix protein (FN) on PLA/PLGA scaffolds, studies were then undertaken to examin the possibility of using osteoinductive proteins to modify the surface microenvironment of PLGA and to modulate human bone marrow stromal cell proliferation, differentiation and bone formation.

BMP's, members of the TGF-β super family, are known to be osteoinductive factors important in the commitment and differentiation of bone progenitors (Urist, 1965; Wozney and Rosen, 1998). However, it is still unclear which BMP or which combination of BMP's will be the most efficacious and cost-effective for bone induction and regeneration in clinical practice. In the current study, an admix of BMP's produced by Saos-2 cells, 'retentate', was found to induce human bone marrow stromal cell colony formation and differentiation. The concentrations of 'retentate' required to induce differentiation and mineralisation were approximately 100-fold lower than concentrations of rhBMP's required to achieve similar effects. Furthermore, this study showed the ability of Saos-2 'retentate' to promote human bone marrow stromal cell adhesion, proliferation, differentiation and mineralisation on PLGA scaffold, indicating the ability to use Saos-2 'retentate' as a reagent for surface modification and osteoinduction for bone formation. However, at this stage, which of the BMP's in this admix is optimal for bone cell differentiation and induction is unclear.

Imai and co-workers (1998) reported that PTN, an extracellular matrix-associated protein, is present in the matrices that act as targets for the deposition of new bone (Imai et al, 1998). In the current studies, rhPTN was found to recruit human osteoblasts and osteoblast precursors, which migrated to areas of intact rhPTN, to promote cell adhesion as well as differentiation on PLGA scaffolds. Recombinant human PTN was chemotactic to human osteoprogenitors at concentration of 50μg/ml and significantly stimulated alkaline phosphatase activity at concentrations as low as 10 pg/ml. Expression of the bone markers *cbfa-1*, alkaline phosphatase, Type I collagen, osteocalcin and subsequent mineralisation confirmed differentiation of the osteoprogenitor cells along the osteogenic lineage. These results indicated rhPTN is a useful factor to modulate human bone marrow stromal cell adhesion, chemotaxis, proliferation and differentiation for bone tissue engineering.

To date, BMP-2 remains the most potent inducer of mesenchymal stem cells differentiation towards the osteoblast lineage (Ahrens et al, 1993; Yamaguchi, 1995). In addition, a number of groups have shown BMP-2 modulates osteoblast proliferation and maturation (Wozney et al, 1990; Katagiri et al, 1990; Rickard et al, 1994). However, the effect of endogenous biologically active BMP-2 is about 100-1000 fold higher than recombinant BMP-2 *in vivo*. Thus, a number of studies in recent years have examined the potential of using gene therapy to deliver the BMP-2 gene to target cell populations (Uusitalo et al, 2001; Laurencin et al, 2001). Partridge et al (2002) have shown the ability to transfer the BMP-2 gene into human bone marrow cells using an adenoviral vector. Infected human bone marrow cells expressed active BMP-2 as confirmed by the differentiation of promyoblastic C2C12 cells. In the current studies, human bone marrow stromal cells were transduced with the BMP-2 gene using an adenoviral vector and seeded on PLGA scaffolds. Transduced cells maintained the

osteoblast phenotype and bone formation was observed. These studies demonstrated the possibility of using adenoviral gene transfer to manipulate bone stem cells to produce specific osteoinductive growth factors. However, before adenovirus gene therapy can be used in the clinic, a number of issues need to be resolved, including: 1) the potential for an immune response, 2) clearance and safety of the virus and, 3) quantification and duration of protein production (Partridge et al, 2002).

Despite the limitations of using adenovirus clinically, the bioactivity of growth factors, such as the BMP's, have also restricted their clinical use. However, the current advance in protein stabilisation has made it possible to generate sustained-release forms of several therapeutic proteins (Putney and Burke, 1998). Thus, this study has examined the possibility to deliver rhBMP-2 using encapsulation protocols.

Following the demonstration that rhBMP-2 promoted human bone marrow stromal cell adhesion, spreading, proliferation, and differentiation on PLGA porous scaffold, powdered PLA and rhBMP-2 were mixed using a supercritical fluid mixing method to generated PLA porous scaffolds encapsulating rhBMP-2. The rhBMP-2 encapsulated PLA scaffolds were shown to have the ability to induce the differentiation of C2C12 promyoblast cell line into osteogenic lineage. *In vivo* studies demonstrated the evidence of primary human bone marrow stromal cells adhesion, proliferation, differentiation and bone formation on/in rhBMP-2 encapsulated PLA scaffolds. These studies established the development of innovative biomimetic biodegradable scaffolds incorporating osteoinductive factors (rhBMP-2) and demonstrated the generation of an osteoinductive/osteoconductive template for bone tissue engineering.

To date, animal models remain the most reliable method to evaluate bone formation. However, animal studies are the subject of moral, ethical and financial considerations.

The current studies have generated an easy, reproducible and cost-effective *ex vivo* assay (CAM) model, which provides a primary vascular invasion microenvironment for the evaluation of innovative bone formation regimes. The application as a pre-screening assay prior to *in vivo* evaluation in future research and pharmaceutical industry programmes will be an avenue to be exploited.

Summary: This thesis has demonstrated the ability to modify surface chemistry of biodegradable PLA/PLGA materials with extracellular matrix proteins and cell binding motif peptides to create biomimetic microenvironments for human bone marrow stromal cell growth. The work was extended to examine the effect of bone growth factors including Saos-2 cell 'retentate' extraction, rhPTN and BMP's on human osteoprogenitor adhesion, proliferation and differentiation on 3-D polymer scaffolds. The results indicate the potential to generate biomimetic environment using these growth factors for bone formation. Adenoviral gene therapy and growth factor encapsulation will need to be further modified before their clinical application can be realised. However, at this stage, which growth factor and which method is the most efficiencious for bone formation cannot be concluded without further in vivo evaluation in critical defect models.

In conclusion, the use of peptides/proteins and 3-D structures to provide positional and environmental information, as in these studies, indicates the potential for biomimetic structures coupled with appropriate osteoinductive factors and human osteoprogenitor cells in the development of protocols for de novo bone formation.

Future Work

A number of avenues are open for further research as a result of data generated in this thesis including:

1) Encapsulation of bone growth factors or human bone marrow stromal cells: Further study will use the super critical fluid mixing method to encapsulate different bone growth and transcription factors as well as human bone marrow cells to investigate the cell-matrix-factor interactions.
2) Development of *in vivo* 'clinical' models for the assessment of bone formation using tissue engineering appraoches: such as the fracture non-union model and segmental bone defect model.

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LIST OF PUBLICATIONS AND ABSTRACTS

Publications

1. X.B. Yang, R. Tare, K.A. Partridge, H.I. Roach, N.M.P. Clarke, S.M. Howdle, K.M. Shakesheff, and R.O.C. Oreffo. Induction of human osteoprogenitor chemotaxis, proliferation, differentiation, and bone formation by osteoblast stimulating factor-1/pleiotropin: osteoconductive biomimetic scaffolds for tissue engineering. Journal of Bone and Mineral Research 2003;18(1):1-11. (In press).

2. D. Howard, K.A. Partridge, X.B. Yang, N.M.P. Clarke, Y. Okubo, K. Bessho, S.M. Howdle, K.M. Shakesheff and R.O.C. Oreffo. Immunoselection and adenoviral genetic modulation of human osteoprogenitors: *in vito* bone formation on PLA scaffold. Biochemical and Biophysical Research Communications 2002. (In press).

3. X.B. Yang, D.W. Green, H.I. Roach, N.M.P. Clarke, K.M., H.C. Anderson, S.M. Howdle, Shakesheff and R.O.C. Oreffo. Novel osteoinductive biomimetic scaffolds stimulate human osteoprogenitor activity -- implications for skeketal repair. Connective Tissue Research. (In press).

4. K.A. Partridge*, X.B. Yang*, N.M.P. Clarke, Y. Okubo, K. Bessho, W. Sebald, S.M. Howdle, K.M. Shakesheff, and R.O.C. Oreffo. Adenoviral BMP-2 Gene Transfer in Mesenchymal Stem Cells: In Vitro and in Vivo Bone Formation on Biodegradable Polymer Scaffolds. Biochemical and Biophysical Research Communications 2002;292:144–152. (*Joint first author).

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Abstracts

6. X.B. Yang, H.I. Roach, N.M.P. Clarke, R.S. Bhatnagar, S. Li, R.O.C. Oreffo. Biomimetic Collagen Scaffolds from Human Bone Tissue Engineering. Tissue engineering 2002 (Third Smith & Nephew International Symposiom. Atlanta, 13-16 Oct. 2002, USA. (Oral presentation).

7. D. Green, D. Howard, X.B. Yang, K. Partridge, N.M.P. Clarke, R.O.C. Oreffo. Marine Invertebrate Skeletons: Promising Tissue Engineering Scaffolds Particularly for Orthopaedic Applications. Tissue engineering 2002 (Third Smith & Nephew International Symposiom. Atlanta, 13-16 Oct. 2002, USA. (Oral presentation).

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9. X.B. Yang, H.I. Roach, N.M.P. Clarke, S.M. Howdle, K.M. Shakesheff, and R.O.C. Oreffo. Induction of bone formation *in vivo* using human osteoprogenitor and osteoblast stimulating factor-1 adsorbed scaffold constructs. Bone and Tooth Society Annual Meeting, Cardiff, June 2002, UK. (Oral Presentation).

10. K. Partridge, X.B. Yang, N.M.P. Clarke, Y. Okubo, K. Bessho, S. Howdle, K. Shakesheff, R.O.C. Oreffo. Bone and Tooth Society Annual Meeting, Cardiff, June 2002, UK. (Oral Presentation).

11. R.S. Tare, K. A. Partridge, X.B. Yang, N.M.P. Clarke, R.O.C. Oreffo, H.I. Roach. Osteoblast-stimulating factor-1 enhances osteogenic differentiation of bone marrow stromal cells, but is not osteoinductive. Bone and Tooth Society Annual Meeting, Cardiff, June 2002, UK.

12. X.B. Yang. Collagen scaffold for bone tissue engineering. British Orthopaedic Research Society Travelling Fellowship Report. British Orthopaedic Research Society Meeting, 8^{th}-9^{th} April, 2002. Leeds, U.K. (Oral presentation).

13. X.B. Yang, H.I. Roach, N.M.P. Clarke, S.M. Howdle, K.M. Shakesheff, H.C. Anderson and R.O.C. Oreffo. Novel Osteoinductive Biomimetic Scaffolds Stimulate Human Osteoprogenitor Activity Implications for Skeletal Repair. Seventh International Conference On The Chemistry And Biology Of Mineralized Tissues. November 4-9, 2001 Sawgrass Marriott Resort Ponte Vedra Beach, Florida, USA. (Oral presentation).

14. K. Partridge, X.B. Yang, N.M.P. Clarke, Y. Okubo, K. Bessho, S. Howdle, K. Shakesheff, R.O.C. Oreffo. Bone tissue engineering – adenoviral BMP-2 gene transfer into mesenchymal sten cells on biodegradeable polymer scaffolds. The second european meeting on cell engineering. Spain. Oct. 25th –28th, 2001. (Oral Pesentation).

15. X.B. Yang, H.I. Roach, N.M.P. Clarke, S.M. Howdle, K.M. Shakesheff and R.O.C. Oreffo. Biomimetic collagen scaffolds for human bone cell growth & differentiation. 3nd Annual Symposium of the Tissue and Cell Engineering Society, Keele, UK. September, 2001:P17.

16. K. Partridge, X.B. Yang, N.M.P. Clarke, Y. Okubo, K Bessho and R.O.C. Oreffo. Ex vivo bone formation using adenoviral BMP-2 gene transfer into mesenchymal stem cells. 3nd Annual Symposium of the Tissue and Cell Engineering Society, Keele, UK. September, 2001. (Oral Presentation).

17. X.B. Yang, H.I. Roach, N.M.P. Clarke, S.M. Howdle, K.M. Shakesheff and R.O.C. Oreffo. Osteoconductive biomimetic scaffolds and human mesenchymal stem cells -- cell-matrix interactions. International Sun Valley Hard Tissue Workshop, Sun Valley, U.S.A. Aug. 5-10, 2001. (Journal of Musculoskeltal & Neuronal Intreactions 2001;2(1):102-P25).

18. X.B. Yang, H.I. Roach, N.M.P. Clarke, S.M. Howdle, K.M. Shakesheff and R.O.C. Oreffo. Induction of human osteoprogenitor chemotaxis, proliferation and differentiation by a novel bone factor, osteoblast stimulation factor-1. Bone and Tooth Society Annual Meeting, Warwick, July 2001, UK (Oral Presentation). (Journal of Bone and Mineral Research 2001;16(6):1179-OC22).

19. X.B. Yang, H.I. Roach, N.M.P. Clarke, R. Quirk, S.M. Howdle, K.M. Shakesheff and R.O.C. Oreffo. Cbfa1, Osf-1 Expression and Ex Vivo Mineralisation by Human Osteoprogenitors on 3-Dimensional Porous Biodegradable Structures. 47th Annual Meeting of Orthopaedic research Society, San Francisco, USA. February 2001:P617. U.S.A.

20. X.B. Yang, H.I. Roach, N.M.P. Clarke, R. Quirk, K.M. Shakesheff and R.O.C. Oreffo. Growth and differentiation of human osteoprogenitors on biodegradable biomimetic structures and films. 22nd Annual Meeting of the American Society of Bone and Mineral Research.Toronto, Canada. 2000. (Journal of Bone and Mineral Research 2000;15:SA221)

21. X.B. Yang, H.I. Roach, N.M.P. Clarke, R. Quirk, K.M., S.M. Howdle, Shakesheff and R.O.C. Oreffo. CBFA1, OSF-1 espression and ex vivo mineralisation by the human osteoprogenitors on 3-dimensional porous biodegradable structures. 2nd Annual Symposium of the Tissue and Cell Engineering Society, Nottingham, UK. 2000:P27.

22. X.B. Yang, H.I. Roach, N.M.P. Clarke, R. Ouirk, K.M. Shakesheff and R.O.C. Oreffo. Biomimetic structures for human osteoprogenitor growth. The 50th Anniversary Meeting of The Bone and Tooth Society. 2000 Cambridge, UK. July 2000:P55. (Journal of Bone and Mineral Research 2000;15(6):1234-P55).

LIST OF AWARDS AND PRIZES

2002	1)	First prize, British Orthopaedic Research Society Annual Meeting 2002 (Third author).	U.K.
	2)	Commendation (oral presentation), Postgraduate Conference 2002, University of Southampton.	U.K.
	3)	First prize (oral presentation), Bone and Tooth Society Annual Meeting 2002 (Joint first author).	U.K.
	4)	First prize (poster presentation), Bone and Tooth Society Annual Meeting 2002 (Second author).	U.K.
	5)	Postdoctoral research fellowship, University California San Francisco.	U.S.A
2001	6)	First prize (poster presentation), 3rd Annual Symposium of Tissue & Cell Engineering Society, Keele.	U.K.
	7)	British Orthopaedic Research Society Travelling Award.	U.K.
	8)	International Chinese Hard Tissue Society Travel Award.	U.S.A.
	9)	Alice Jee Young Investigator Award, Sun Valley.	U.S.A.
2000	10)	First prize (poster presentation), 2nd Annual Symposium of the Tissue and Cell Engineering Society. Nottingham.	U.K
1999	11)	Three year Ph.D. studentship, University of Southampton.	U.K.