ORTHOPEADIC TISSUE ENGINEERING UTILISING IMMUNO-SELECTED HUMAN MESENCHYMAL STEM CELLS

Daniel Howard

Doctor of Philosophy

(October 2003)

University of Southampton

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This thesis is dedicated to Liz, my family, friends and

all the people who tried to educate me.

"Engineering is about the application of knowledge and experience to produce something, or to design a practical solution to a problem. In many ways it is the friendly face of science, because it is all about putting science to work for the benefit of humanity."

Professor Ann Dowling,

Mechanical engineering, University of Cambridge.

University of Southampton

ABSTRACT

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Skeletal disease accounts for approximately five million pounds of NHS spending per day and with an increasingly aging population this figure can only be expected to rise. A material that is able to fully repair damaged skeletal tissues would therefore be of great importance and tissue engineering has the potential to produce a material sufficient for repair. The use of mesenchymal stem cells for tissue engineering would provide a versatile cell choice, as they have been shown to be easily isolated from marrow and can be cultured to produce the characteristics of osteoblasts and chondrocytes. Furthermore, a defined population of mesenchymal stem cells can be isolated using the antibody STRO-1.

In this study a mesenchymal cell population was isolated using the antibody STRO-1 and tested for the ability to generate bone and cartilage. The chondrogenic ability of the isolated cells was tested by generation of aggrecan and type II collagen using micromass culture. For osteogenic tissue generation, selected mesenchymal stem cells were modified to produce BMP-2 and cultured on PLA scaffolds within *in vivo* diffusion chambers. For alternative tissue engineering strategies, PGA fleece, *Porifera*, supercritical CO_2 formed PLA and alginate cell supports were used as growth conduits. Growth conditions were optimised by using different seeding strategies, combined with perfused and rotating culture vessels to enhance nutrient exchange.

The selection of STRO-1^+ cells resulted in a population consisting of 7%±3% of the nucleated marrow cells. In culture the STRO-1^+ CFU-F colonies were larger in diameter and were able to express more alkaline phosphatase activity than unselected control cells. Using an *in vivo* diffusion chamber assay, STRO-1 mesenchymal stem cells formed mineralised, radio-opaque, alkaline phosphatase active, organised type I collagen material within the biodegradable PLA scaffold. In rotating culture with PGA fleece, the continuous cell lines ATDC5 and pZIP were able to produce coherent tissue-engineered constructs, thereby proving the viability of the rotating culture system. Furthermore, STRO-1^+ mesenchymal stem cells could be grown throughout PGA fleece scaffolds in rotating and perfused culture. However, culture conditions will have to be optimised for the use of aged primary human cells.

In conclusion, the methods used were able to support a scheme of cartilage and bone tissue engineering and to enhance progenitor cell numbers *in vitro,* although modification and integration of the constructs will have to be further investigated.

Acknowledgements

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I wish to thank my supervisors Richard Oreffo and Trudy Roach. The good people of University Orthopaedics; Kris Partridge, Xuebin Yang, David Green, Stephanie Inglis, Carol Roberts, Anne Linge, Debbie Collins, Rahul Tare and to Mr Nicholas Clarke for supporting academic work at University Orthopaedics. Collaborators and helpers; Yasanori Okubo, Kazuhisa Bessho, Steven Howdle, Kevin Shakesheff, Karina Stewart, Jon Beresford, Marcus Smith, Julian Chaudhuri, Bruce Caterson and Clare Curtis, and the financial support of the BBSRC, as without every last one of them it simply would not have been possible.

CONTENTS

ABBREVIATIONS

A/S	Alcian blue/ Sirius red
ABC	Avidin Biotin conjugate
AC	Articular cartilage
ACI	Autologous cell implantation
AdBMP2	Adenoviral - bone morphogenetic protein
AEC	3-Amino-9-ethyl-carbazol
AER	Apical ectodermal ridge
ALCAM	Activated leukocyte cell adhesion molecule
ALP	Alkaline phosphatase
ANOVA	Analysis of variation
BCIP/NBT	5-Bromo-4-chloro-3-indolyl phosphate/ nitro blue tetrazolium chloride
BMP	Bone morphogenetic protein
BMU	Bone multi-cellular unit
BSA	Bovine serum albumin
CBFA1	Core binding associated factor 1
CFU-E	Colony forming unit - Erythrocyte
CFU-Eo	Colony forming unit - Eosinophil
CFU-F	Colony forming unit fibroblastic
CFU-GEMM	Colony forming unit
CFU-GM	Colony forming unit - granulocyte monocyte
CFU-MEG	Colony forming unit
Chm-1	Chondromodulin-1

CILP	Cartilage intermediate-layer protein
CMFDA	5-Chloromethylfluoroscein diacetate
c-MYC	Chicken myelocytic
COMP	Cartilage oligomeric matrix protein
CrtL	Control Link
CS	Chondrotin sulphate
CsCl	Caseum chloride
DHEA	Dehydroepiandrosterone
DMEM	Dulbeco's modified Eagles media
DMMB	Di-methyl-methylene-blue
DPX	Distyrene plasticiser xylene
DTT	Dithiothreitol
ECM	Extra cellular matrix
EDTA	Ethylenediaminetetraacetic acid
EH -1	Ethidium homodimer 1
EtBr	Ethidium bromide
FCS	Fetal calf serum
FDA	Federal drug administration
FGF	Fibroblastic growth factor
FITC	Fluorescein isothiocyanate
GAG	Glycos amino glycan
GDF 9	Growth and differentiation factor - 9
GLUT	Glucose transporter
H & E	Haematoxylin and Eosin
HA	Hyaluronic acid

HA	Hydroxy appatite
HA/TCP	Hydroxy appatite/Tri calcium phosphate
HARP	Heparin affinity regulatory peptide
HARV	High aspect ratio vessel
HBBM	Heparin-binding brain mitogen
HB-GAM	Heparin binding growth associated molecule
HBNF	Heparin-binding neurotrophic factor
HBSS	Hanks buffered saline solution
HEPES	Hydroxyethylpiperazine ethanesulphonic acid
HOX	Homobox
IGD	Inter globular domain
IGF	Insulin like growth factor
IGF-BP	Insulin like growth factor - binding protein
IHC	Immuno histo chemistry
Ihh	Indian hedge hog
ITS	Insulin Transferrin Selenium
KS	Keratin sulphate
LTBP	Latent binding protein
MACS	Magnetically activated cell sorting
αMEM	Alpha modification of eagles medium
MSC	Marrow stromal cell
MSC	Mesenchymal stem cell
MSX-1	Msh homeo box homolog 1 (Hox 7)
NASA	National Aeronautics and Space Administration
NC4	Non collagenous protein 4 (of type IX collagen)

OA	Osteoarthritis
OB	Osteoblast
OC	Osteoclast
OP	Osteoporosis
PAGE	Poly acrilamide gel electrophoresis
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
PFA	Paraformaldehyde
PGA	Poly-glycolic acid
PHSC	Pluripotent haematopoietic stem cells
PLA	Poly-lactic acid
PLGA	Poly lactic co glycolic acid
PMSF	Phenylmethanesulfonyl fluoride
PNP	P-nitrophenyl phosphate
POB	Pre-osteoblast
PRELP	Proline/arginine rich end leucine rich repeat protein
PTH	Parathyroid hormone
PTH-rp	Parathyroid hormone-related peptide
PTN	Pleiotrophin
RER	Rough endoplasmic reticulum
RGD	Arg Gly Asp
rhBMP-2	Recombinant human bone morphogenetic protein
RUNX 2	Runt domain transcription factor (CBFA1)
SDS	Sodium dodecyl sulphate
SMA	Smooth muscle actin

SMAD	Small, mothers againsed decapentaplegic
SOX 9	Sex determining region Y with homobox features
STK	Serine threonine kinase
TBS	Tris buffered saline
TCP	Tri calcium phosphate
TE	Tris EDTA
TGF	Transforming growth factor
THR	Total hip replacement
TTBS	Tween- Tris buffered saline
VEGF	Vascular endothelial growth factor
Wnt	Wingless integration
ZPA	Zone of polarising activity

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1. INTRODUCTION

1.1. The skeletal system

The skeletal system is a hinged physical framework designed primarily for movement and mechanical support. The skeleton also serves to provide an important reserve of lipids, calcium and phosphates as well as a hardened protective covering for delicate systems such as brain, spine and marrow. Development of bone is a multiphase process originating from different embryonic cell groupings to provide a load adaptive (Mosley *et al.*, 1997), low weight, high strength alloy of mineral and interwoven collagen fibre, aided by using dense outer layers and a weight saving inner structure. At joints, bone is surfaced with cartilage, a flexible, impact damping and low friction material. Articular cartilage is a mixture of collagen fibrils and a proteoglycan pre-stressing agent. Due to the composition of bone and cartilage the joints such as at the hip can resist intermittent normal joint pressures of 5-15 MPa ($5\text{-}15 \times 10^6$ N/m^2; (Wong *et al.*, 2003)) often for more than 70 years of use.

1.1.2. Long bone structure

Long bone nomenclature is divided according to the location and structure of the components. The middle of a long bone is referred to as the diaphysis, the ends are epiphysis and between the two is the metaphysis. The features are described as proximal or distal in relation to the torso (figure 1.1). The bone structures are composed of two types of bone - solid cortical and sponge-like cancellous bone.

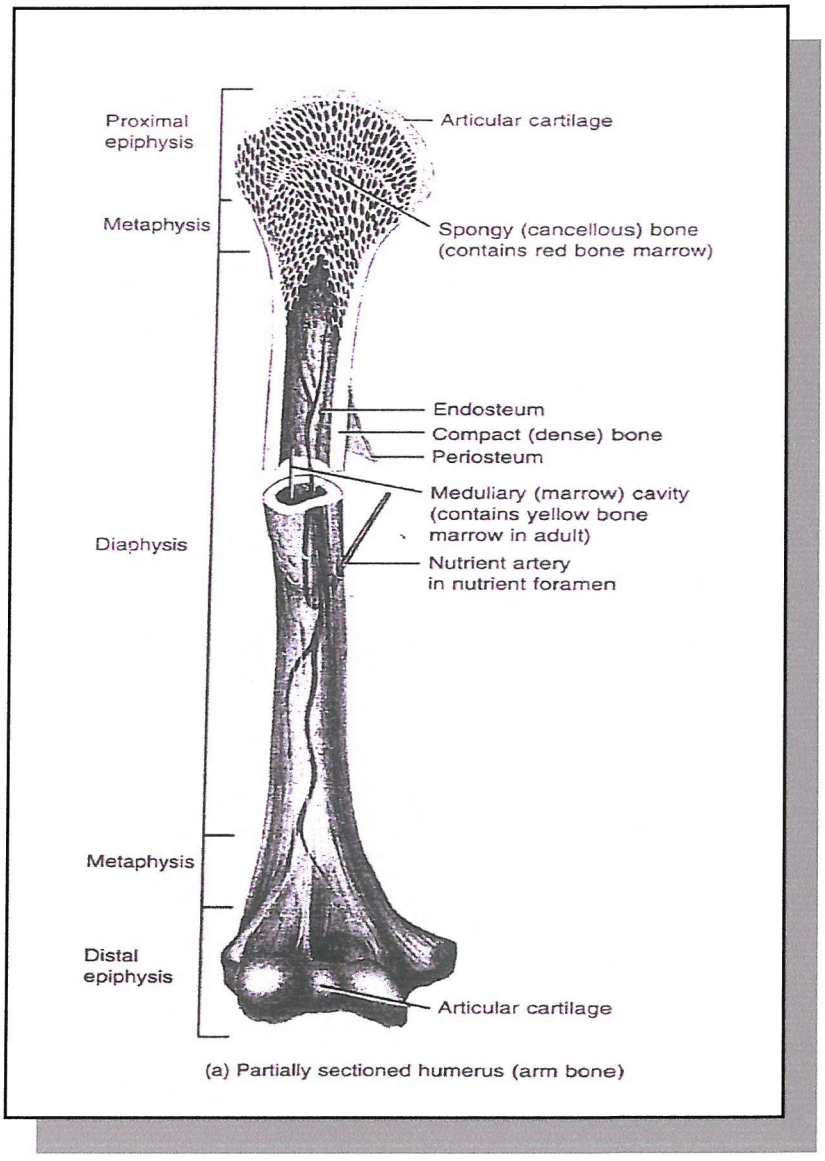

Figure 1.1. Structure of a long bone (from www.bmb.psu.edu)

1.1.3. Bone type

1.1.3.1. The strength of **cortical bone** is derived from a lamellar structure of mineralised collagen fibres orientated into bundles of 5-100μm Haversian columns. Each Haversian column is an organisation of mineralised collagen fibre layers running parallel around a central vessel. Perforating the Haversian lamellae are Volkman's canals (figure 1.2) allowing solute transport between cells in an otherwise impervious bone matrix.

1.1.3.2. Trabecular bone fills the semi-load bearing internal structure of the cortical bone. The architectural organisation of trabecular bones is able to reduce weight and still maintain most of the mechanical strength by aligning the trabecular struts along the areas stress lines. Trabecularisation also allows marrow and blood to infiltrate.

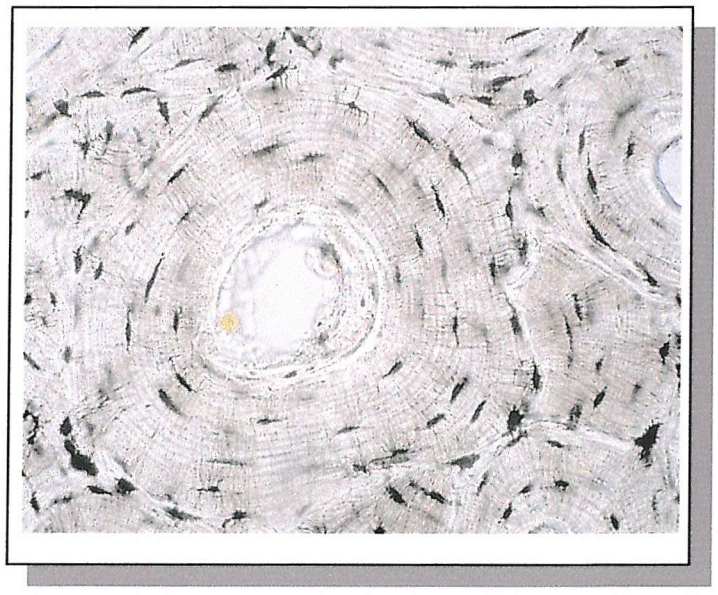

Figure 1.2. The lamellar haversian system in cross section with dark stained cells (osteocytes) with radiating canaliculi (from www.udel.edu/Biology).

1.1.4. Long bone morphology

The **diaphysis** is the tubular midshaft of cortical bone with an inner cavity of trabecular bone.

The **medullary cavity** is contained within the diaphysis of cortical bone occupied by cancellous bone. Within the cavity is the marrow, a diverse group of cells containing haemapoietic stem cells, stromal stem cells and marrow adipocytes.

The **Metaphysis** is a transition zone where the bone changes from long diaphysis to the wider structure of the epiphyses.

The **epiphyses** comprise the distal and proximal ends of the long bone. They are composed of dense trabecular bone covered by a layer of cortical bone. Epiphysis have an increased volume compared to the diaphysis and are covered by articular cartilage to form the articulating joints. In the growing long bone the region between the calcification centres of the diaphysis and epiphysis remains cartilaginous and is responsible for longitudinal growth of bones during childhood. These growth plates eventually calcify and form the **epiphysial line** in adult long bone.

The **periosteum** is a toughened fibro-cartilaginous cell-rich membrane that surrounds the bone, adding minor structural support, providing a rich source of bone progenitor cells and growth factors. It is composed of a bi-layer of cells, the outer layer consisting of fibroblasts surrounded by collagen filaments and the inner of an osteogenic layer from which cells can differentiate into osteoblasts. It is supplied with nerve fibres, lymph and blood vessels. In addition the periosteum is able to protect bone integrity, as loss of periosteum can result in loss of bone material.

The internal surfaces of bone are covered by **endosteum,** a single layer of flattened osteoprogenitor cells and a small amount of connective tissue.

1.1.5. Cells of bone and cartilage

The matrix of bone is maintained and cycled by a series of interacting bone cell types present throughout the matrix. These cells are A) osteoclasts, B) osteoprogenitor cells, C) osteoblasts, D) osteocytes, E) bone lining cells and F) chondrocytes.

A) Osteoclasts are formed from the fusion of blood monocyte/macrophage progenitor cells and function to degrade the bone matrix. They are large cells up to 20-100μm (0.1mm) in diameter, highly motile, capable of travelling 100μms and excavating material equivalent to the matrix deposited by 100-1000 osteoblasts (Rubin., 1997). The cell dies by apoptosis after approximately 3 weeks (Mundy., 1999). Osteoclasts resorb bone by attaching on one side of the cell via a characteristic actin-rich ruffled border, moving over bone and creating cavities (Howship's lacunae) via the secretion of an acidic environment with degrading enzymes including collagenases and acid phosphatases.

B) Osteoprogenitor cells provide a source of undifferentiated cells from which osteoblasts, osteocytes and chondrocytes derive. Osteoprogenitors are an undifferentiated pool of cells found on most bone surfaces and within the marrow, they also have the potential to produce adipocytes, reticular cells and fibroblasts (Oreffo *et al.*, 1999; Owen *et al.*, 1988).

C) Osteoblasts are bone forming, alkaline phosphatase-rich cuboidal cells able to lay down organic bone matrix at bone forming surfaces for mineralisation. As mineralisation proceeds osteoblasts can become encapsulated within the abundant matrix, once within the calcified matrix the osteoblast cell performs a maintenance function and is referred to as an osteocyte.

D) Osteocytes are maintenance cells and comprise the most abundant group of cells within bones. Osteocytes morphology varies with age and functional activity comparable in composition to their osteoblast counterpart, to the final form of a condensed cell with reduced rough endoplasmic reticulum (RER) and golgi. Osteocytes are connected to each other via a canaliculae enabling cell to cell communication (Figure 1.3) and are proposed to function as actuators of mechanical stimuli to signal bone remodelling. The mechanical signalling is transduced, at least in part, by glutamine and receptors more commonly associated with brain signal transduction (Skerry *et al.*, 2001).

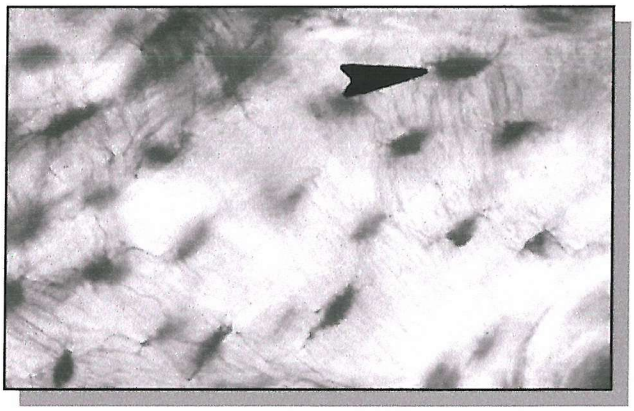

Figure 1.3. Silver nitrate stained osteocytes (arrowed; 400x magnification: www.usc.edu)

E) Bone lining cells a layer of thin flattened inactive cells play a key role in preventing bone re-absorption. Removal of bone lining cells usually results in re-absorption of the underlying bone.

F) Chondrocytes form the cartilage template by which most bones develop. Bone template chondrocytes hypertrophy and die and are eventually replaced by bone. Chondrocytes remain at the growth plate during growth and at the ends of bones as articular cartilage.

1.1.6. Composition of Bone

Bone provides support by a composite structure analogous to reinforced concrete, with the inorganic mineral component for hardness and resilience from protein based organic interwoven matrix of fibrous collagen (Glimcher., 1990).

1.1.6.1. Bone inorganic content

The bone mineral is carefully deposited on collagen. Mineral precipitation is initiated by nucleating proteins, bone sialoprotein and osteocalcin (reviewed in (Roach., 1994)). Calcium phosphate in the form of ~100Å crystalline hydroxy apatite ($Ca_{10}[PO_4]_6[OH]_2$) constitutes approximately 65% of the inorganic mass of bone (reviewed in (Boskey., 2003; Glimcher., 1990)). Organisational maturation of the mineral content results in an increase in bone strength with age (Su *et al.*, 2003) and varies in strength with varying morphology, dimension and distribution of apatite crystals (Boivin *et al.*, 2003). Alternate forms of calcium phosphate and carbonates with small amounts of sodium, magnesium and fluoride comprise the rest of the inorganic component.

1.1.6.2. Bone organic content

The organic component of bone includes the cells, secreted osteoid and is composed predominantly (~90%) of type I collagen. Collagens provide the structural framework for connective tissues by utilising their fibrous tensile nature. Collagens, including type I, organize from an initial pro-protein into trimerous chords, which in turn form much larger bundles complexed by other ancillary molecules. Other organic constituents comprise approximately 10% by weight of the organic matrix, and include bone sialoprotein, osteocalcin, osteonectin, chondroitin sulphate, albumin, glycoprotein, lipids, laminin and fibronectin (Roach., 1994).

1.1.7. Skeletal development

The skeleton is composed of organised cells and tissues that exists throughout the body, the regulation of such a structure is therefore complex and has a plethora of controlling genes regulated temporally and spatially through development.

The skeleton derives from three tissue groupings (not including some of the smaller elements of jaw) essentially i) the neural crest for the cranium, collar bones and sternum ii) the spine and disks (axial) form from paraxial somite mesoderm, iii) the limbs then form around this from separate mesodermal developmental buds, all of which intercalate to give the final singular appearance (Hall *et al.*, 1992; Olsen *et al.*, 2000).

Limb development is controlled by zones of polarising activity (ZPA) with the apical ectodermal ridge (AER) zones responsible for limb bud growth. ZPA/AER can be multiple along the embryo but are very tightly controlled by the Hox (HomeoBox) group of genes controlling limb patterning and T-box genes regulating limb differences.

Skeletal cells differentiate down the skeletal route by induction of two key genes, *sox9* and *cbfa1*. The sox genes (*sry* (sex determining region Y) with Homobox features) are associated with chondrocyte development and *cbfa1* (core binding associated factor -1) with osteoblast development. In *sox9* knockout mice, no chondrocyte specific markers are expressed with resulting effects on the endochondral bone formation. *Sox9* functions by binding a *Col2a1* transcription site directly activating collagen expression (Lefebvre *et al.*, 1998; Olsen *et al.*, 2000; Yan *et al.*, 2002).

As well as *sox9, cbfa1* plays a pivotal role in osteogenesis (Komori *et al.*, 1998) highlighted by studies in which a lethal *cbfa1* mutation results in absence of osteoblast differentiation and bone formation (Komori *et al.*, 1997; Otto *et al.*, 1997). *Cbfa1* is

able to increase bone matrix formation and mineralisation by up-regulating the expression of bone matrix maturing genes, including type I collagen, osteopontin, bone sialoprotein, osteocalcin, and fibronectin. (Ducy., 2000; Kern *et al.*, 2001; Prince *et al.*, 2001).

1.1.8. Histogenesis and development of bone and cartilage

As different elements are formed via different embryonic cell groups, so the skeleton also forms in different ways – the two distinctive forms are 1) intramembranous ossification via calcification of a membrane and 2) endochondral ossification via the calcification of a cartilaginous template.

1) Intramembranous ossification (Figure 1.4) starts with a mesenchymal condensation where cells differentiate directly into osteoblasts without a cartilaginous template. Centres of ossification develop and produce trabecular bone, which is penetrated by growing blood vessels and mesenchyme forming marrow. Connective tissues on the outer surfaces then form the endosteum and periosteum. It is the source of most flat bones and contributes to the growth of short bones and thickening of the long bones.

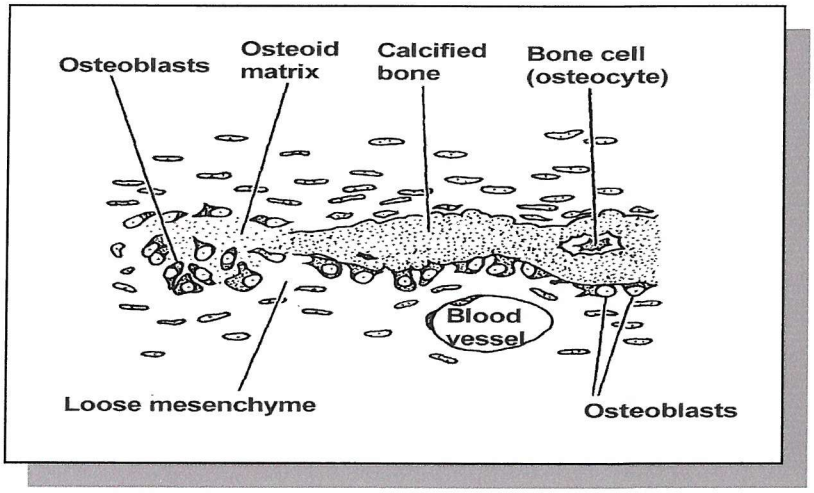

Figure 1.4. Intramembranous ossification (Adapted from Gilbert 1997).

2) Endochondral ossification (figure 1.5) is responsible for the formation of short and long bones via a chondrogenic template. Endochondral ossification occurs in three main stages – i) condensation of mesenchymal tissue, ii) differentiation into chondrocytes which pattern the skeletal tissues and iii) replacement with bone via ossification centres aided by vascularisation. The condensation of aggregates of cells occur with the development of the foetus, forming the final cartilaginous skeletal features (Delise *et al.*, 2000). The cartilage template subsequently partially calcifies around the diaphysis leading to invasion by a vascular supply forming buds of osteoprogenitor cells. The buds of osteoprogenitor cells expand, penetrate the cartilage template and secrete bone matrix. The bone matrix calcifies by spreading from the ossification centres formed from the vascular invasions.

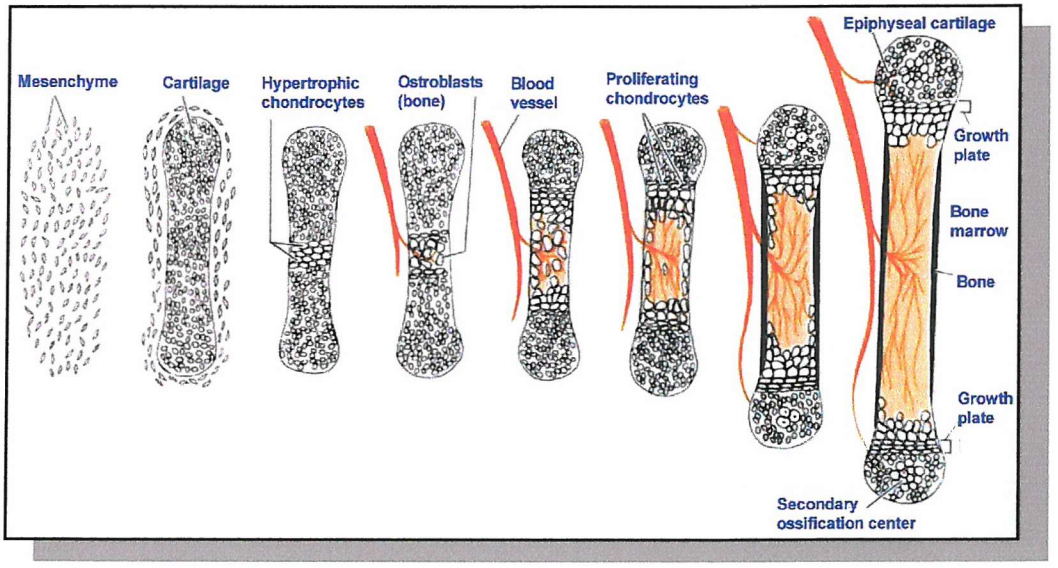

Figure 1.5. Endochondral ossification of the long bones (from classes.aces.uiuc.edu).

1.1.9. Bone remodelling

Bone is constantly remodelling and adapting, allowing regulation of the body's mineral content and bone density (figure 1.6). Skeletal remodelling can be triggered by changes in mechanical forces, damage and hormonal responses to changes in calcium and phosphorus. Overall, control of remodelling is by calcium regulating hormones parathyroid hormone (PTH), parathyroid hormone-related peptide (PTH-rp) and vitamin D, which regulate rates of cell differentiation and mineral transport. A variety of other hormones including calcitonin, leptin, growth hormone, thyroid hormones, glucocorticoids and oestrogen also have major effects on skeletal remodelling as does signalling similar to neurons (Skerry *et al.*, 2001).

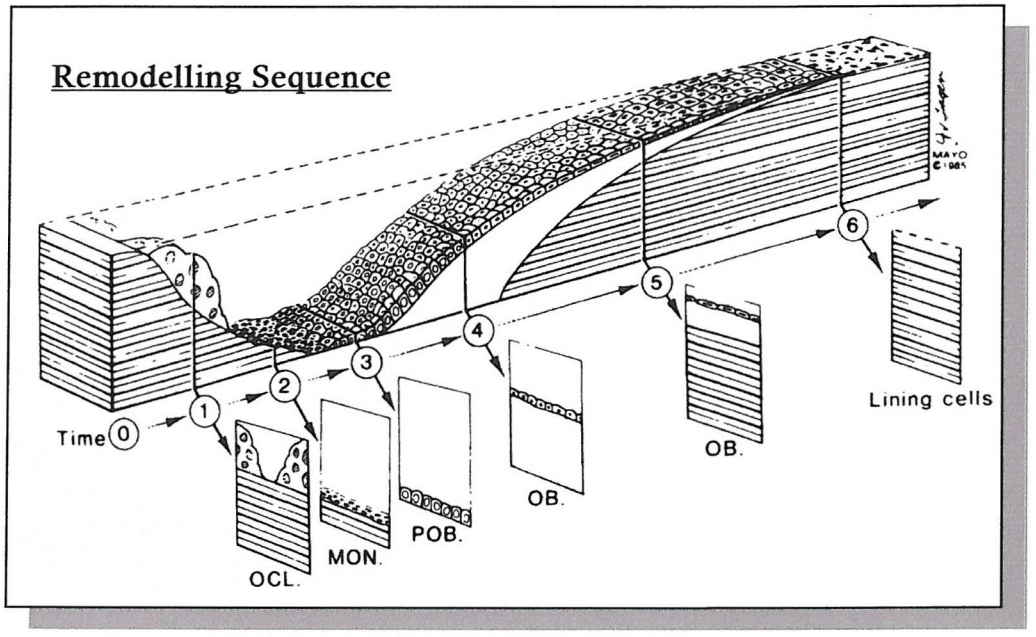

Figure 1.6. Bone remodelling.

1) osteoclast (OCL) resorption of the bone matrix, 2) reversal, 3) pre-osteoblast (POB) attraction, 4) osteoblasts (OB) form bone matrix, 5) mineralisation of the osteoid occurs, 6) mineralised bone structure is completed with a layer of lining cells (Eriksen *et al.*, 1994).

Once initiated, remodelling involves bone matrix degradation followed by the matrix being filled in with new osteoid, this process refreshes the skeleton approximately every 10 years. The series of events which result in renewal of bone material can be broken down as follows:

1) Once the signal for bone degradation has been recognised, osteoclast precursor cells are chemo-attracted to the site of degradation.
2) The osteoclasts form from the monocyte/macrophage cells and are able to produce an extremely acidic environment containing degrading enzymes on the surface attached to the bone, which is then able to dissolve bone matrix leaving an open channel.
3) Osteoclasts apoptose after approximately 3 weeks leaving an exposed channel resulting in release of factors able to recruit osteoprogenitor cells to the site.
4) Attracted osteoprogenitors differentiate to osteoblasts and synthesise new bone matrix filling the lacunae.
5) The matrix mineralises and bone lining cells adhere. Histological observation shows the events occurring in synchrony as bone multi-cellular units (BMU) within Howship's lacunae (cutting cones; figure 1.6).

1.1.10. Growth plate of long bones

In the epiphyses of long bones a disk of cartilage continues to grow and transform to bone, under the protection of the epiphyses, until adulthood (figures 1.7 and 1.8). The epiphysial growth plate maintains an un-mineralised cartilaginous region, where chondrocytes can continue to grow in size and number, enabling an increase in the size of the bone by continually supplying new cartilage material for mineralisation.

Elongation occurs by providing seed cells which rapidly multiply, increase in size and produce matrix, predominantly in columns arranged in the longitudinal plane so providing maximal lengthening per cell (Hunziker., 1994). Elongation at the growth plate is regulated primarily by growth hormone (Siebler *et al.*, 2001) with controlling gradients and feedback of Indian hedgehog protein at the endochondrium, combined with parathyroid-releasing hormone and parathyroid-related protein from the upper growth plate inducing proliferation and effecting hypertrophy (figure 1.7: (Karsenty., 2003; Stevens *et al.*, 1999; Volk *et al.*, 1999; Wagner *et al.*, 2001; Williams *et al.*, 1998)).

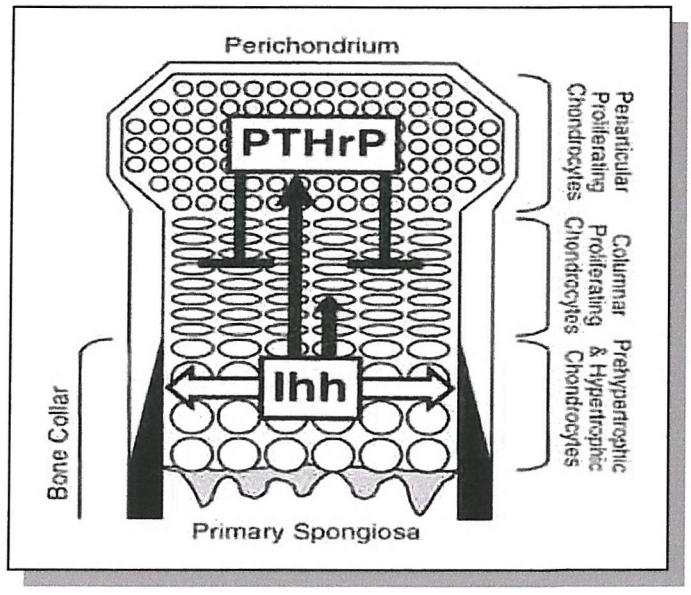

Figure 1.7. Regulation of the growth plate (from Karsenty 2001)

The bias between cell number, cell volume and matrix accumulation shows growth attributable to approximately 10% by cell proliferation, 30% by matrix deposition and a surprising 60% by chondrocyte enlargement (Wilsman *et al.*, 1996). The growth rate times from proliferation to terminal differentiation vary but, typically, occurs over about 20 days in humans dependant on their age (Kember *et al.*, 1976).

The growth plate can be divided into histological zones (figure 1.8), which also elegantly highlights the function and progression of the growth plate development through time.

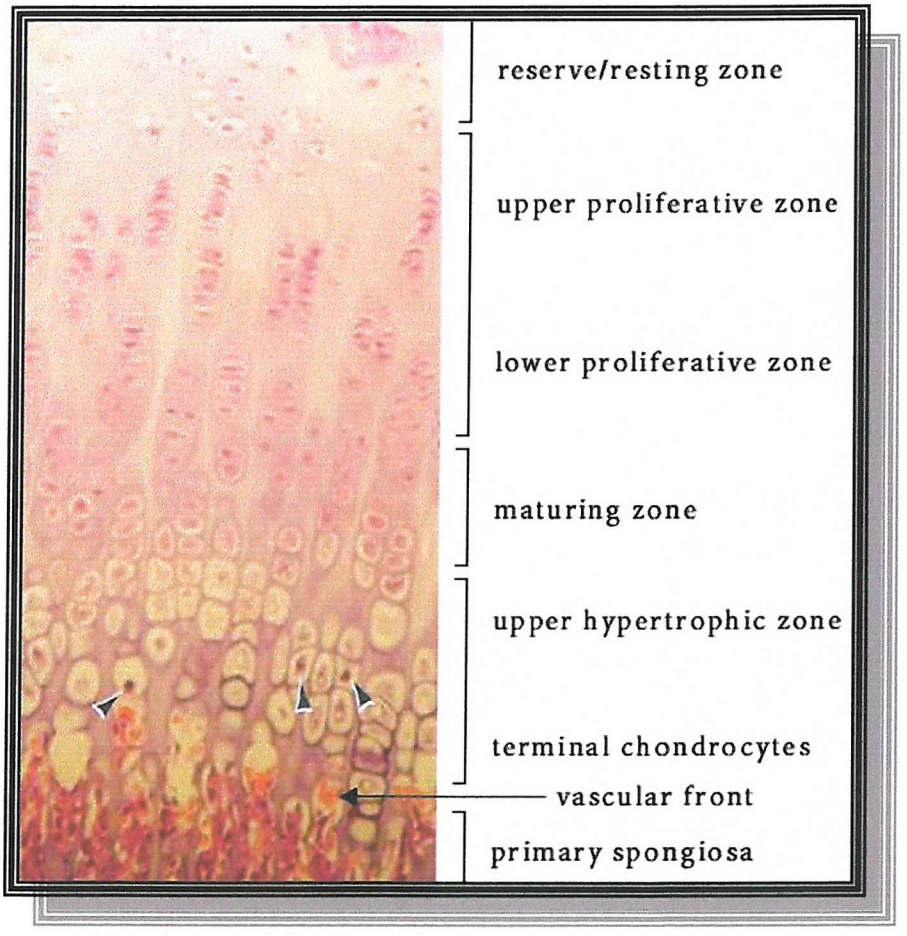

Figure 1.8. Murine growth plate (Arrows show apoptotic cells:

Used with permission from Dr Trudy Roach).

The histological appearance of the growth plate allows it to be divided as follows; **reserve zone** is a region populated by sparse, small, round chondrocytes and pre-chondrocytes that serve as a pool of new cells for proliferation.

In the **proliferative zone** cell numbers increase generating a population of differentiating chondrocytes expressing type II collagen and aggrecan (Kosher *et al.*, 1986). Chondrocytes grow in an ellipsoid morphology which then grow in longitudinal columns due to aligned successive mitotic divisions (Hunziker., 1994).

Maturing zone, increase in total volume occurs via simultaneously occurring tissue growth parameters, but not proliferation. The chondrocytes increase in cell volume by changing from flattened ellipsoid cells to columns of rounded cells and actively produce more matrix in order to increase the size of the growth plate. The growth in size and matrix production can be seen to occur with an increase in size of rough endoplasmic reticulum (RER) and Golgi (Farquharson *et al.*, 2000; Hunziker *et al.*, 1999).

Within the **hypertrophic zone** chondrocytes increase in size and formations of irregular calcium phosphate spicules can be observed along with strong expression of type X collagen.

In the **terminal zone,** cells down regulate type II collagen expression but alkaline phosphatase, osteonectin, osteopontin, VEGF and vitamin D receptor increase. Chondrocytes degenerate and die by apoptosis before replacement by bone tissue (Farquharson *et al.*, 2000; Gerber *et al.*, 1999).

In the **calcification zone,** calcified cartilaginous matrix is invaded by osteogenic cells and capillaries from the bone marrow cavity. Osteogenic cells gather on calcified spicules of cartilaginous matrix and begin to deposit bone matrix. Cells from the

metaphysis then resorb parts of the mineralised matrix, and lay down new mature bone on the remaining mineralised cartilage.

1.1.11. Bone fracture repair

After trauma resulting in breaking of the bone, repair is initiated by the disruption of the blood supply causing a haematoma, which provides the initial supporting material for subsequent stages. Infusion by a large number of inflammatory cells then release factors that initiate repair, as well as growth factors from surrounding tissues. The torn periosteum initiates intramembranous bone growth and within 24 hours cells differentiate into osteoblasts with osteoblast numbers peaking at day 10. "Soft" mixed fibrous and cartilaginous callus forms increasing the structural integrity with chondrocytes producing a cartilaginous matrix for approximately 3 weeks with hypertrophy of these chondrocytes starting after the first week (Barnes *et al.*, 1999). The cartilaginous matrix is replaced with immature load-bearing bone matrix, which is then remodelled into mature bone. The events of fracture repair occur with many conserved features of endochondral formation (Ford *et al.*, 2003).

1.2. CARTILAGE

Cartilage can be found throughout the body including in the following - nose, ears, bronchi, oesophagus, trachea, larynx and lumbar intervertebral disks and forms the template for the adult skeleton during development as well as during the growth phase of long bones at the epiphysial plates.

Cartilage can be characterised as clusters of cells trapped within their own excess of secreted collagen and glycoprotein matrix with no blood vessels or nerves, only receiving nutrients through a hydrated matrix. There are a variety of cartilage subtypes with their own distinct characteristics, commonly divided into the following;

Fibrous cartilage (figure 1.9A) is present in intervertebral disks, menisci and perichondrium. Fibrous cartilage is comprised of organized layers of matrix within which cells are trapped between large fibrous bundles of type I collagen making it strong under tension.

Elastic cartilage (figure 1.9B) also known as tendinous or reticular is present in tendons, epiglottis, larynx, auditory tube, ears and the pubic symphysis. It is comprised of a high content of twisted elastic elastin fibres and collagen type II, making it strong yet elastic.

Hyaline cartilage (figure 1.9C) is rich in type II collagen and proteoglycan. The smooth hard glassy cartilage is found at articulating joints. Chondrocytes within the hylane matrix are more sparsely distributed, with an abundance of matrix, than either fibrous cartilage or elastic cartilage.

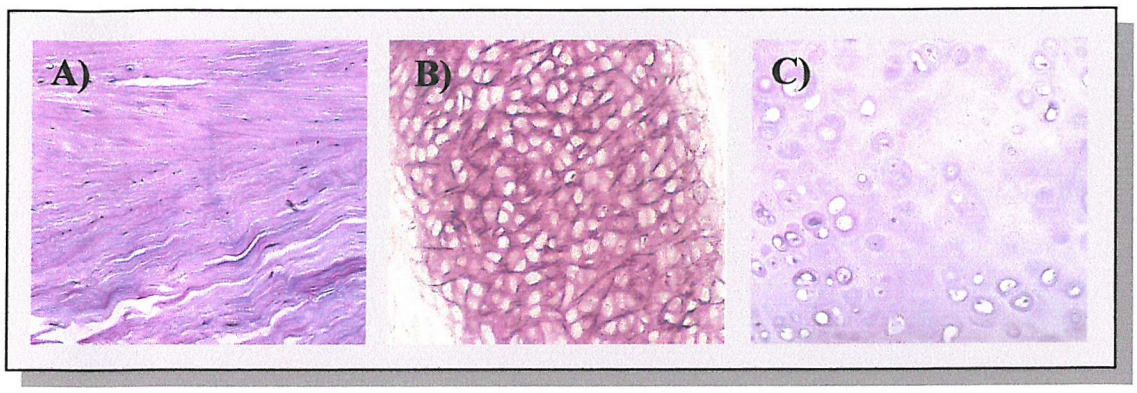

Figure 1.9. Different cartilage subtypes: A) Fibrous cartilage, B) Elastic cartilage and C) Hyaline cartilage (Modified from www.cellbio.utmb.; Edu and Young 2000).

1.2.2. Chondrocytes are the cells that secrete and maintain cartilage matrix, chondrocytes are typically oval and trapped in the cell matrix, often without contact to other chondrocytes. They are found in different densities in the different types of cartilage and even different regions of the same cartilage, resulting in a variety of chondrocyte phenotypes. Histological features of chondrocytes within hyaline cartilage are distinct, the cells have multiple small caveolae processes leading into the matrix and occupy lacunae. They are surrounded by a chondron "basket" of collagenous pericellular matrix, which separates the cells from the rest of the matrix. The matrix zones around the chondrocytes can be divided into the closer territorial matrix, which tends to stain differentially, to the interterritorial matrix of the cartilage tissue. Chondrocytes typically have large synthetic organelles associated with matrix synthesis and glycogen droplets for energy storage in the avascular matrix (Eggli *et al.*, 1988).

1.2.3. The composition of cartilage

Cartilage although tough, comprises up to 80% water, however the manner in which water is bound within the collagen meshwork gives cartilage properties of strength, low friction and impact damping (Maroudas *et al.*, 1987). Unlike most other tissues the matrix forms over 90% of the dry weight of the tissue (Hardingham *et al.*, 1992). Cartilage matrix is definable through the production of two main constituents – fine (20-50nm; (Hunziker *et al.*, 1987)) fibrous type II collagen and the large highly glycosylated proteoglycan pre-stressing material, aggrecan, which make up approximately 40% and 60% of the matrix respectively. Other multiple ancillary but important molecules such as decorin, biglycan and fibromodulin allow the collagen fibres to form and allow interactions with proteoglycans and cells (figure 1.10; (Knudson *et al.*, 2001)).

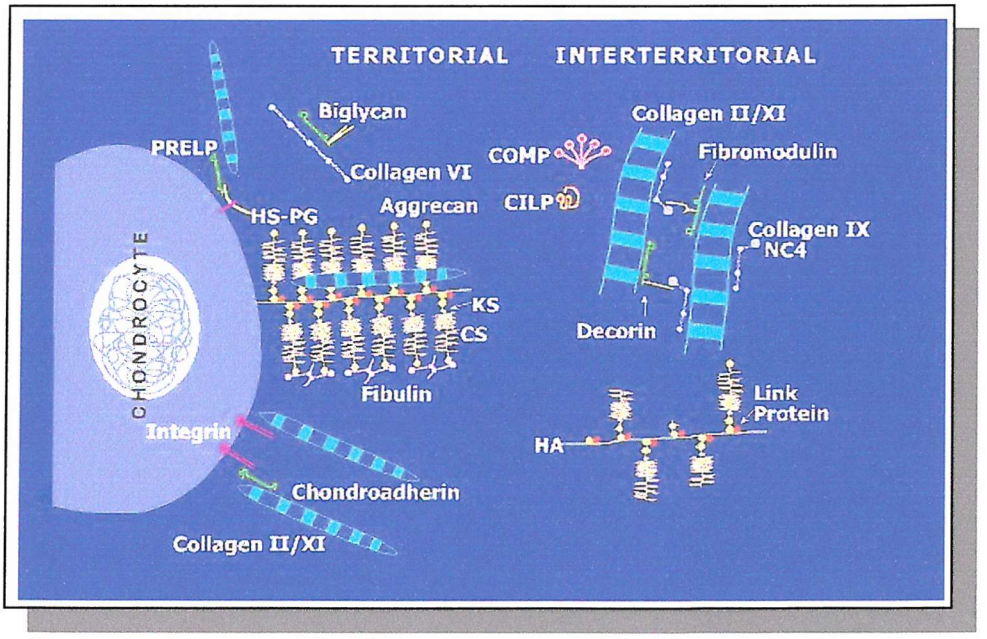

Figure 1.10. The main components of cartilage matrix (from www.cmb.lu.se/ctb).

1.2.3.1. Type II collagen

Type II collagen, found almost exclusively in cartilage, consists of a fine network of fibrils that provide cartilage with great tensile strength. Type II B $[\alpha 1(II)]_3$ (alpha 1 type II homotrimer) collagen is the predominant form (Ottani *et al.*, 2002). Type II collagen can be found in heterotypic fibres, associating with other connector proteins such as decorin, which are able to modify the properties of collagen via regulation of fibril diameter and attachment to other proteins (Knudson *et al.*, 2001). Type II collagen is also found in a modified lengthened version within the cornea (Linsenmayer *et al.*, 1977).

1.2.3.2. Aggrecan (Large aggregating proteoglycan) is a branched proteoglycan which when combined with hyaluronan develops many levels of branching, analogous to the trunk, branches and fronds of a pine tree (figure 1.11). The protein branch component of the proteoglycans is a ~230kDa core protein with several distinct functional regions (Caterson *et al.*, 2000; Watanabe *et al.*, 1998) with one end complexing via folded disaccharide covered globular domains (G1) to hyaluronan molecules resulting in a complex of over $3x10^6$ kDa (Heinegard *et al.*, 1985). The protein core is covered in fronds of long chains of negatively charged glycosaminoglycan (GAG) chondroitin and keratin sulphate chains composed of 40-50 repeats of glucosamine and galactose sugars (glucaronic and uronic acid) (Heinegard *et al.*, 1977). Aggrecan is intimately linked with the compression resistance of cartilage and is a highly indicative marker of the chondrocyte phenotype.

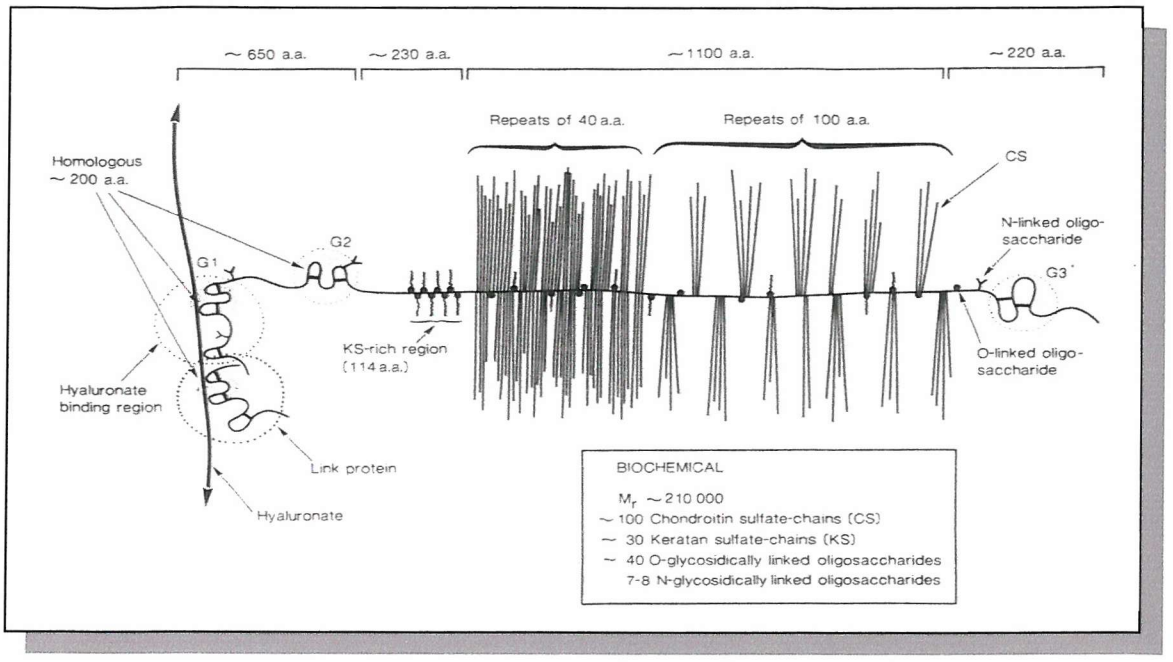

Figure 1.11. Aggrecan (from (Heinegard *et al.*, 1993)).

1.2.4. Other matrix components of articular cartilage

Proteoglycan molecules bind to long **Hyaluronan** (hyaluronic acid) chains forming super aggregates of over 3GDa. They are comprised of repeats of 2 linked sugars - glucaronic acid and glucosamine, these disaccharides are then repeated up to 10,000 times resulting in large molecules up to 10μms in size (Hardingham *et al.*, 1992).

Link protein aids attachment of proteoglycans to hyaluronan and is similar in structure to the globular binding region of aggrecan. Link is essential to cartilage stability, with knockout mice of the link controlling gene, *crtl1,* resulting in severely stunted growth (Watanabe *et al.*, 1999).

Small (40kDa) lysine rich proteoglycan's named **biglycan** and **decorin** are also found in cartilage matrix. Their molecules bind to the surface of collagen fibrils, where they regulate collagen diameter (Kavanagh *et al.*, 1999; Neame *et al.*, 1989; Visser *et al.*, 1998).

1.2.5. Cartilaginous Di-Athrodal Joints

Di-arthrosis are covered in articular cartilage, which in turn is surrounded by a synovial membrane able to secrete a lubricating fluid, thus keeping the friction levels of the articulating joints low. The joints are stabilised by strong ligament connections to stop any potentially damaging separations of the joint. Knee joints are aided by a menisci, a thick crescent shaped wedge of tough fibrous cartilage material, acting to further support and cushion the joint. The articular cartilage of the joints is organised at the molecular level to cope with the high mechanical stresses and strains of movement under load, by layering into different zones (figure 1.12). The different zones are able to cope with shear at the surfaces and compression in the lower zone. The zones contain different collagen organisation as well as different amounts of load resisting proteoglycans (Muir., 1995; Treilleux *et al.*, 1992). In later life the zone may become less clear or not present at all as the surface zones are eroded by wear and enzymatic damage and the lower zones succumb to calcification. In knee and hip joints the cartilage is layered into four zones:

1) The **tangential** (superficial/gliding) zone (figure 1.12) comprises 10-20% of the cartilage thickness, covering the surface and merges with the perichondrium at the sides of the joint. The tangential zone only contains a limited number of flattened chondrocytes cells. The abundant (85% by dry weight) fibrillary collagen in this region is aligned perpendicular to the surface along the lines of stress and has less proteoglycans for shear and abrasion resistance (Freeman., 1979).

2) **Intermediate** (middle/transitional) zone is characterised by an excess of rounded chondrocytes, fibrillary collagen organised randomly and an abundance of glycosaminoglycans. This layer constitutes approximately 60% of the cartilage

thickness and has increased synthetic activity over the other layers, evidenced by the increased sizes of the Golgi and RER (Eggli *et al.*, 1988).

3) **Radial** (deep) zone cartilage contains chondrocytes organised into columns of cells nested together in chondrons with thicker more aligned collagen fibres. The cells of this level tend to have more glycogen stores and constitutes approximately 30% of the total cartilage thickness.

4) The **Calcified** subchondral region interfaces with the bone and is distinguishable by the differentiation into the bone matrix accompanied by a calcification "tide mark".

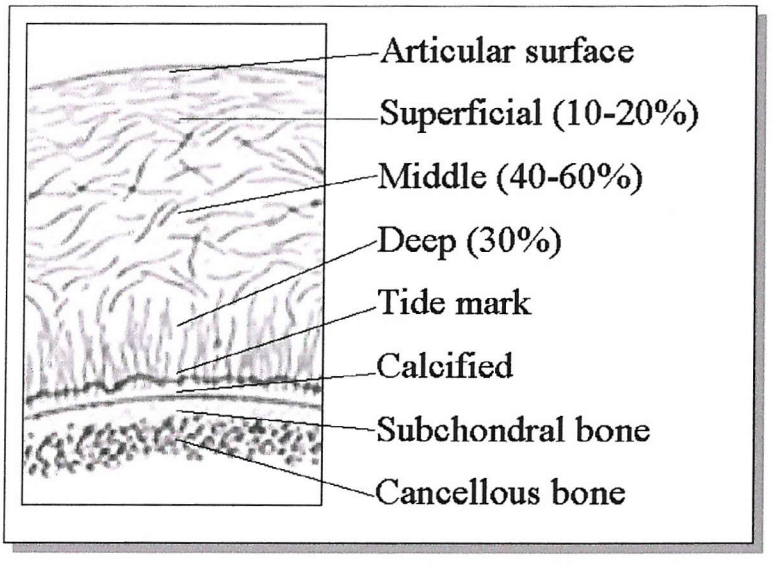

Figure 1.12. Articular cartilage construction with reference to collagen fibre orientation (modified from (Tyyni *et al.*, 2000)).

1.3. BONE MARROW

Marrow occupies the internal trabecularised cavities of the bones and is highly vascularised by thin walled vessels with a specialised sinus system. The marrow is comprised of red haematopoietic cells and fatty yellow marrow supported by a fibrous stroma of cells (figure 1.13).

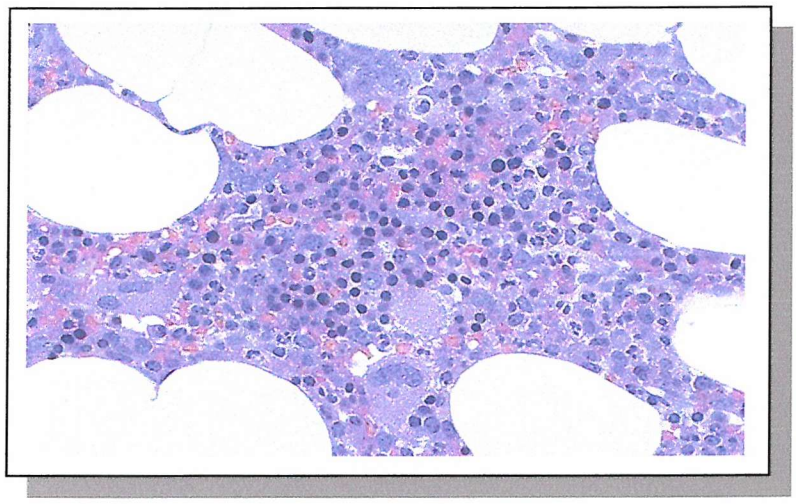

Figure 1.13. Sectioned marrow of a normal individual

(large spaces are adipocytes; from www.aamdsglossary.htm).

Red haemopoietic bone marrow contains the myeloid cells that make red blood cells, white blood cells and platelets. Red marrow has been shown to contain the pluripotent haematopoietic stem cells (PHSC).

Yellow marrow contains more adipocytes than red marrow and is postulated to provide a lipid store, heat, energy and a source of fat derived factors (Gimble *et al.*, 1996). With an increase in weight and age, the balance between red and yellow marrow shifts toward yellow marrow as the two can interconvert (Maniatis *et al.*, 1971; Meunier *et al.*, 1971).

1.3.1. Cells of marrow

Pluripotent haemopoietic stem cells (PHSC) differentiate into the multitude of morphologically distinct cells including erythrocytes, granulocytes, lymphocytes, thrombocyte cells (platelets), megakaryocytes, granulocytic precursors as well as providing the source of the multinucleated osteoclasts which reabsorb bone (figure 1.14).

Stromal cells provide growth factors and physical support to the blood producing cells (Bianco *et al.*, 2001b).

Adipocytes form more of the marrow volume in later life typically displaying large vacuoles filled with lipids.

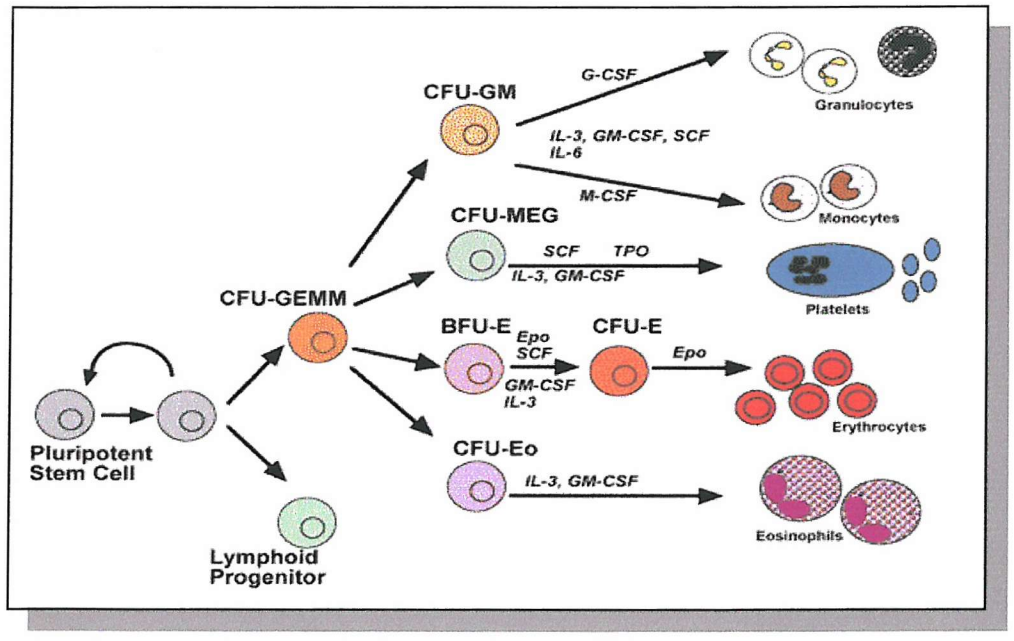

Figure 1.14. Differentiation of blood cell types (from web.wi.mit.edu).

1.3.2. Histogenesis and development of marrow

During development, stem cells located in the dorsal mesentery near the primitive aorta migrate to the yolk sac. The progeny rapidly divide and develop into blood cell precursors of erythroid, myeloid, monocyte, lymphoid, megakaryocytic and endothelial cell types. The location of haematopoiesis shifts from the yolk sac to the developing liver (Rossant *et al.*, 2002), to the spleen, then forms the marrow within the medullary cavity. The major sites of blood production continues to shift within the marrow groupings during adult life from long bones to mostly the flat bone marrow cavities in later life.

1.4. MARROW STROMAL CELLS

In bone, marrow stromal cells (MSC) act in a supporting role where they are thought to be part of a haematon, a specific organisation of haematopoietic and supporting cells (figure 1.15; (Bianco *et al.*, 2001a; Sullivan *et al.*, 1989)). Fibroblastic colonies forming in *in vitro* cultures of human bone marrow are thought to represent the stromal cells and further cultures of these cells have been shown to be convertible to adipocytes, chondrocytes, osteoblasts and reticular cells. Thus the marrow stromal cell, represented in culture by the fibroblastic colony, is thought to represent an undifferentiated pool of stem cells (Friedenstein., 1995; Pittenger *et al.*, 1999).

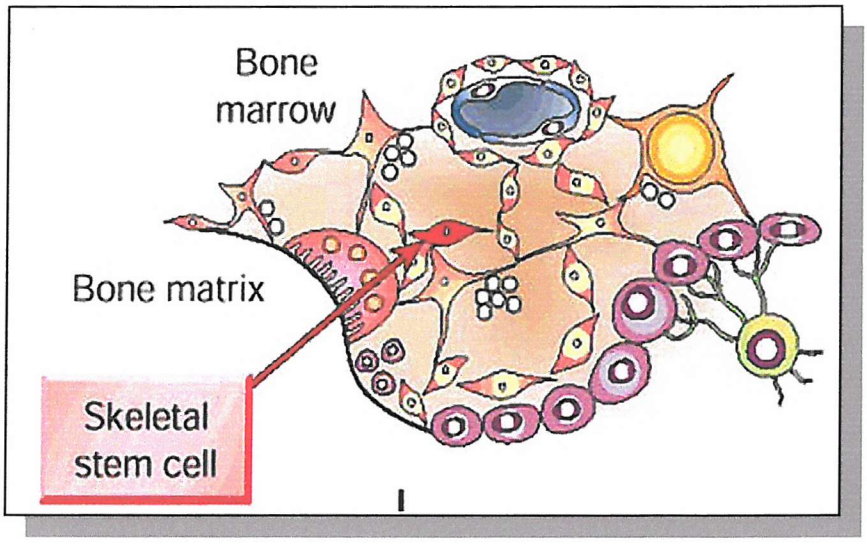

Figure 1.15. Marrow stromal cells in the haematon (from Bianco and Robey 2001).

1.4.2. Marrow stromal cells as adult stem cells of the skeletal system

Marrow stromal cells can be isolated from marrow as adherent colony forming units - fibroblastic (CFU-F(Friedenstein *et al.*, 1966)). CFU-F when subject to appropriate stimuli are able to differentiate into reticular cells, fibroblasts, osteoblasts, adipocytes and chondrocytes (figure 1.16; (Ashton *et al.*, 1980; Bruder *et al.*, 1998; Friedenstein

et al., 1966; Pittenger *et al.*, 1999)) these cells therefore represent a stem cell population present within the marrow (Owen *et al.*, 1987).

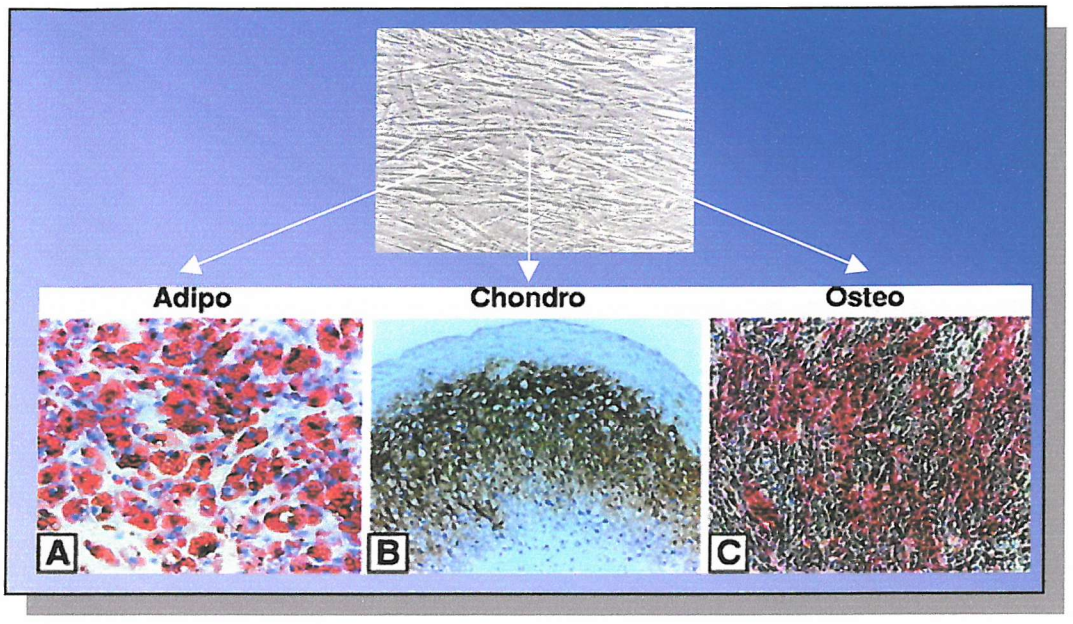

Figure 1.16. Multipotential nature of stromal cells with different *in vitro* culture conditions (Modified from Pittenger *et al* 1999).

1.4.3. Marrow stromal cell population

Marrow stromal cells are present at a very low density within bone marrow representing less than 1 in 3 x 10^4 nucleated cells (Aubin., 1999; Oreffo *et al.*, 1998b). Variation in numbers is not age significant in adults (14-87 years male and female; (Oreffo *et al.*, 1998a)) but is affected by a reduction in differentiation ability, as *in vitro* chondrogenic potential has been found to drop with age (Murphy *et al.*, 2002), as does colony size, an indicator of proliferation potential and the numbers of alkaline phosphatase colonies in osteoporotic patients (Oreffo *et al.*, 1998a). Diet *in utero* has also been shown to have an effect on CFU-F numbers present (Oreffo *et al.*, 2003).

1.4.4. Committal and differentiation of MSC

MSC have two phases of life cycle, in phase one they regenerate (self renewal), in phase two the daughter cells are prompted towards growth and a more mature phenotype (differentiation). In the early phase of differentiation MSC indicate a committed progenitor phenotype towards their potential outcomes by up regulation of specific master/early genes. *Cbfa1* (*Aml3, Runx2*) and s*ox9* are some of the earliest genes to be expressed signifying the leaving of the self-regeneration cycle and into the differentiation cycle. *Cfba1* signifies a move towards the osteoblast pathway and *sox9* further towards the chondrocytic pathway (De Crombrugghe *et al.*, 2001). Once committed, further developmental stages take place with expression of later genes and proteins, such as fat accumulation in adipocytes, type I collagen, alkaline phosphatase activity in osteocytes and type II collagen and glycosaminoglycans in chondrocytes.

1.4.5. Markers of mesenchymal stem cells

CFU-F are heterogeneous in size, morphology and potential for differentiation, evidenced by the heterogeneity of alkaline phosphatase activity (Aubin., 1998). Colonies with lesser potentials are thought to represent alternative stages of differentiation (Owen *et al.*, 1987). Defined stem cell populations can be cultured by group selection using identifying antibodies (Gronthos *et al.*, 1994; Majumdar *et al.*, 2000; Simmons *et al.*, 1991; Stewart *et al.*, 1999). A number of antibodies have been developed for use in identifying early mesenchymal stem cells by a variety of laboratory groups including HOP-26 (Human osteoprogenitor-26, CD-68, (Joyner *et al.*, 1997)), STRO-1 (cell surface glycoprotein, (Simmons *et al.*, 1991)), SH-2 (CD105, (Haynesworth *et al.*, 1992)), SB-10 (ALCAM, (Bruder *et al.*, 1998)), and Sca-1 (stem cell antigen, (Spangrude *et al.*, 1988)). These antibodies have been shown to identify

smaller marrow cell groupings containing the majority of mesenchymal progenitors from marrow although there are no definitive markers as yet (Stewart *et al.*, 2003).

1.5. Current bone and cartilage treatments

1.5.1. Arthroplasty

If for a variety of reasons such as osteoarthritis, wear or underlying bone conditions, the joint becomes unstable or unusable, the joint can be removed and replaced with a mechanical joint (figure 1.17). Most problems occur with the attachment of the joint to the remaining bone with loosening from the cement resulting in strong focal forces and subsequent particle loosening, degrading the surrounding bone. It is typically 15 years before remedial surgery should be anticipated which may be an issue in younger patients.

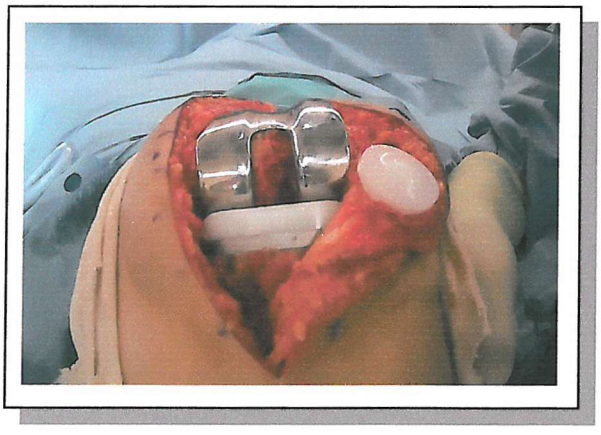

Figure 1.17. Total knee replacement (www.genufix.com)

1.5.2. Autogenous bone graft

Autografts are the first choice for filling of defects in bone material, typically from larger bones such as iliac crest. Autograft procedures have a high success rate (typically 90%) with a low risk of immune rejection or disease transfer, which makes it the preferred choice. However, there are anatomical limits to supply and shape, with the potential for donor site morbidity. Allografts and, potentially, xenografts may also be used but have the potential to transmit unknown pathogens (Goldberg *et al.*, 1993).

1.5.3. Distraction osteogenesis (Ilizarov technique)

In cases of shortened bones due to disease and damage, bone can be lengthened via the surgical separation of the bone and use of external fixators to stretch the healing zone which results in formation of a large healing callus (Ilizarov *et al.*, 1971). This techniques risks infection via the external fixators and is not suitable for all cases.

1.5.4. Lavage

In joints the loss of the meniscus and reduction of synovial fluid result in tissue damage. Washing of joints, often with an antimicrobial solution to remove any degraded tissue, appears to help. Hyaluronan injections also may serve to restore better functioning of joints (Lindsay *et al.*, 1969).

1.5.5. Debridement

Healing can be aided at bone and joint surfaces by removing the damaged or infected tissue from around the diseased area leading to a reduction in pathogenic signals and therefore a better cell environment (Santavirta., 2003).

1.5.6. Mosaic Arthroplasty

To resurface a joint, a core of cartilage is removed at the damage site and replaced with smaller cartilage and bone cores (osteochondral plugs) from non (or less) load-bearing areas (figure 1.18). This technique works well but is surgeon dependant, does not result in a full healing of the defect and may not be used over larger areas due to necrosis (Bobic., 1996).

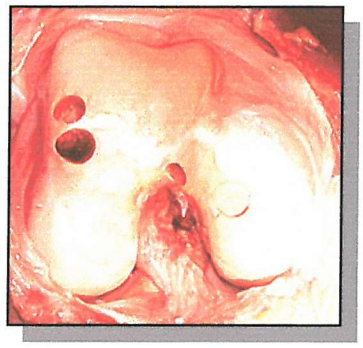

Figure 1.18. Mosaic arthroplasty (www.isakos.com).

1.5.7. Regeneration enhancement by micro-fracture

The procedure starts with removal of damaged cartilage and then holes are punched through to the marrow below to allow blood and bone marrow to permeate (figure 1.19). The holes allow marrow cells through that can differentiate to cartilage, however it appears in the majority of cases a fibrous tissue rather than articular cartilage is formed (Buckwalter *et al.*, 1994; Steadman *et al.*, 1999).

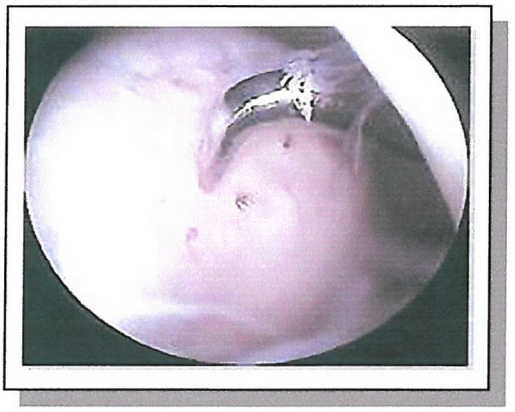

Figure 1.19. Micro-fracture (From www.kneeclinic.info)

1.5.8. Cell based augmentation of mechanical replacements

Cultured marrow CFU-F cells are used to coat implants with a suitable biological adhesion matrix *in vitro* outside the reaches of immune response and of other cell types. Successful integration of human ankle ceramic bone implants have been augmented by culture with marrow stromal cells before implantation, thereby improving initial implant attachment and subsequent integration although only over the surface of the implant (Ohgushi *et al.*, 1999).

1.5.9. Autologous Cell Implantation (ACI)

Repair of cartilage defects can be achieved by removal of a small cartilage biopsy, the chondrocytes isolated and ex *vivo* culture expanded. The increased number of cells can then be placed into a focal defect under a retaining flap of periosteal lining which is sutured in place (figure 1.20). Implanted cells result in localised growing cells, which are able to repopulate and regenerate the cartilage material. This repair strategy is able to replace useable tissue in 75% of cases, although only 25% with fully "articular" type cartilage (Ashton *et al.*, 2001). An essentially similar method has also been employed using mesenchymal cells which are added to the articular cartilage defect. These cells are able to differentiate into cartilage under the influence of localised factors, the subchondral cells are also able to differentiate into bone material (Wakitani *et al.*, 2002a).

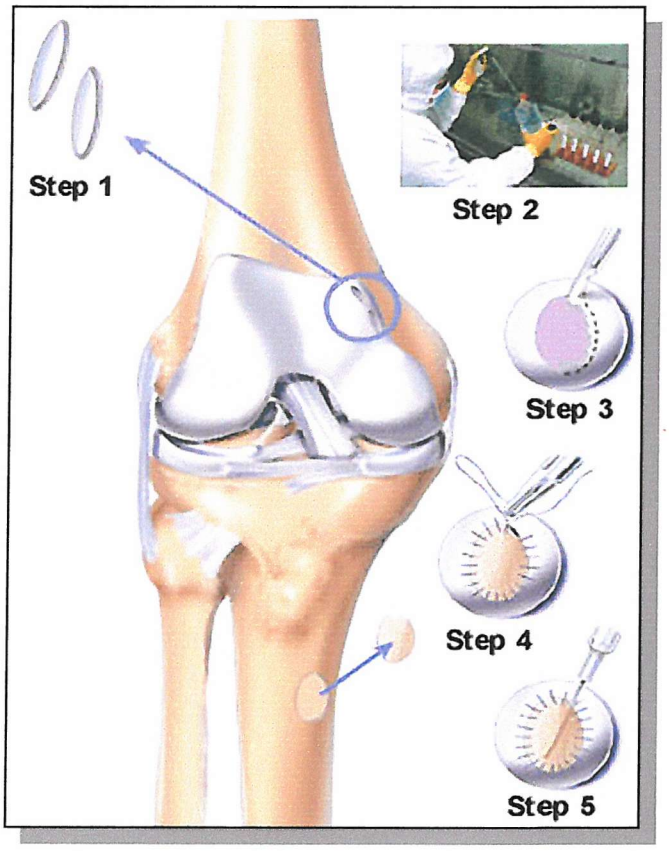

Figure 1.20. Autologous cell implantation (from www.scoi.com/carticel)

1.5.10. Tissue engineered cartilage implants

Chondrocytes have been isolated from the nasal septum, cultured, then seeded onto hyaluronic cell scaffolds and have been shown to produce the morphological and phenotypic features of cartilage. These tissue engineered constructs have been press fitted into knee defects using a standardised fitting system (Hollander., 2002). Tissue engineered constructs, unlike mechanical constructs, may be susceptible to the same disease process as the original tissue and attempts to reduce this effect are being sought (Kafienah *et al.*, 2003).

1.5.11. Enhancement of defect filling

Bone defects can be filled with a hydroxyapatite/tri-calcium phosphate (HA/TCP) scaffold over which the new bone can form. Addition of marrow to HA/TCP material can speed the bone healing process (Connolly., 1995) and a system to enhance this process during surgery has been developed (Kadiyala *et al.*, 2002). *In vitro* cultured marrow cells have been added to mineral scaffolds also using deliberately mismatched allogenic cultured marrow cells (Livingstone *et al.*, 2001; Petite *et al.*, 2000).

1.6. TISSUE ENGINEERING

Tissue engineering in its broadest sense is the replacement of tissues with newly created tissue and can be defined as "*Application of scientific principles to the design, construction, modification and growth of living tissues using biomaterials, cells and factors, alone or in combination*" (Langer *et al.*, 1993).

Morphology and development of cells is highly dependant upon the composition of it's surrounds, relying on solute factors, contact with surfaces or cells and nutrient supply. Tissue engineering can be reduced to the material requirement for 1) cells, 2) conducive growth factors, 3) shape forming scaffolds and 4) a culture method to bring all these factors together (figure 1.21). Each of the component parts will be described in the following sections.

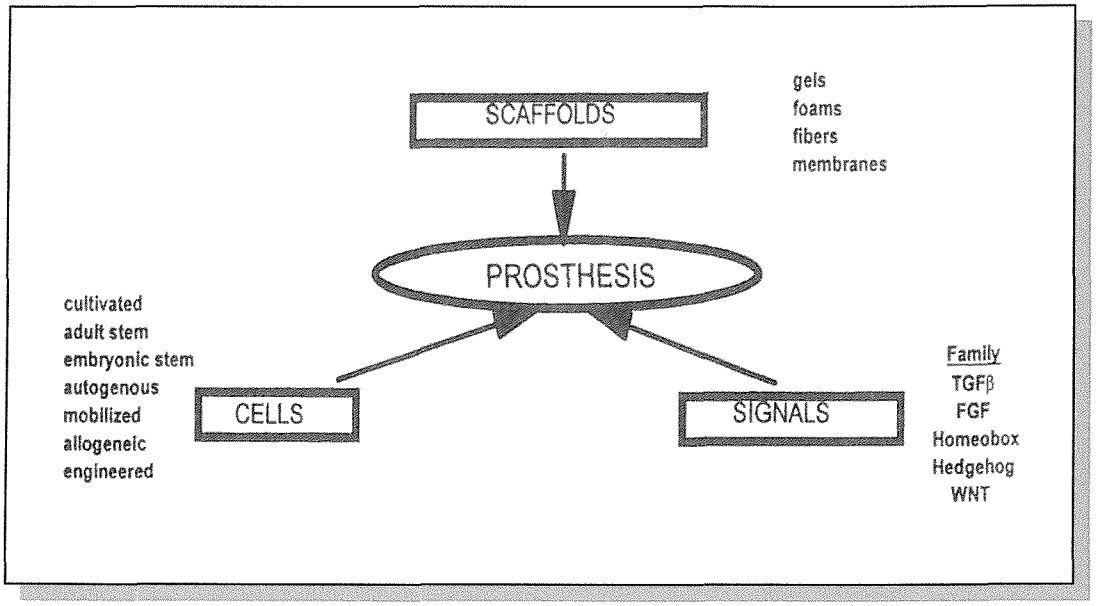

Figure 1.21. Tissue engineering strategy (Bell., 2002).

1.6.1. CELLS FOR TISSUE ENGINEERING

Skeletal tissue is generated *de novo* by mesenchymal progenitor cells differentiating into a variety of distinct cell types. The path of differentiation is considered to include multiple stages and restriction points (Aubin., 1998). Differentiation can be observed via typical markers of each, with adipocytes -fat accumulation, chondrocytes- type II collagen, Osteocytes- alkaline phosphatase, Osteocalcin and bone sialoprotein. For orthopaedic tissue engineering, mesenchymal stem cells (MSC) have many advantages over other cell types as they can be stored un-differentiated, display tripotency (Pittenger *et al.*, 1999) and allogenicity (Livingstone *et al.*, 2001) as well as being relatively easy to harvest and culture *in vitro* with high growth rates (figure 1.22).

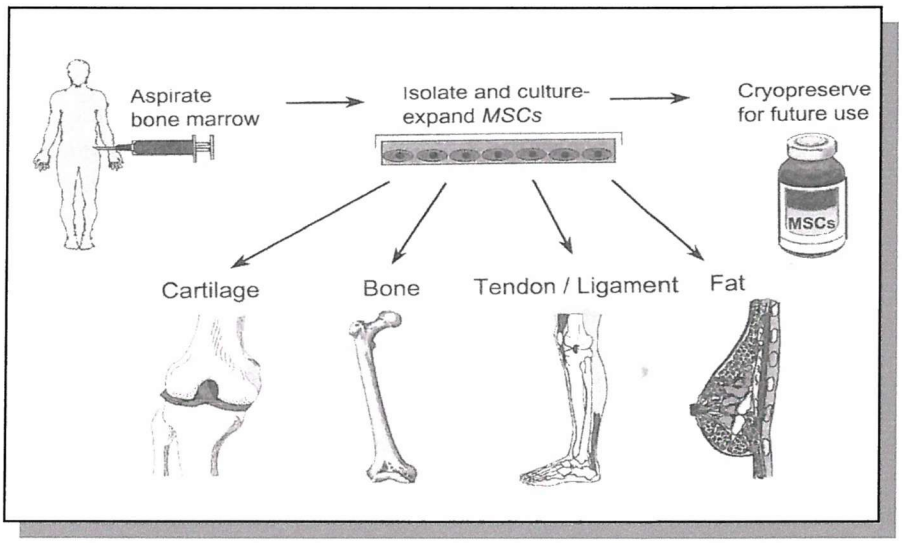

Figure 1.22. Potential uses of MSC in tissue engineering (Bruder *et al.*, 2000).

1.6.2. GROWTH FACTORS

In order to modulate the behaviour of tissue constructs a variety of growth factors can be utilised. The effects of factors may not be the same in tissue engineering studies as many factors have been studied in serum free monolayer cultures of cells, however, an overview of the effects of transforming growth factor beta (TGFβ), bone morphogenetic proteins (BMPs), pleiotrophin (PTN), insulin, fibroblast growth factor 2 (FGF-2), and the variety of components present within serum have been compiled below.

1.6.2.1. Transforming growth factors (TGF)

Transforming growth factors (TGF) are a large super family and include bone morphogenetic proteins (BMPs), activins, inhibins and growth and differentiation factor 9 (GDF-9). TGF are among the most prevalent growth factors in bone matrix, found bound to matrix proteins such as decorin and attached to a specific sequestering molecule known as latent TGF binding protein (LTBP) a 190kDa protein. TGFβ is contained within adult cartilage at relatively high levels (300-500ng/g cartilage) with most being in the inactive 25kDa opposed homodimer form requiring proteolytic processing before activation. Activated TGF initiates part of the re-modelling cycle of bone (Bonewald *et al.*, 1991) and is expressed at high levels in embryonic cartilage. TGFβ is considered to be an important growth factor for the differentiation of mesenchymal cells into cartilage cells (Cancedda *et al.*, 1995). It works via two main receptors type I and type II with additional binding receptor III combined with endoglin regulating its availability (Miyazono *et al.*, 2000). Type I and II are serine threonine kinase type (STK) and use the SMAD pathways for cell functions, type III receptor (betaglycan) binds TGFβ via a GAG domain. Effects are pleiotrophic depending upon cell type, differentiation state and culture conditions, for example

MSCs cultured in the presence of TGFβ1 with no serum are growth stimulated whereas cultures with 10% serum are inhibited except at low concentrations (Locklin *et al.*, 1999). In general when considering TGFβs they augment the synthesis/secretion of matrix proteins and protease inhibitors, whilst decreasing the synthesis of matrix degrading proteins. Chondrocytes transfected with TGFβ show a dramatic increase in proteoglycan and type II collagen synthesis over controls (Moller *et al.*, 2000). Administration of TGFβ1 stimulates bone formation and promotes healing of fractures and skeletal defects, suggesting that it affects the marrow stromal stem cell population *in vivo* (Bonewald., 1999; Gazit *et al.*, 1999).

1.6.2.2. Bone Morphogenetic Protein (BMP)

BMPs are a large (30+) subgroup of the TGF superfamily. BMP-2 is considered essential for bone development (Wozney *et al.*, 1988) acting in combination with growth factors in embryogenesis such as *wnt* and other homobox genes to stimulate chondrocyte maturation. Mature BMP-2 has a relative molecular mass of 18kDa (and dimers of 36kDa) with many similarities to TGFβ, the active form is a homodimer joined by a disulphide bridge. Full length BMP-2 is a 396 amino acid, glycosylated protein of which 114 are the mature protein with a 19 aa signal region and a 263 aa pro-region. BMP-2 initiates chondrogenesis in undifferentiated chondrogenic ATDC-5 cells (Shukunami *et al.*, 1998) and in C3H10T1/2 mesenchymal cell lines in pellet culture (Carlberg *et al.*, 2001). BMP-2 has been shown to up-regulate GAG production on PGA/chondrocyte constructs and culture expanded in the presence of FGF-2 (Martin *et al.*, 2001).

1.6.2.3. Pleiotrophin

Pleiotrophin (PTN) also known as heparin binding growth associated molecule (HB-GAM), heparin affinity regulatory peptide (HARP), heparin-binding neurotrophic factor (HBNF), heparin-binding brain mitogen (HBBM), p18 and osteoblast stimulating factor 1 (OSF-1), is found in high levels in both brain and bone (Tezuka *et al.*, 1990). In brain PTN is associated with neurite extension, whereas in the skeletal system it is associated with mesenchymal integration (Mitsiadis *et al.*, 1995). Other cells reported to express either PTN or PTN mRNA include osteoblasts, foetal chondrocytes, astrocytes, neurons, schwann cells, embryonic mesoderm, neuroepithelium and some tumour cell sources and is a potential marker of lung cancer (Jager *et al.*, 2002). PTN binds to a member of the proteoglycan family, syndecan (Raulo *et al.*, 1994). Concentration is an important factor in PTN activity as low concentrations (10 pg/ml) of PTN stimulate osteogenic differentiation but higher concentrations (ng/ml) have no effect (Yang *et al.*, 2003). When added after the osteoinductive growth factor BMP-2, PTN is able to enhance osteogenic differentiation (Tare *et al.*, 2002). PTN (50 μg/ml) is chemotactic to human osteoprogenitors and has been shown to stimulate total colony formation, alkaline phosphatase-positive colony formation, and alkaline phosphatase-specific activity at low concentrations (10 pg/ml; (Tare *et al.*, 2001; Yang *et al.*, 2003). PTN has been shown to enhance chondrogenesis in micromass cultures of chick limb bud cells (Dreyfus *et al.*, 1998) as well as inducing up regulation of the phenotypic markers - type II collagen, GAG and proteoglycans in chondrocytes (Tapp *et al.*, 1999).

1.6.2.4. Insulin

Insulin is secreted by the pancreas and is required by cells for glucose uptake from the blood. Insulin is a basic requirement in defined cell culture media modulating growth rate and cell size. Insulin increases production of glycogen (up to 2 fold with a corresponding 2 fold uptake of glucose) and up regulates glucose transporters GLUT-1 to 4 and induces *c-myc* mediated pathways.

1.6.2.5. Fibroblastic growth factor -2 (FGF-2)

FGF-2 (basic FGF: bFGF) has been shown to increase the ratio of STRO-1 positive cells in cultures (Walsh *et al.*, 2000) and to be able to significantly improve the outcome of the micromass cultures and bone formation (Martin *et al.*, 2001). However addition of FGF-2 to media for attachment isolation of fibroblastic marrow cells (CFU-F) showed a marked 30% drop in CFU-F formation (Walsh *et al.*, 2000). It is believed that FGF-2 promotes a less defined phenotype and that these "immature" stem cells must therefore be inhibited from attachment. FGF-2 has also been shown to reduce smooth muscle actin in chondrocytes and reduce their contractile fibroblast nature on fleece scaffolds (Martin *et al.*, 2001).

1.6.2.6. Serum components

Fetal calf serum (FCS) is often used to increase growth in cell culture. FCS is a semi defined source of protein and growth factors including many factors which otherwise would need to be added separately e.g. albumin, aldosterone, apolipoprotein, calcitonin, calcium, carotene, cholesterol, cortisol, creatine, dehydroepiandrosterone (DHEA), erythropoietin, estradiol, fatty acids, ferritin, fibrinogen, folic acid, glucagon, glucose, growth hormone, insulin, iron, parathyroid hormone, phosphorous, progesterone, prolactin, serum protein, pyruvate, serotonin, somatomedin (IGF), thyroxine, testosterone, transferrin, and vitamins A,B,C,D,E (Berkow *et al.*, 1993).

1.6.3. SCAFFOLDS

In order to provide three dimensional structure and support for cultured cells in tissue engineering, a scaffolding material is required. Scaffold structure can be designed as to allow modification of the densities of the cells, access to nutrients and growth factors. It has now become possible to incorporate other materials such as growth factors and surface modifications to the scaffold (biomimetic scaffolds) to modulate the cell attachment and tissue development.

Scaffolds have been generated from a variety of materials, including permanent materials such as titanium fleece (van den *et al.*, 2003), or biodegradable materials like collagen (Gargiulo *et al.*, 2001). Biodegradable synthetic polymer scaffold materials include the FDA approved polymers polyglycolic acid (PGA; Dexon), poly lactic acid (PLA) and composites of the two as poly-lactic-co-glycolide polymer (PLGA; Vicryl) which can be shaped into a variety of different formats (figure 1.23).

Favoured naturally occurring scaffolds include collagen, hyaluronan, tri-calcium phosphate (TCP) and hydroxyapatite (HA). Natural scaffolds such as demineralised bone are ideally suited to cells by the design of nature. However, they were not designed with tissue engineering in mind and may still illicit immune reaction, lack mechanical strength, contain pathogens in allogenic source material or still have problems with integration such as with the use of cortical bone where the cells cannot easily enter and necrosis is induced.

Synthetic polymers can be tailored to create a biomimetic environment but there must be a balance between the access of cells to the scaffold, structural integrity, potential degradation rates and accumulation of breakdown products (Freed *et al.*, 1998). The scaffold should be shapeable or produced in a suitable shape, general format guidelines for scaffolds state that there should be a porosity of between 75%-90% with

pore sizes between 100-400 μm on average, however this does not hold true for all cell scaffolds, as gels or scaffolds with the cells printed (Mironov *et al.*, 2003) or cast (Shakesheff *et al.*, 2001) within them do not follow these rules.

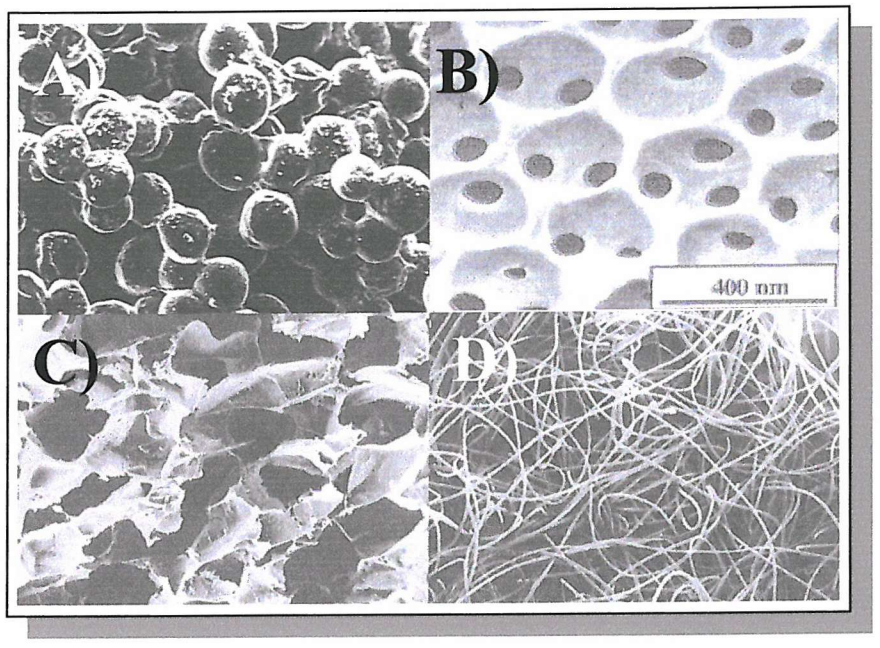

Figure 1.23. PGA formed into a variety of architectures. A) beads, Porous scaffolds formed with B) regular and C) irregular porogens, D) Fleece. (Modified from;(Kim *et al.*, 1998; Lu *et al.*, 2003; Ma *et al.*, 2001)

Advances in scaffold surface chemistry allow structures to be modified to modulate cell attachment and activities, modifications such as RGD and P-15 peptide coatings (Bhatnagar *et al.*, 1999; Yang *et al.*, 2001). Scaffolds can be used as a growth factor delivery device by incorporating growth and other factors into the scaffold that are released upon degradation (Shakesheff *et al.*, 1998; Yang *et al.*, 2003). Refinements in the micro and nano-topography of the scaffold can be used to alter cell responses and to orientate cell growth.

Many scaffolds have been developed by different groups for differing requirements. As an example of the diversity in scaffold architecture and composition the following

list is far from exhaustive but includes - Gels (Alsberg *et al.*, 2001; Atala *et al.*, 1994), porogen cast polymers (Ma *et al.*, 2001), supercritical CO_2 formed polymers (Shakesheff *et al.*, 1998), spun fibre polymers (Glowacki *et al.*, 1998), composite spun polymers (Chung *et al.*, 2002), bound fleece (Mikos *et al.*, 1993), matrix printed (Mironov *et al.*, 2003; Sherwood *et al.*, 2002), fibrin-fibrinogen (Gorodetsky *et al.*, 1998), synthetic diatoms and vatarite spheres (Green *et al.*, 2002), specially designed and engineered protein hydro gels (Kisiday *et al.*, 2002), chitosan (Elcin *et al.*, 1998), HA and TCP (Ohgushi *et al.*, 1999), gelatines (Kang *et al.*, 1999), de-proteinised bone hydroxyapatite, calcium carbonate, calcium sulphate, bio-glass, demineralised bone matrix (Urist *et al.*, 1970), coralline hydroxyapatite (Holmes., 1979), collagen (Ponticiello *et al.*, 2000), collagen GAG copolymers, collagen gel, agarose (Mauck *et al.*, 2000), alginate gel (Alsberg *et al.*, 2001), hyaluronic acid (Aigner *et al.*, 1998), self forming biotin/avidin bead mixes (Shakesheff *et al.*, 2001). Due to the diverse nature of these materials they have not been tabulated, as each compound and each format should be considered individually, as small differences in composition and shape have profound effects in complex systems.

1.6.4. BIOREACTORS

In monolayer culture, cells have a high surface area allowing good access to nutrients and gases. Tissue engineering still relies upon the high growth rates of monolayer culture to supply the starting cell population before addition to the scaffold, followed by bioreactor culture, for the formation of the final tissue.

However in tissue engineered constructs the depth of tissue decreases the access to nutrients and reduces the rates of exchange that occur, even in the thinnest of tissues, as observed over only 2 mm constructs (Freed *et al.*, 1998). A bioreactor which allows a high exchange rate is often employed to enhance growth parameters (Freed *et al.*, 1993) and comparisons between stationary cultures and rotating bio-reactor cultures have been shown to increase the number of proliferating cell nuclear antigen (PCNA) positive cells within PGA constructs, clearly demonstrating the increase in growth rate (Glowacki *et al.*, 1998). Supplying nutrients of sufficient and correct composition is a key point in the accumulation of cells and matrix for tissue engineering, although practical aspects of cell damage, contamination resistance and cost have also to be addressed. Bioreactors tend to rely upon variations and combinations of three main modes of nutrient supply; 1) perfusion, 2) stirred vessel or 3) moved (rotating) vessel.

1.6.4.1. 1) Perfused

Perfusion is where the construct is constantly bathed in media by some form of pumping system or gravity feed (Huckle., 2001) typically the media is pumped from a chilled media store through silicone tubing, which due to the nature of silicone rubber allows warming of the media and efficient gas exchange, this is then pumped throughout the chamber nourishing the tissue construct before being removed to a media waste bottle, although in some systems the media is recycled. For example hollow fibre liver and kidney cell systems work on perfusion with the cultured cells

attached to a mass of fibres within a movable vessel. Other systems pass media up a column thereby suspending constructs in an upward flow of media, modifications of this design can be used to accommodate more constructs and give a more versatile system (figure 1.24; (Sittinger *et al.*, 1997)). However perfusion systems tend to require large volumes of media to operate which if they contain multiple growth factors can be prohibitive in cost. These systems may also suffer from blockage and high focal pressure with the potential to damage or strip cells from the scaffold.

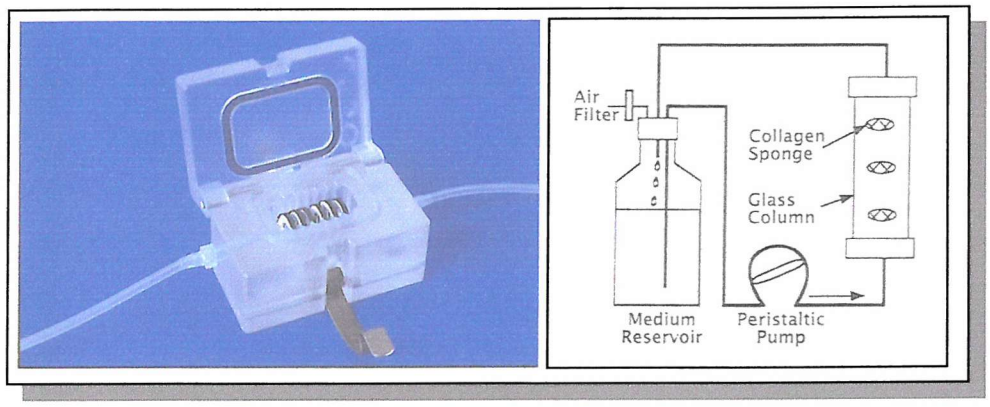

Figure 1.24. Perfused culture systems (from

(Glowacki *et al.*, 1998; Sittinger *et al.*, 1997))

1.6.4.2. 2) Stirred vessels

Stirred vessels rely upon re-circulation of media via mechanical movement such as spinner flasks and suspension culture systems initially developed for microbial work. Stirred vessels rely upon propeller like systems to circulate media and cells in addition mini-loop reactors can be employed which use a small centrifugal pump to re-circulate media through a permeable inner vessel containing the construct.

1.6.4.3. 3) Moving vessel

In moving vessel systems the vessel is able to induce movement in the media within the chamber. NASA used a rotating system in order to culture cells constantly with limited maintenance for space flights (figure 1.25). This system also produces a low shear exchange of nutrients and has now been adapted for tissue cultures of a variety of cell types (Freed *et al.*, 1997). In essence the culture system consists of a rotating drum which tumbles the constructs constantly through media, with one end covered in a silicone membrane providing a high surface area for gas exchange. Other systems use attached biomaterials moved throughout the media (Saini *et al.*, 2003). Rotating bioreactors can also be combined with a perfusion system for long term maintenance and maturation of cultures.

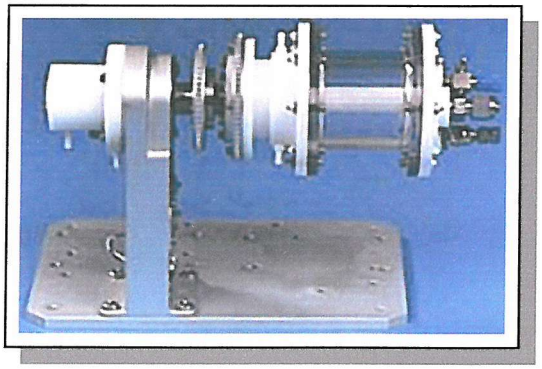

Figure 1.25. Rotating culture vessel (Synthecon.com).

1.6.4.4. Modulation of bioreactor conditions

It is well established that chondrocytes and bone cells are load responsive *in vivo* (Skerry *et al.*, 2001) and chondrocytes have been shown to increase extra cellular matrix (ECM) secretion *in vitro* (Smith *et al.*, 1996). Bioreactors which produce dynamic stress, up-regulate extra cellular matrix production, above that produced by static compression, which seems to be more detrimental when compared to dynamic pressure (Korver *et al.*, 1992; Sah *et al.*, 1989; Smith *et al.*, 1996). Limits for the stresses of chondrocytes show an upper maximum limit of 1 MPa (145 PSI) dynamic compression and 0.5 MPa (72.5 PSI) static compression (Burton-Wurster *et al.*, 1993; Guilak *et al.*, 1994). Glycosaminoglycan (GAG) synthesis is increased in chondrocytes when exposed to 5 MPa (725 PSI) at 0.25 Hz and at a variation of 1-5% at frequencies of 0.001 - 1 Hz for 24 h but not when compressed statically. There is variation in the literature regarding appropriate conditions for cell growth with reports using pressures as high as 10 MPa (1450 PSI) were shown to increase cell matrix production in cartilage explants (5-15 MPa is physiological for the hip joint). Dynamic flow also appears to be affected by its associated parameters of fluid flow and streaming potentials (Buschmann *et al.*, 1995). Culture with no gas exchange ability in embryonic mesoderm showed that cartilage formation was much quicker (Duke *et al.*, 1993).

1.7. Clinical requirement for skeletal repair

By the year 2050 it is estimated that over 10% of the population of the UK will be over 80 years old, with an average age of over 40 (UN census, unstats.un.org) and one of the rewards of a long life is osteoarthritis (OA) and osteoporosis (OP) (Robbins *et al.*, 1976). It is estimated there are greater than 3 million musculo-skeletal procedures per year in the US including approximately 500,000 grafting procedures (www.aaos.org). In 1994, orthopaedic surgeons in the UK treated on average 908 patients each (www.bbc.co.uk). The cost of 50,000 primary hip operations is around £250 million, with orthopaedic treatment costing the NHS and government around £5 million a day (National Osteoporosis Society 2002, nos.org.uk).

Artificial mechanical joints are currently the "gold standard" method of dealing with severely worn or damaged joints, however - wear particles, localised tissue damage and mechanical loosening due to material mismatch means that joint replacements last approximately 15 years before remedial surgery may be needed. In younger patients joint replacement needs careful consideration due to the limited lifespan of artificial joints where further tissue damage may impair any further useful management. Distraction osteogenesis (Ilizarov *et al.*, 1971) can be used to lengthen existing bones but cannot make complex shapes. Autografts and allografts have been used extensively in bone repair, autografts are associated with donor-site weakening and morbidity, and is constrained by anatomical limitations, whilst allografts are limited by the potential risks of rejection and infection (Gazit *et al.*, 1999). Trabecular bone grafts are favoured as cortical bone results in poor integration and necrosis (Goldberg *et al.*, 1993). Therefore a substitute material for bone and cartilage repair, which is shapeable, integrates well and results in a full biologic repair would offer many advantages over current techniques.

1.8. AIMS AND OBJECTIVES

The aim of these studies is to ultimately generate cartilage and bone constructs appropriate for repair of defects. The scheme of work to be undertaken is outlined in figure 1.27. The cell population to be used is a defined marrow stromal cell population with the ability to produce phenotypes indicative of both cartilage and bone. Using the selected cells and applying tissue engineering strategies, this thesis will examine the following;

- Selection and characterisation of a STRO-1^+ marrow CFU-F population.
- Development of methods to enhance CFU-F numbers.
- Use of alginate cell encapsulation methods for generation of potential tissue engineered constructs.
- Culture of genetically modified STRO-1^+ CFU-F on supercritical CO_2 expanded PLA scaffold to produce a bone substitute.
- Generation of a cartilage phenotype by using a micromass differentiation culture system on control and marrow stromal cells.
- Development of a method for tissue engineering constructs using primary human CFU-F.

Schematic overview of the work undertaken

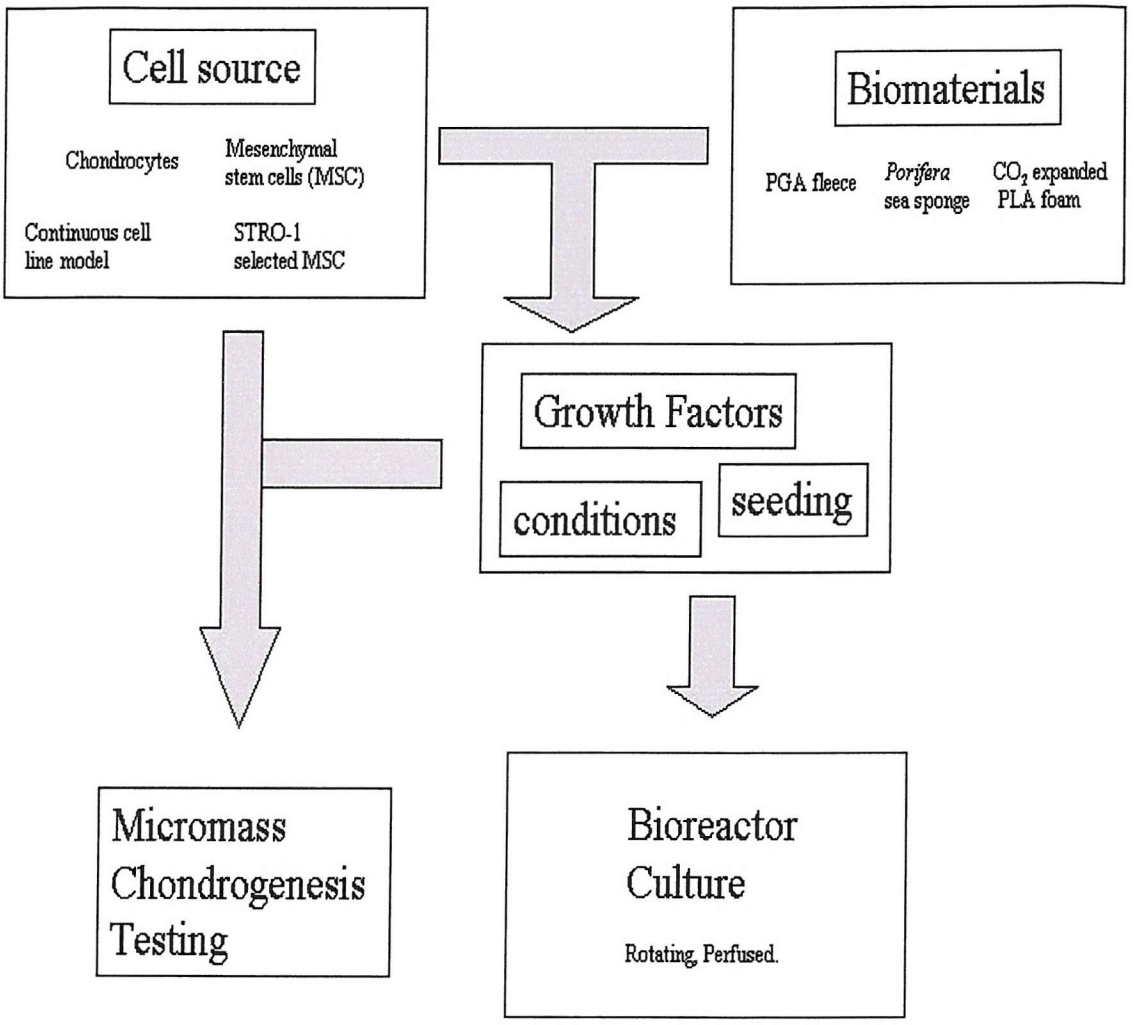

Figure 1.26 Work scheme

2. MATERIALS AND METHODS

2.1. Materials

The rotating bioreactor was purchased from Synthecon (Houston, Texas, USA). The perfused bioreactor was a gift from Dr Julian Chaudhuri, Dept of Chemistry, University of Bath. PGA fleece was purchased from Cellon (Arlon, Luxembourg). Supercritical fluid produced PLA scaffolds were supplied by Professor Kevin M Shakesheff, school of Pharmacy, University of Nottingham. STRO-1 hybridoma and MACS protocol were from Dr Jon Beresford and Dr Karina Stewart, Dept of Pharmacology, University of Bath. Chondrogenic ATDC5 cells were a gift from Dr Agamemnon Grigoriadis, Dept of Orthodontics, Guy's Hospital, Kings College London. Lymphoprep cell separation solution was purchased from Robins Scientific (Solihull, UK). MACS reagents and equipment was supplied by Miltenyi Biotech (Bisley, Surrey, UK). Histological stains, alcian blue, sirius red and toluidine blue were from Gurr (BDH, Poole, Dorset). Fetal Calf serum was from Meldrum International (Bourne end, UK). 130 µl diffusion chambers (Millipore, UK). Media reagents were purchased from Invitrogen (Glasgow, UK). ITS premix (insulin (10 µg/ml) transferrin (5.5 µg/ml) selenium (5 ng/ml; Sigma, Poole, UK).CMFDA1 (5-chloromethylfluorescein diacetate) and ethidium homodimer-1 (EH-1; Molecular probes, Leiden, Netherlands). All other reagents were purchased from Sigma chemical company (Poole, UK) unless otherwise stated.

2.2. Scaffold

Supercritical CO_2 expanded PLA (University of Nottingham), PGA fleece (Cellon, Luxemboug) and Sea sponge (commercially prepared by drying to remove cells and fixing in potassium permanganate and sodium metabisulphide; www.qmuseum.qld.goc.au) were prepared by cutting into 2-5mm^3 pieces. PLA and PGA were sterilised by UV light for 1 hour. Porifera sea sponge was sterilised by immersing in 4°C 70% ethanol overnight. PLA and sea sponge were soaked in serum free medium overnight at 4°C before use.

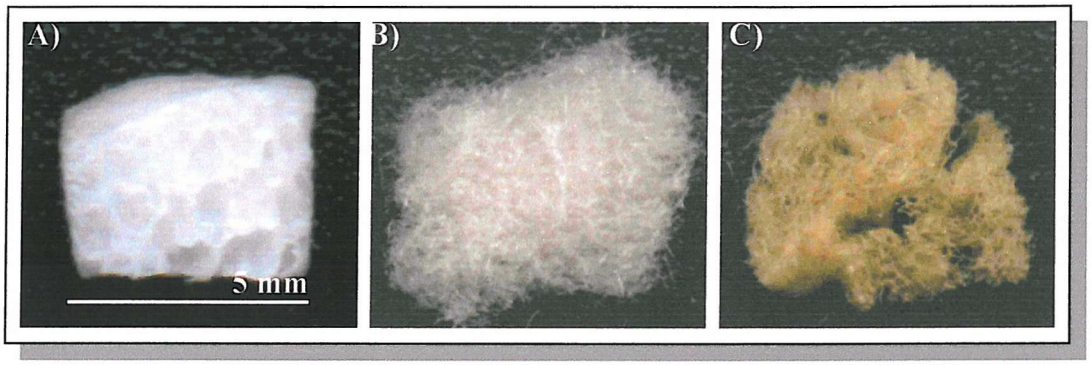

Figure 2.1. A selection of scaffolds including A) 5mm^3 CO_2 expanded PLA courtesy of Professor Kevin Shakesheff, University of Nottingham B) 5mm^3 PGA fleece (Cellon) and C) 5mm^3 Sea sponge (*Porifera spp*).

2.3. Cell culture

2.3.1. Isolation and culture of human bone marrow cells

Bone marrow samples were obtained from haematologically normal patients undergoing total hip replacement procedure. Tissue which otherwise would be discarded was used with approval of the Southampton hospital ethics committee (LR194/99). Human bone marrow was shaken vigorously in 10mls of αMEM media and supernatant was removed to a separate tube, this was repeated 4 times. The

suspension was then centrifuged at 200 g for 4 minutes and the cells retained. The pellet was re-suspended in 10mls of αMEM and filtered through a 75 μm gauze (Becton–Dickinson, Oxford, UK) to remove any large marrow debris. For the purpose of counting, an aliquot of cells were diluted 1/10 and red blood cells disrupted with 0.1% acetic acid and counted on a haemocytometer. Nucleated marrow cells were plated out at 2-3x10^7 per 80cm^2 flask or 2-3x10^8 per 180cm^2 flask. Culture expansion was in αMEM supplemented with a selected lot of serum (previously shown to culture CFU-F from marrow) at 10% (v/v) and incubated until confluent in a humidified incubator at 37°C supplemented with 5% CO_2. Typical expansion time was 1 week to attach to the culture plastic and expansion of cell number for a further 2 weeks with media changes twice a week, as previously described (Oreffo *et al.*, 1998a). Cells were used at no more than two passages.

2.3.2. STRO-1^+ marrow cell isolation using MACS

In order to supply a defined marrow population an antibody labeling system was used whereby marrow stromal cells could be identified in a group of cells and isolated by the use of a magnetic removal system. To remove marrow cells from the surrounding trabecular bone material from the reamings of the bone shaft approximately 5g of marrow was added to 10 ml of αMEM and shaken vigorously. Media was then decanted to a fresh universal tube, the process was repeated four times. The resulting cell suspension was spun at 200g for 4 minutes at 18°C. The pellet was re-suspended in 10ml of αMEM then passed through a 75μm filter to remove large particles including bone chips, this stage resulted in isolation of cells from the surrounding bone material.

To remove the excess of erythrocytes the cell suspension was made up to 25 ml with αMEM and gently poured onto 20ml of lymphoprep (Robins scientific, Solihull, UK)

in a 50 ml tube. The sample was spun at 800g for 20 minutes at 18°C. The buffey coat (cell layer) was removed to a fresh 50 ml tube. To remove the lymphoprep, an excess of Hank's buffered saline solution (HBSS) was added, followed by spinning the cells at 250g for 4 minutes, twice. Cells were added to 10 ml of HBSS and counted using a haemocytometer.

To magnetically label the cells they were pelleted at 200g for 5 minutes and re-suspended at $1x10^8$ cell per 10mls of blocking solution, (HBSS with 5% (v/v) FCS, 5% human normal AB serum and 1% BSA fraction 5). The cells were incubated with blocking solution in a refrigerator for 30 minutes. To remove the blocking solution cells were pelleted at 200g for 5 minutes and then rinsed in 50mls of de-gassed MACS buffer (HBSS 1% BSA fraction 5) repeated twice. To the cell pellet, primary STRO-1 antibody supernatant (10mls) was added and incubated in a refrigerator for an hour. After incubation with the STRO-1 antibody the cells were washed of excess antibody, which would interfere with subsequent stages, with 3 washes of cold MACS buffer as before. Cells were re-suspended (gently) in 800 µl of de-gassed MACS buffer, then incubated with 200 µl of MACS anti-IgM with attached para magnetic particles (Miltenyi biotech; surrey, UK) for 45 minutes. Anti IgM FITC (15 µl) was added at 45 minutes and incubated for a further 15 minutes.

Once the cells were labelled the cells were then separated using a magnetic column as follows: Degassed MACS buffer was used to pre-wet the MACS columns. Labelled cells were then added to an un-magnetised column and collected as control unsorted cells or separated as follows. Antibody incubated cell suspension was added to a column within the magnet and the elutant collected as STRO-1 negative. To remove non STRO-1^+ magnetically labelled cells the column was washed a further 3 times with 500µl of MACS buffer. Finally, out of the magnet, 1 ml of MACS buffer was

added and collected as the positive fraction. For each fraction a cell count was performed. Cells were cultured for 1 week to show separation of the majority of CFU-F. For alkaline phosphatase positive colony counts of the groups, $2x10^6$ nucleated cells were cultured in a $25cm^2$ flask in the presence of ascorbate and dexamethasone (asc dex) for 14 days before staining and counting. Cells were cultured in monolayer for 3 weeks in 10% FCS αMEM supplemented with ascorbate and dexamethasone to look for alkaline phosphatase expression and without ascorbate dexamethasone for observation of type I and type II collagen by immuno- histochemistry.

2.3.3. Isolation and culture of human chondrocytes

Femoral heads were obtained from haematologically normal total hip replacement (THR) patients. In order to supply a comparison to the marrow stromal cells chondrocytes were isolated and cultured according to the oscell™ procedure (courtesy of B Gargulio and B Ashton, RJAH orthopaedic hospital, Oswestry) as follows: In a sterile class II hood the cartilage was stripped using a scalpel and placed into 1ml of DMEM/F12 media. Using a new scalpel the cartilage was then further sliced into $1mm^3$ cubes. Approximately 200 mg of cartilage mince was added to 10 ml of digest medium consisting of 10 ml of HEPES buffered DMEM/F12 containing 10 mg of collagenase (Boehringer Mannheim) and 1mg of DNAse (Sigma, Poole UK), this was left overnight in an humidified incubator at 37°C and supplemented with 5% CO_2. After incubation the cell capsules were broken open by passing the solution through a 19G needle. Chondrocytes were rinsed in 10% FCS supplemented DMEM/F12 and centrifuged into a cell pellet at 400g for 10 minutes twice to remove the enzymes. The cells were then re-suspended into 10% FCS supplemented DMEM/F12 and plated into 25 cm^2 culture flasks at a cell density of $1x10^5$ for maximum growth rate, or $1x10^6$ for

minimum culture length, until confluence. Culture for the expansion of cell numbers was at 37 °C 5% CO_2 for approximately 2 weeks, changing the media every 2-3 days.

2.3.4. Culture of ATDC-5 cell line

Immortalised murine ATDC-5 cells which are a primative cell line previously shown to transform from a primative cell to a chondrocyte cell in overconfluent monolayer cultures, were cultured by incubation on 75 cm^2 culture flasks in 5% FCS supplemented DMEM with insulin (10 μg/ml) transferrin (5.5 μg/ml) selenium (5 ng/ml; ITS) premix (Sigma, Poole, UK) the cells were trypsinised at no later than 60% of confluence and split 1:8 at passage and not allowed to attain full confluence as this could result in transformation. These cells were supplied courtesy of Dr Agememnon Grigoriadis and were culture expanded and used at a passage number of n+9 to n+12.

2.3.5. Culture of MG63 cell line

MG63 human osteosarcoma cells (ECCAC. No. 86051601 passage = 107, used at n+4) were cultured in order to provide a positive control for STRO-1 antibody binding as they have previously been shown to be strongly positive (Stewart *et al.*, 1999). Cells were cultured in 10% FCS supplemented DMEM and the cells passaged weekly 1:8, using trypsinisation to passage the cells.

2.3.6. Culture of C2C12 cell line

To provide an assay for BMP-2 a cell line was used. The C2C12 cell line responds to BMP-2 by up-regulating alkaline phosphatase activity (Katagiri *et al.*, 1994; Okubo *et al.*, 1999). Alkaline phosphatase activity was then assayed in comparison to known amounts of BMP-2. C2C12 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FCS and passaged 1:8 regularly, before confluence, to prevent myogenic differentiation. C2C12 cells (ECACC. No. 91031101 passage =

13, used at n+ 10) are a primative murine cell line which when allowed to grow confluent will transform to a muscle cell type.

2.3.7. Culture of pZIP transformed stromal cell line

To provide a continuous stromal cell line pZIP cells were used. PZIP cells were specially transformed from cultured marrow stromal (CFU-F) cells derived from a Legg-Perthes patient using a specially designed plasmid. PZIP cells were cultured in DMEM containing 10% FCS and selection agent G418 to maintain only the transformed cells. These cells were supplied courtesy of Dr Jon Beresford and were culture expanded and used at a passage number of n+4.

2.2.8. Cell Trypsinisation

Monolayers of cells were rinsed of serum with two washes of PBS and incubated with 0.05 % (w/v) Trypsin/ 0.02 % (w/v) EDTA solution at 37 ^{O}C for 7 minutes to breakdown the cells attaching matrix so releasing the cells. The cell suspension was then centrifuged at 250g for cell lines and marrow stromal cells/CFU-F and at the higher 400g for chondrocytes. Cells were then rinsed in serum supplemented αMEM and centrifuged at 200g for 5 minutes to remove the trypsin activity, cells could then be used.

2.3. Culture vessel use

2.3.1. Perfused

In order to increase the nutrient and metabolite exchange rate in three dimensional tissue constructs a versatile reactor was produced by Dr Julian Chaudhuri and Dr Marcus Smith in the Department of Chemistry at the University of Bath. The reactor consists of a central chamber with 4 inlet/outlet ports into which pre-seeded scaffolds

were placed (figure 2.2 A). Gravity feed of media passes through 2 meters of fine diameter silicone tubing, which allows exchange of heat and gasses before entering the culture vessel. A 2 meter long outflow prevents any micro organisms from gaining access. A valve controls the flow rate in a range of approximately 1-50 ml per day through the vessel, typically a flow rate of 1ml per hour (24 ml per day) was used.

2.3.2. Rotating culture

The rotating bioreactor produced by Synthecon (Texas, USA) was utilised, the vessels used in this series of experiments were the 50ml disposable units which had a silicone membrane for gas exchange and were based on the high aspect ratio design (HARV) in that the cylinders are taller then they are long (figure 2.2 B). The vessels, when used, were opened at the large access port and a sterilised scaffold placed into the vessel using sterile forceps, the vessel was then filled with 50 ml of serum free media, serum free media was initially used in order to reduce any bubbles as this effects the orbit of the scaffold within the chamber. The serum could then be added via one of the syringe luer ports with excess collected via the other syringe port. For continued culture 10 ml of media was exchanged every 2-3 days via the syringe ports.

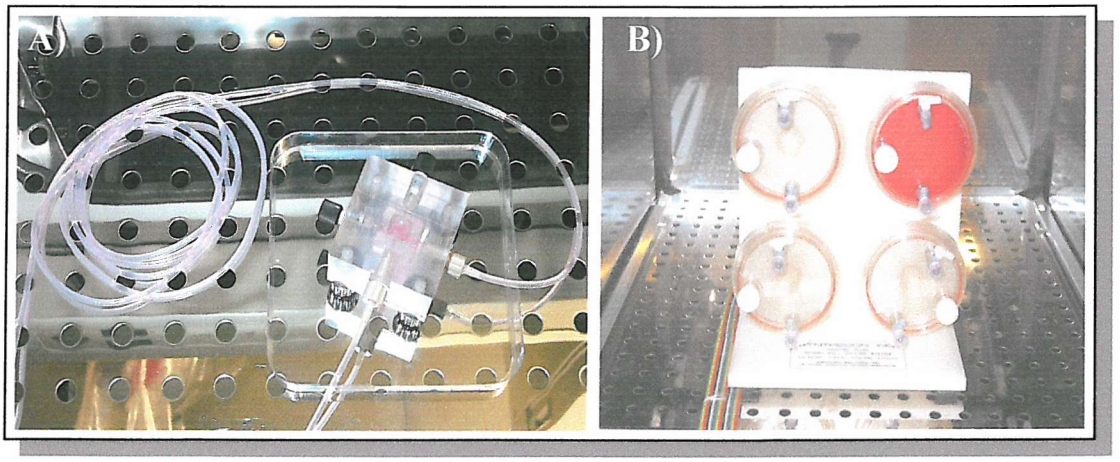

Figure 2.2. Bioreactors used in the study include A) the perfused chamber bioreactor from the University of Bath and B) a disposable HARV rotating bioreactor system from Synthecon.

2.4. Biochemistry

2.4.1. Alkaline phosphatase

Cells were rinsed in an excess of PBS and frozen at –80°C until assayed. Cell monolayers were scrapped into a solution of distilled water containing 0.001% (v/v) Triton X-100 detergent. For 6 well plates 0.6mls of solution for a single well of a six well plate was used and 1.0ml for a 25cm^2 flask. The cells were lysed by freeze thawing 3 times (10 minutes freezing in a –80°C freezer and 15 minutes in a 37°C incubator). In a microplate 50µl of cell lysate was incubated for 20 minutes with 100 µl of 2-amino-2-methyl-1-propanol buffer containing 100mM p-nitrophenyl phosphate (PNP). The reaction was stopped using 100 µl of 1M NaOH. Resulting PNPP formed by alkaline phosphatase on PNP was measured at 410nm on a Elx800 BioTech spectrophotometer. The assay results were expressed as nM, PNPP/min/µg DNA.

2.4.2. DNA assay

Cells were rinsed in an excess of PBS to remove media then rinsed in distilled water to remove salts and frozen at –80°C until assayed. Cell layers were scrapped into a solution of distilled water containing 0.001% (v/v) Triton X-100 using a 30 cm cell scrapper (Orange scientific, Belgium). The cells were lysed by freeze thawing 3 times (10 minutes freezing in –80°C freezer and 15 minutes in an 37°C incubator). Then in a 96 well Cyto-fluor plate (Becton-Dickenson, Oxford, UK) 50 µl of lysed cell solution was diluted with 50 µl TE buffer (10 mM Tris-HCL (Sigma, UK) 1mM EDTA (Ethylenediaminetetraacetic acid ; BDH, UK) pH 7.5) and 100µl of 1/200 (5 µl per 995 µl TE) picogreen diluted in TE (Molecular Probes, Leiden, Netherlands) was added. Fluorescence was observed using 480nm excitation and 520nm emission on a Flx800 BioTech fluorometer (Winooski, USA).

2.5. Histology

2.5.1. Sample preparation

Due to the degradable nature and solubility in chloroform, biodegradable scaffold constructs were fixed in either cold 95% ethanol or soaked for two hours in formol calcium. Samples were dehydrated in graded cold ethanol (50%, 90%, 100%, 100% for 5 minutes each) then prepared for wax embedding by soaking in 50 % histoclear/ethanol for an hour and then 100 % histoclear for one hour. Samples were then soaked in 60^{O}C wax for an hour to allow relatively cool saturation of wax into the sample and then embedded in fresh wax blocks for sectioning.

Human articular cartilage or *porifera* sponge constructs were sectioned by embedding in Cryo-M-Bed solution (Bright, Huntingdon, England) and freezing at –80^{O}C overnight. Sectioning was on a cryostat (Reichert Jung) at –25 ^{O}C at a thickness of 7-10 μm.

2.5.2. Alkaline Phosphatase stain

Alkaline phosphatase activity was detected by using a colour development solution reactive to the alkaline phosphatase enzyme activity. In brief, cell cultures were rinsed in PBS and then distilled water followed by fixing in cold 95% ethanol, the samples were then kept in a freezer (-20°C) until needed. Flasks were stained with alkaline phosphatase reactive staining solution (0.4ml Napthol AS-MX phosphate, alkaline solution diluted in 9.6ml of distilled water, 2.4mg of fast violet-B salt). Fixed cells were incubated with enough solution to cover the sample (6 well plate = 1ml per well, 25cm^2 = 2ml, 80cm^2 = 8ml) and incubated at 37^{O}C for 20 minutes before being rinsed in distilled water to terminate the reaction. For the purpose of CFU-F counting an Anderman colony counter was employed as previously described in Oreffo *et al* (1998b).

2.5.3. Alcian blue and Sirius red staining.

Sectioned paraffin slides had the paraffin removed from the sections by dipping in baths of citroclear (BD supplies, Aylesbury, UK) for 7 minutes each, then through graded alcohols (100% 5 min, 100% 5 min, 90% 5 min, 50% 5 min (BDH)), then to water for 10 minutes in order to remove citroclear and rehydrate the sections. Sections were stained for 10 minutes with Weigerts haematoxylin which was differentiated by dipping into acid ethanol (1 ml of acetic acid glacial per 100ml ethanol) and rinsed for 10 minutes in water before incubating for 10 minutes in (0.5%w/v) Alcian blue. Sections were prepared for the next stage by dipping sections for 20 minutes in molybdo-phosphoric acid before rinsing for 1 minute in water and then to picro-sirius red for an hour. Finally slides were rinsed in water for 1 minute before being dehydrated in graded alcohols (50% 30 s, 90% 30 s, 100% 30 s, 100% 30s) and histoclear twice for 30 seconds before mounting in DPX (distyrene plasticiser xylene, HD supplies, UK).

2.5.4. Toluidine Blue staining

Sectioned paraffin slides were taken to water as above. The slides were then placed onto a hotplate at 50^{O}C and stained with pre warmed 1% toluidine blue in 100mM sodium acetate solution for 1 minute, rinsed briefly in water for 1 minute and differentiated in 90% ethanol for 1 minute. Sections were allowed to dry for a minimum of 2 hours before mounting in DPX.

2.5.5. Live/Dead viability stain

Cells were stained for viability by the use of a dye, Cell-Tracker™ green, which becomes fluorescent green by activation utilising the metabolic activity within the cell. In addition ethidium homodimer-1 was used to stain the nucleus red since this stain could only gain access to the nucleus if the cell membrane was leaky and hence identify a dead cells. Cells therefore stain green for live and active and red for dead. In brief; warmed cell culture samples (cells or tissue culture constructs) were incubated with a covering of a pre-warmed solution of the two fluorophore dyes, 25 μg of 5-Chloromethylfluroscein diacetate (CMFDA; Molecular Probes, Leiden, Netherlands) dissolved in 10 μl of DMSO (Di methyl sulphoxide), combined with 1mg Ethidium homodimer (EH-1; Molecular Probes, Leiden, Netherlands) in 5 ml of media, for 1 hour at 37^{O}C in a humidified cell culture incubator. Residual dye, which would interfere with the observations, was washed from the samples with two rinses of culture media at half hour intervals and observed by fluorescence microscopy with the samples in PBS to reduce any background colouration from media.

2.5.6. Marrow CFU-F assay

MSC cells were counted using a haemocytometer and the average of five counts used. Cells were then seeded at 2x10^{6} nucleated cells per T25cm^{2} cell culture flask in αMEM supplemented with 10% FCS, 100 μM ascorbate-2-phosphate and 10nM dexamethasone. Cells were cultured for 14 days at 37°C 5% supplemented CO_2 in a humidified incubator. After culture the cells were rinsed briefly in phosphate buffered saline (PBS) and fixed in 4°C 70% ethanol. Cells were stained for alkaline phosphatase activity and colonies were then counted on an Anderman colony counter (Oreffo *et al.*, 1998a).

2.5.7. Cell surface area measuring

Cells were counted manually for cell surface area by taking a standardised 50X photograph of toluidine blue stained cells using a camera equipped inverted microscope. Measurement was performed by overlaying a grid on which 10 random small dots were placed, the cells closest to the marks were measured by counting the number of squares which the cells covered. Units were therefore arbitrary units of area.

2.5.8. Colony diameter

Toluidine blue stained colonies were observed and measured under a dissection microscope and the graticule calibrated for size, using an electron microscopy grid, between each sample. Colonies were selected by the use of an overlayed acetate of 10 randomly placed dots, with the colonies nearest the mark being measured.

2.5.9. Microscopy and image analysis

Images were taken using an inverted microscope (Leica DMIRB/E) equipped with fluorescent imaging and a Zeiss Axiophot utilising an Axiovert 200 digital imaging system. Cells labelled with 5-Chloromethylfluroscein diacetate (CMFDA1), Ethidium homodimer 1 (EH1), Fluorescein isothiocyanate (FITC) and Ethidium bromide (EtBr) were recorded on a Leica Leitz DM RBE with a water immersion objective.

2.5.10. X-ray

Before removal from diffusion chambers, samples were subject to x-ray in order to determine if any radio opaque material was present, this was performed using an HP 43806 X-ray machine.

2.6. Histochemistry and immuno-histochemistry.

Prior to immuno - histochemical analyses, PLA scaffold samples were fixed with 4% para-formaldehyde or 95% ethanol, dependent on the staining protocol and, as appropriate, processed to paraffin wax and 5 μm sections prepared. Controls (omission of primary antibody) were included in all studies.

2.6.1. Type I and II collagen immuno histochemistry

Sectioned paraffin slides had the paraffin removed from the sections by dipping in baths of histoclear (Citrolene – BDH) for 7 minutes each, then through graded alcohols (100% 5 min, 100% 5 min, 90% 5 min, 50% 5 min) then to water for 10 minutes in order to remove citroclear and rehydrate the sections. Sections were incubated in 3% H_2O_2 for 15 minutes to stop endogenous enzyme activity followed by blocking in 1% BSA solution in PBS for 30 minutes. The sections were then dipped briefly into water to remove the excess blocking solution then incubated with 100μl of primary anti type I collagen antibody (LF-67 courtesy of Dr Larry Fisher) or anti type II (α1(II)) collagen (Calbiochem, UK) at a 1:300 diluted in PBS. The primary antibody was incubated overnight at 4°C in a humidified chamber alongside controls without the primary antibody. The primary antibody was rinsed off using a brief dip in water and subsequent rinsing with High salt (1M NaCl, 50mM tris, 0.05% tween), low salt (0.5M NaCl, 50mM tris, 0.05% tween) and tris (0.1M tris, 0.1% tween) solutions to ensure all excess antibody was removed. Detection started with addition of a 2° anti rabbit IgG biotinylated antibody raised in goat (Sigma, Poole, UK) which was used to detect the primary antibodies via incubation with a biotin binding, avidin conjugated peroxidase enzyme (Extra avidin peroxidase, Sigma, UK). Detection of the peroxidase antibody complex was performed by incubating the sections in a colour precipitating solution of AEC (3-amino-9-ethyl-carbazol 0.01g into 1250 μl of dimethylformamide)

in acetate buffer (500 µl of AEC in 9500 µl of acetate buffer: acetic acid, sodium acetate, pH 5.0) activated beforehand with 5µl of 30% H_2O_2.

2.6.2. Detection of STRO-1 antibody

Sections were incubated in 3 % H_2O_2 to stop endogenous enzymes before blocking in 3% albumin fraction V, the sections were incubated overnight at 4°C and detected using biotinylated anti murine IgM µ chain specific antibody and secondary ExtraAvidin Peroxidase™ (Sigma, Poole Dorset) with a AEC (3- amino-9-ethyl-carbazol) colour developer solution. Sections were counterstained with alcian blue and light green for 20 seconds each, in order to better identify the surrounding tissues.

2.6.3. STRO-1 immuno histochemistry of CFU-F

STRO-1^+ marrow cells were cultured for 1 week in αMEM supplemented with 10% FCS. Cultures were rinsed in PBS before blocking with HBSS containing 5% (v/v) FCS, 5% (v/v) human normal AB serum, and 1% BSA fraction V. Incubated with STRO-1 antibody for 1 h at 4 °C, and exhaustively washed in HBSS containing 1% BSA fraction v. Detection of STRO-1 binding was via anti-IgM FITC (1:500). To highlight the cell nuclei, cultures were briefly stained with 0.002% (w/v) ethidium bromide in PBS before rinsing and observed using fluorescence microscopy.

2.7. Detection of STRO-1 and type II collagen by Western blotting

Samples were prepared by rinsing in PBS to remove BSA and then rinsed in dH_2O to remove excess salts, the samples were then homogenised in loading mix (50 mM Tris, 100 mM dithiothreitol, 2% (w/v) SDS, 0.1% (w/v) bromophenol blue, 10% glycerol). Samples, molecular weight markers and controls (10 µl) were loaded onto denaturing SDS, 10% polyacrylamide gel with a 5% stacking gel and electrophoresed at 75V through the stacking gel (approximately 1 hour) and 2 hours through the resolving gel at 150V in SDS tris glycine running buffer (25 mM tris, 0.25 mM glycine and 0.1% (w/v) SDS). Resulting gels were added to transfer buffer (20% methanol in running buffer) and transferred at 45mA per gel (0.8 mA per cm^2) for 1 hour onto a nitrocellulose membrane. The membrane was blocked using 5% (w/v) marvel milk powder in 0.05% (v/v) tween-tris buffered saline (TTBS) for 1 hour, washed 3 times in TTBS 5 minutes each and incubated with primary antibody, 1:1000 for type II collagen and 1:1 with supernatant for STRO-1 in TTBS overnight at 4°C. Detection of STRO-1 antibody was via the use of an anti IgM µ chain specific biotinylated goat IgG and extra avidin™ alkaline phosphatase with BCP/NBT (5-Bromo-4-chloro-3-indolyl phosphate/ nitro blue tetrazolium chloride) colour development solution.

2.8. Statistical analysis

The students *t* test, paired Student–Newman–Keuls test, Welch's corrected unpaired and paired students *t* test, ANOVA and repeat measure ANOVA were used using the Graphpad Instat statistics package (San Diego, Ca, USA) depending upon the experiment. Values are typically expressed as means ± SD (standard deviation) or SEM (standard error of the mean) derived from 4 replicates unless otherwise stated. Graphical representation of probabilities are *= $p<0.05$, **= $p<0.01$ and ***= $p<0.001$.

CHAPTER 3

Immuno selection of marrow CFU-F using antibody STRO-1

3.1. Introduction

Originally adult marrow stem cells (MSC) were isolated *in vitro,* by adhesion as fibroblastic colonies to cultureware from marrow aspirates, these cells were subsequently shown to be able to convert to a variety of phenotypes, including those expressing the markers of chondrocytes and osteocytes (Ashton *et al.*, 1980; Bruder *et al.*, 1997b; Friedenstein *et al.*, 1966; Pittenger *et al.*, 1999). The ability of CFU-F cells to be cultured *in vitro* and to be transformed to either the chondrogenic or osteogenic pathway should make them adaptable cells for skeletal tissue engineering applications. Direct isolation of stem cells from marrow can be achieved using antibodies to epitopes expressed on groups of cells containing the stem cells. In this study, to isolate a more defined group of marrow cells, the monoclonal antibody STRO-1 was utilised. The antibody STRO-1 has previously been shown to isolate a group of cells containing all the marrow CFU-F (Gronthos *et al.*, 1994; Simmons *et al.*, 1991). Furthermore Oreffo *et al* (1996) demonstrated that the number and proliferative capacity of CFU-F osteoprogenitor cells are maintained during ageing from adulthood, as well as in patients with osteoporosis. Unfortunately, although the numbers of CFU-F are not affected with patient age, they do vary in differentiation potential for chondrogenesis with age and disease (Murphy *et al.*, 2002; O'Driscoll *et al.*, 2001). As well as the reduced differentiation potential, CFU-F do not represent a homogeneous group of cells, as observed by heterogeneity represented by differential expression of markers, such as alkaline phosphatase activity (Aubin., 1998), and after extensive culture *in vitro* most marrow stromal cell clones are only able to be prompted to express character pairings of chondrocyte osteocyte (Muraglia *et al.*, 2000). From previous studies, it appeared that CFU-F represent a grouping of the same cell type, but at

alternate, early differentiation points, maintain a reserve of cells for tissue turnover and repair (Aubin., 1998).

A range of antibodies have been developed to study marrow cells and some recognise epitopes expressed on stem cells during differentiation to a mature cell, these can potentially be used to identify, as well as isolate, the different developmental stages (figure 3.1) (Aubin *et al.*, 1982; Bruder *et al.*, 1997a). Antibodies to epitopes expressed during the stem cells development to a mature cell could therefore be used to isolate and characterise cells at various stages, and some could potentially identify the earliest progenitor cells (figure 3.1).

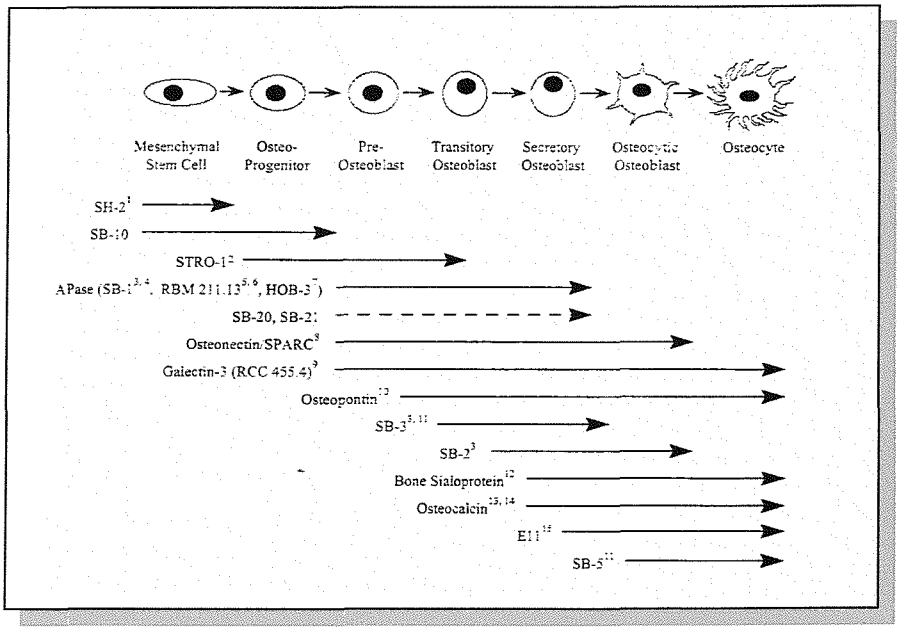

Figure 3.1. Progression of mesenchymal stem cells to osteocytes phenotype and markers expressed (Bruder *et al.*, 1997a).

Several antibodies have displayed efficacy in identifying a CFU-F cell population, including antibodies SB-10, STRO-1, SH-2, and HOP-26 (Barry *et al.*, 1999; Bruder *et al.*, 1998; Joyner *et al.*, 1997; Simmons *et al.*, 1991). Selecting antibodies, such as SB-10, often identify common epitopes on whole groups of bone marrow cell types,

which include the fibroblastic colonies when cultured *in vitro*. SB-10, for instance, has been shown to react with activated leukocyte-cell adhesion molecule 99 (ALCAM), SH-2 with CD105 (Endoglin; TGF receptor type III) and the epitope for HOP-26 shown to be the cell surface and lysosomal enzyme CD63 or melanoma associated antigen, a member of the tetraspan glycoproteins (Barry *et al.*, 1999; Bruder *et al.*, 1998; Zannettino *et al.*, 2003).

With the CFU-F identifying antibody STRO-1, the epitope remains undiscovered. Although it has been shown to isolate the osteogenic/ chondrogenic precursors and not to bind to myeloid cells, megakaryocytes, macrophages, or cells that either enhance or inhibit the production of CFU-F (Dennis *et al.*, 2002; Gronthos *et al.*, 1994; Simmons *et al.*, 1991). STRO-1 cells in culture have previously been characterised as not exhibiting factor VIII, CD14 positive macrophages and limited CFU-GM, BFU-E, CFU-Mix and adipocytes which can normally be found in marrow cultures grown *in vitro* (Gronthos *et al.*, 1994; Simmons *et al.*, 1991). The antibody, STRO-1, was developed by using a population of $CD34^+$ bone marrow cells, because the $CD34^+$ population contains a variety of cell groups it was considered that antibodies derived from these cells could be used to further divide the bone marrow cell populations (Simmons *et al.*, 1991). Further investigations showed that the marrow cell population not expressing glycophorin (negative erythroid fraction) but with the STRO-1 epitope, had an increased ratio of 1:100 CFU-F to nucleated cells compared to approximately 1: $1x10^4$ normally present within the marrow (Simmons *et al.*, 1991). STRO-1 binding in peripheral blood showed minimal binding except to a group of CD20 positive B cells (Simmons *et al.*, 1991), however it should be noted that STRO-1 binding has been reported in the cells of the vasculature (Bianco *et al.*, 2001a).

As STRO-1 could provide a way of isolating a specific group of marrow stromal stem cells, and that these cells could have the potential to form characteristics of both bone and cartilage, STRO-1 selected marrow cells would represent an ideal cell group for the purpose of orthopaedic tissue engineering.

Therefore the aim of this work was to isolate a more defined cell group by using the monoclonal antibody STRO-1. Specifically to isolate STRO-1^+ cells from the marrow of a group of total hip replacement patients using a magnetic antibody isolation system. The cells could then be checked for CFU-F content and observed for the features of the isolated CFU-F *in vitro*.

3.2. Method

3.2.1. Samples.

Bone marrow samples were obtained from haematologically normal patients undergoing routine total hip replacement surgery. Only tissue, which would have been discarded, was used, with the approval of the Southampton General Hospital Ethics Committee (LR194/99). A total of 12 samples (five male and seven female of mean age 71±11 years) were prepared.

3.2.2. Magnetic activated cell sorting.

STRO-1^+ cells were isolated as described previously (Stewart *et al.*, 1999). In brief; red blood cells were removed by centrifugation using lymphoprep solution (Robins scientific, Solihull, UK). The nucleated cells from the interface layer were re-suspended at 1×10^8 cells per 10 ml blocking solution, HBSS containing 5% (v/v) FCS, 5% (v/v) human normal AB serum, and 1% (w/v) bovine serum albumin fraction v (BSA v). After blocking, the cells were washed in 50 ml of MACS buffer (HBSS containing 1% fraction v BSA) and incubated with filtered STRO-1 antibody hybridoma supernatant for 1 hour at 4°C (hybridoma was provided by Dr. J. Beresford, University of Bath). After incubation with STRO-1, the cells were washed in 3x 50 ml MACS buffer in order to prevent excess unbound STRO-1 antibody from sequestering the second stage magnetically labelled antibodies. Cells were then incubated with 100 µl of magnetic MACS anti-IgM beads in 900 µl of de-gassed MACS buffer (Miltenyi Biotech, Bisley, UK).

The 1 ml of labelled cells were then subjected to a magnetic separation regime as follows; for a control group of cells for comparison the cells were added to an un-magnetised column and collected as control unsorted cells, or for separations the cells were added to a cell separation column within the magnetic holding device. When the

magnetic field was in place the labelled cells were retained within the column whilst the eluted cells were collected as the un-retained STRO-1 negative fraction. The column was washed through with MACS buffer to remove the majority of unattached STRO-1 negative cells and then with the magnet removed, the magnetically labelled cells could be eluted with 1 ml MACS buffer, as the STRO-1 positive fraction (figure 3.2). For each fraction, five cell counts were performed using a fast read disposable counting chamber (ISL, Paignton, UK).

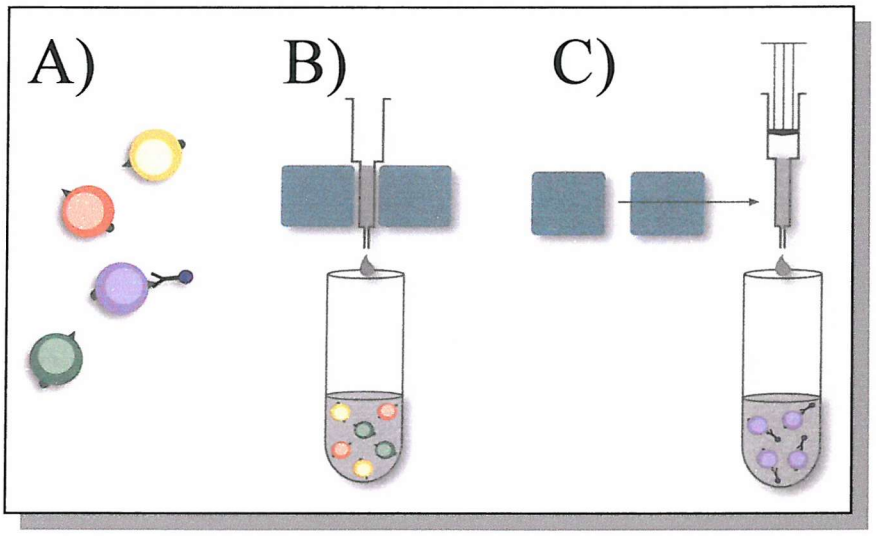

Figure 3.2. Magnetically activated cell isolation procedure A) Control cell fraction with 1° STRO-1 antibody with a 2° para-magnetic particle antibody, B) Focused magnetic force column retain the STRO-1 cells allowing the negative fraction to be eluted and C) removal of the retaining magnetic field and recovery of STRO-1 cell fraction. (Adapted from Miltyni Biotech)

3.2.3. Colony formation assay.

CFU-F formation was examined using 2×10^5 nucleated cells from STRO-1 negative, positive, and control groups cultured in 25 cm^2 flasks. Samples (four female, age 73±9 years) for the CFU-F assay were cultured in αMEM supplemented with 10% (v/v) FCS with dexamethasone (10 nM) and ascorbate-2-phosphate (100 mM), in the presence of penicillin (5000 U/100 ml) and streptomycin sulphate (5 mg/100 ml). Cultures were maintained at 37 °C in a humidified incubator, supplemented with 5% CO_2. The medium was changed after 6 days (to allow attachment of CFU-F) and cultures were stopped at day 10. At completion of cell culture, the medium was removed and the cell layer was washed in phosphate-buffered saline (PBS). Total (toluidine blue stained) and alkaline phosphatase-positive colonies were counted blind (via a numbering system) and the colonies counted using an Anderman colony counter (Anderman, Kingston-on-Thames, UK).

3.2.4. Cell surface area measuring

On triplicates of 3 samples, colony diameters were assessed by measuring 10 random colonies per flask using a random colony location grid. Comprised of a clear acetate with 10 random dots, with the closest colony measured by a calibrated Zeiss dissection microscope. Counts were repeated to confirm reproducibility.

3.2.5. Human marrow sample preparation for STRO-1 histology

For histological location of STRO-1 in total marrow tissue, five different samples were obtained from patients undergoing total hip replacements and a small whole marrow chip from each sample was fixed in each of the following to account for different fixation methods effecting any potential binding; i) 70% ethanol, ii) 5% paraformaldehyde or iii) 4% formol calcium. Fixed samples were then decalcified in a

5% EDTA solution for 3 weeks at 4°C to soften the trabeculae of the marrow samples. Samples were processed to paraffin wax for sectioning before histology.

3.2.6. Murine femur preparation for STRO-1 histology

Murine femurs (8 week old juvenile) were fixed in 4% PFA for 24hrs and decalcified in a 5% EDTA solution for 2 weeks. Samples were then processed in paraffin embedding and 5 μm sections cut, prior to STRO-1 immuno-histochemistry.

3.2.7. Statistical analysis.

CFU-F analysis was performed on quadruplicate samples. Alkaline phosphatase was assayed from four samples of each triplicate, STRO-1 negative, positive, and control fraction. Statistical significance was determined using paired Student–Newman–Keuls test with GraphPad InStat software (GraphPad software, San Diego, CA, USA).

3.3. Results

3.3.1. Immuno-selection of human osteoprogenitors using STRO-1

Isolation of the STRO-1 positive marrow cells by magnetically activated cell separation (MACS) showed that overall, the STRO-1^+ bone marrow nucleated cell fraction comprised 7.12±3.78% of the total nucleated cell population. The negative fraction consisted of 74±16% of the nucleated marrow cell population, with the remainder being removed in the washing phase. STRO-1^+ isolates were all lower than 15% of the starting nucleated cell number (Table 3.1, Figure 3.6 A). Immuno histochemistry with STRO-1, following culture for 1 week in non-osteogenic media, further confirmed the presence of the STRO-1 epitope on the immuno-selected cells (figure 3.3). Negligible staining using STRO-1 monoclonal antibody was observed in the STRO-1 negative fraction (figure 3.3).

Table 3.1. STRO-1 immuno-selection of cells from the nucleated marrow cell population

Group	n	Age in years	% STRO-1^+	% STRO-1^-
All (M+F)	12	71 ± 11	7.12 ± 3.78	74.46 ± 16.06
Female	7	74 ± 9	6.18 ± 3.20	77.17 ± 16.11
Male	5	66 ± 11	8.43 ± 4.49	69.93 ± 18.28

Values are expressed as the mean ± SD

3.3.2. Verification of STRO-1 isolation of cells

Isolation of STRO-1^+ CFU-F was confirmed by staining the STRO-1^+ isolated cells using the STRO-1 antibody with a fluorescent FITC (Fluorescein isothiocyanate) conjugate (Figure 3.3). Cells were cultured for 10 days to develop recognisable CFU-F colonies. The experiments showed that the STRO-1^+ fraction contained CFU-F colonies (Figure 3.4 and 3.5) and that the CFU-F cultures were still detectable as STRO-1 positive by immuno-histochemistry, after 10 days in culture. STRO-1 negative cells also showed a few CFU-F colonies but these were not typically stained using STRO-1 antibody, highlighting the presence of STRO-1 negative CFU-F.

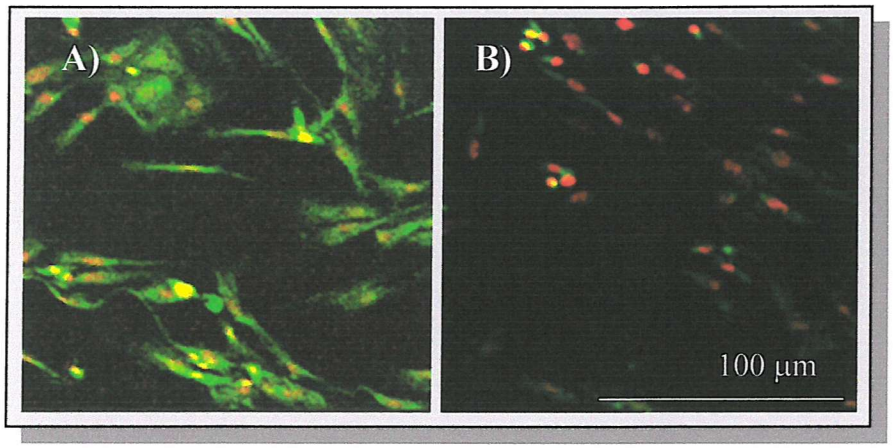

Fig. 3.3. Cultures of adherent human bone marrow cells immuno-stained for STRO-1 using a green FITC conjugate and ethidium bromide nuclear stain (A) STRO-1 selected cells (B) STRO-1 negative cells (original magnification 50×).

3.3.3. Colony forming efficiency

Examination of CFU-F formation in osteogenic media on day 10, showed an enrichment of CFU-F as observed by increased alkaline phosphatase-positive CFU-F in the STRO-1^+ immuno-selected cultures (Figure 3.4, 3.5, 3.6). In the samples examined, no significant differences in the levels of STRO-1 reactivity was observed with respect to male and female donors or with the age of the donors (Figure 3.6). Quantitation of CFU-F number showed a significant (305 ± 86%, $p<0.001$) enrichment of colony number in the STRO-1 immuno-selected fraction. Calculation of the number of CFU-F to initial nucleated cell number showed a ratio of approximately 1 CFU-F in 6000 cells for the STRO-1^+ group, an increase in CFU-F forming ability over the control group which had an average ratio of approximately 1 CFU-F in 20,000 cells. There was an associated reduction in CFU-F in the STRO-1 negative group to 1 colony forming unit in 30,000 cells.

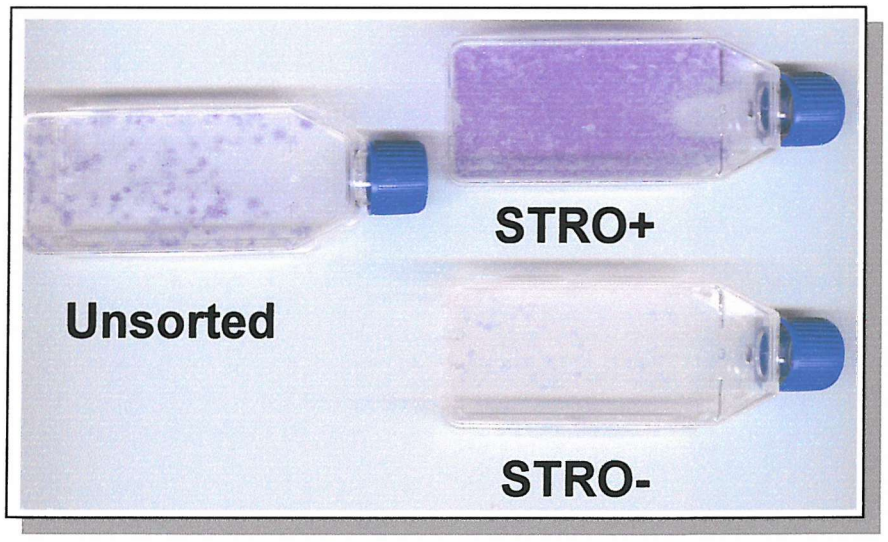

Figure 3.4. Expression of alkaline phosphatase active CFU-F in STRO-1 sorted and unsorted human bone marrow populations cultured for 10 days in osteogenic media each seeded with the same number of nucleated cells (2×10^6 cells/flask).

3.3.4. Colony Diameter

Colony diameters were larger ($p<0.05$: from paired samples, not between groups) in the STRO-1^+ selected group with means of 2900 μm over the control groups 2300 μm (figure 3.5 and 3.6 D). As was expected, a high variation in CFU-F colony sizes between patients were observed as indicated by the high standard deviation (58%; 2900±1600 μm). No significant differences between total CFU-F colony number (toluidine blue stained) and alkaline phosphatase positive CFU-F were observed in the STRO-1^+group.

3.3.5. Alkaline phosphatase activity

An increase in specific alkaline phosphatase activity (190±42%, $p<0.01$) in the STRO-1 positive fractions over unsorted cultures was observed (control = 100%: figure 3.6 C). The STRO-1 negative fraction had a corresponding decrease in specific alkaline phosphatase activity (26±33%, $p<0.01$), when compared to control unselected cultures.

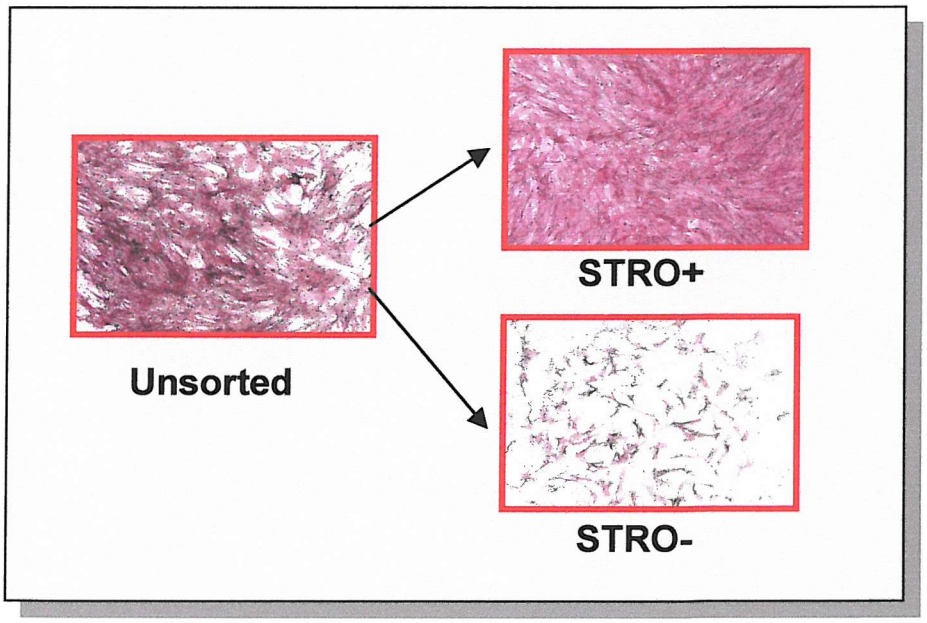

Figure 3.5. CFU-F colonies from different separation groups showing dispersion of each sub division with alkaline phosphatase staining (field of view was approximately 1000 μm).

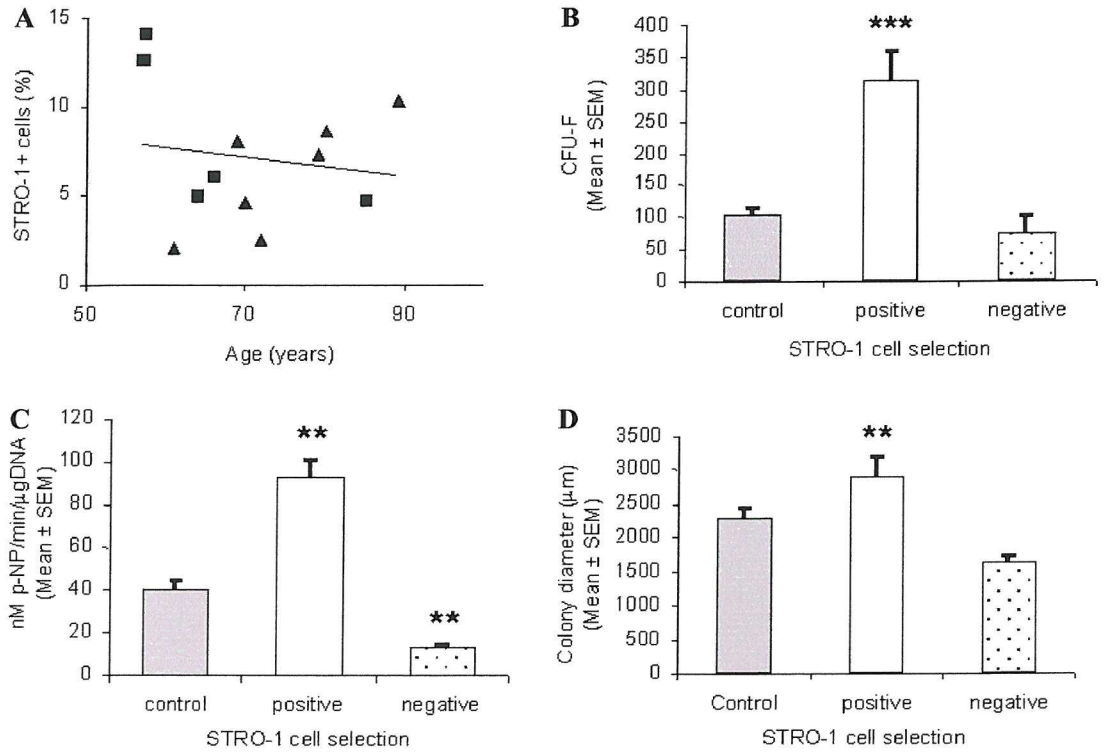

Figure.3.6. (A) STRO-1+ bone marrow cells isolated by magnetically activated cell sorting (■ male, ▲ female). For each of the selection groups of STRO-1 after culture in osteogenic media for 10 days, each seeded with the same number of nucleated cells (B) total CFU-F counts per 25 cm^2 flask, (C) alkaline phosphatase specific activity, (D) mean diameter of 10 random CFU-F colonies from four samples (mean ± SEM, ** = $p<0.01$, *** = $p<0.001$ by paired newman keuls test).

3.3.6. Detection of STRO-1 during *in vitro* culture

Some studies have suggested that STRO-1 disappears from culture over 2 weeks, but can be regained using FGF-2 (Walsh *et al.*, 2000), therefore *in vitro* cultures of CFU-F were checked for expression of STRO-1 over a period of 4 weeks. STRO-1^+ selected human marrow cells were cultured as CFU-F for 7, 14, 21 and 28 days in 10% FCS supplemented αMEM and analysed by both immuno-histochemistry (IHC; figure 3.7) and western blotting detection (figure 3.6) of the epitope. The immuno staining of the STRO-1^+ cells showed that the first few established colonies were brighter (figure 3.7). These bright STRO-1^+ immuno-positive cells then went through an increase in number until week 2, then the STRO-1 staining declined in intensity with increasing number. In the STRO-1 western blots, the day 7 and day 14 time points showed limited expression of epitope due to limited protein, however, the final 28 day time point showed distinct binding of STRO-1 this was probably due to increased cell numbers, even though the immuno histochemistry showed less intensity per cell. The presence of multiple STRO-1 bands at the day 21 and day 28 time points with STRO-1, appeared to bind a high molecular weight protein at over 75 kDa which made it a relatively large protein, the other bands however were much smaller with approximately 8 distinct bands appearing. From the observation of STRO-1 in subsequent *in vitro* cultures it could be seen that STRO-1 was expressed and was continued to be expressed until day 28 although the levels expressed per cell diminish after 14 days.

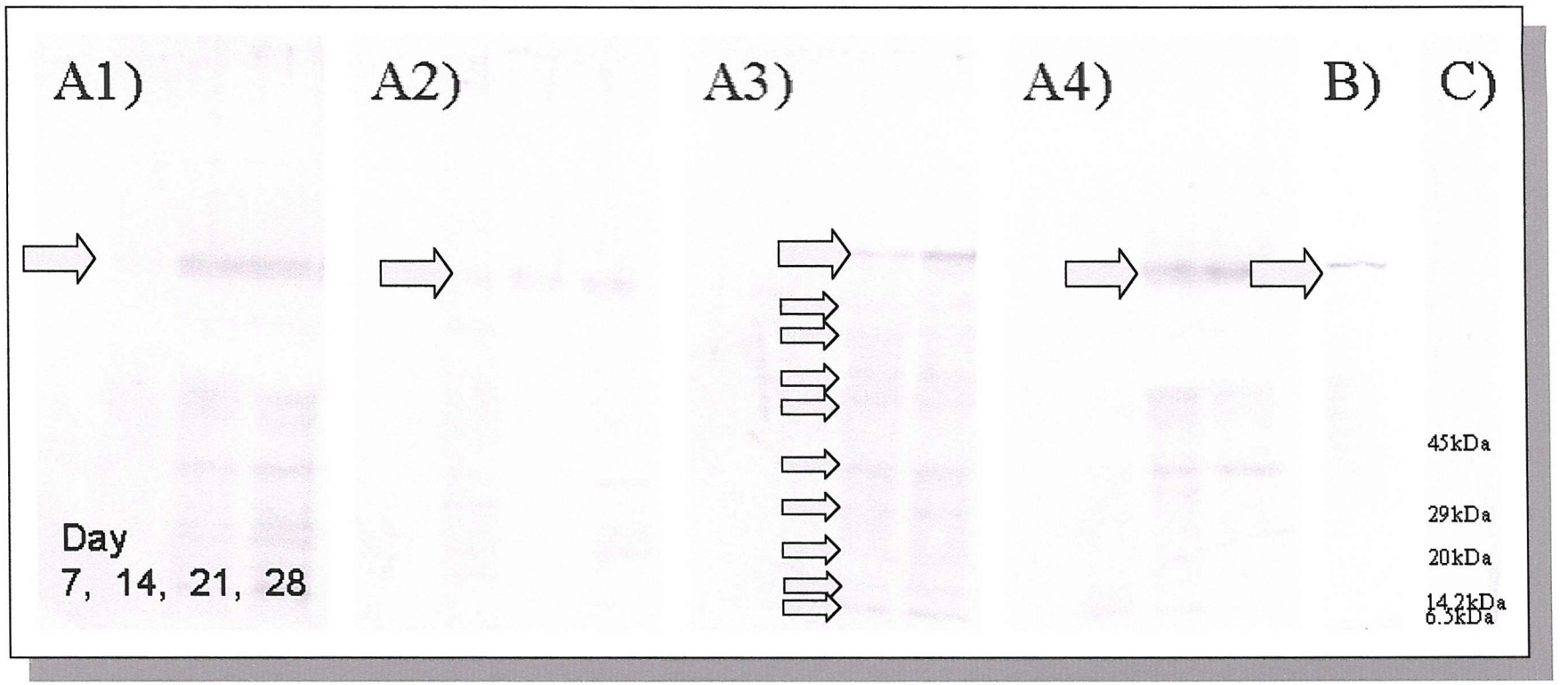

Figure 3.7. Western blots of MSC cell extracts on 7,14,21 and 28 days after seeding. **A-1-4)** Repeats of STRO-1 detection with day, 7,14,21 and 28 running from left to right of each blot, **B)** positive control of Mg 63 cell line previously shown to express STRO-1 epitope and **C)** negative control with omitted primary antibody (arrows show main bands).

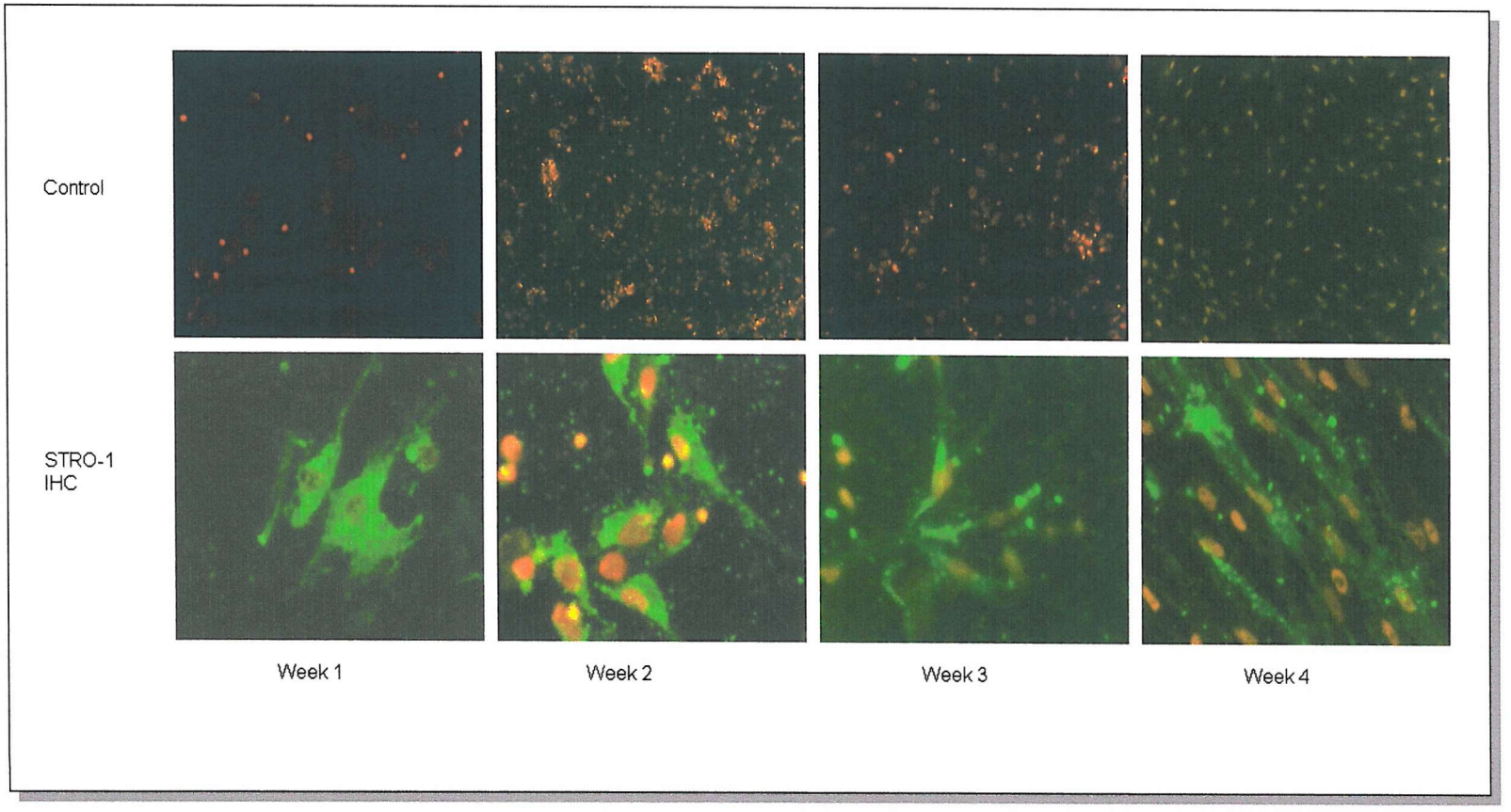

Figure 3.8. FITC labelled STRO-1 with red (EtBr) nuclear stain fluorescent immuno histochemistry on isolated marrow cells at week 1,2,3 and 4 showing controls and corresponding STRO-1 IHC stain

3.3.7. Location of STRO-1 in skeletal tissues

Human marrow did not show any definitive evidence of STRO-1 binding in several differently prepared marrow histology sections, using either dye precipitation (ABC) or fluorescent IHC method. However STRO-1 immuno-histochemistry of week 8 mouse femurs showed distinct binding in the upper layers of cartilage matrix, this appeared associated with the matrix possibly through cross reactivity or trapping, although some of the cells also showed staining over that seen in the control sections (figure 3.9).

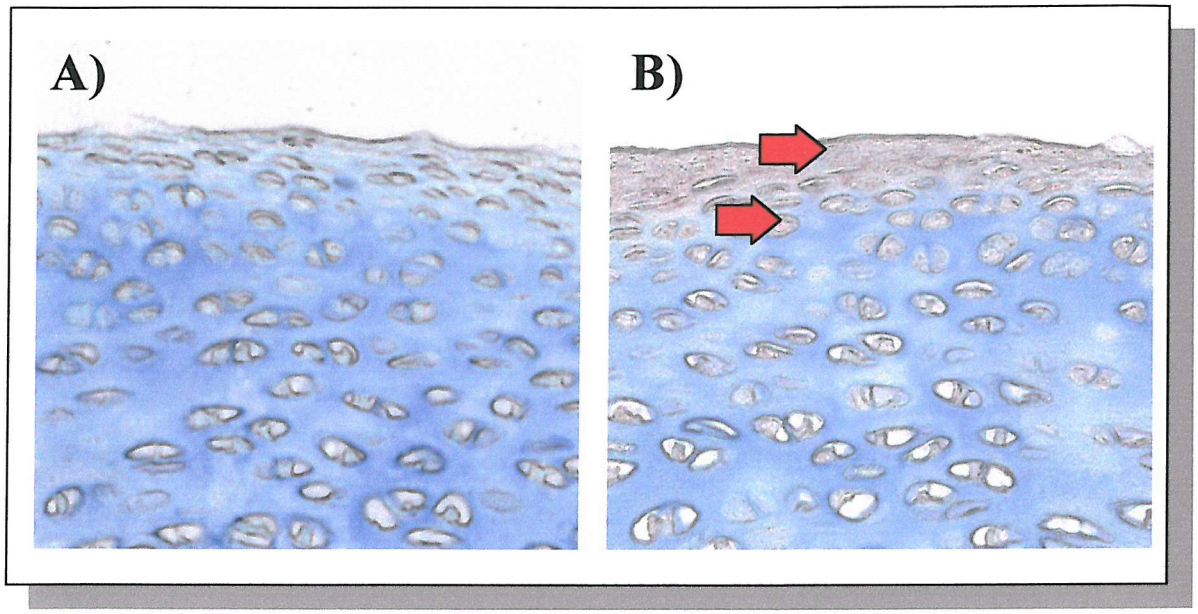

Figure 3.9. 7μm sections of murine femur A) control and B) STRO-1 stained, the arrows indicate staining seen in matrix and in cells (originally 100x magnification).

3.4. Discussion

In the present study, STRO-1^+ marrow cell selection resulted in a significant increase in the ratio of CFU-F cells and, furthermore, indicates different osteogenic potentials present within the different STRO-1 fractions *in vitro*. Different characteristics were observed in the cell subsets, with STRO-1 selection increasing specific alkaline phosphatase activity in the STRO-1^+ group over the negative and control fractions (Figure 3.5). These results are in agreement with the previous studies of Gronthos *et al* (Gronthos *et al.*, 1994), where STRO-1^+ CFU-F showed greater than 90% expression of alkaline phosphatase in each colony. Interestingly, in contrast to our findings, the study also reported that the STRO-1 negative population failed to produce any CFU-F colonies. This discrepancy may be due to the differences between magnetic cell isolation and fluorescence cell sorting, as well as in cell culture and protocols between laboratories.

Immuno-staining with STRO-1 showed CFU-F marrow cells cultured *in vitro* over 28 days changed the expression of STRO-1 with cell morphology, the few cells in early cultures were brighter at week 1 whereas by week 4 the cells had grown into organised sheets and were showing much less intense staining (figure 3.7). Interestingly, the observation of a peak in STRO-1 epitope expression at 2 weeks of culture was observed in the original description of STRO-1 by Simmons and Torrok-Storb (1991) by the separate method of FACS analysis. The western blotting of total protein showed that the predominantly expressed STRO-1 recognition sites were larger than BSA at over 75kDa. There were also several stained bands below this weight, which suggests cross- reactivity and or degradation/aggregation. Simmons and Torok-Storb (1991) reported that occasional cells expressed high levels of STRO-1 in 4 week cultures, and that when cloned these cells apparently behaved in a similar way to the originally

isolated cells with respect to growth rate. Other studies have also observed differences in STRO-1 antigen expression and therefore used the definition of STRO-1^{bright} or STRO-1^{dull} to further sub-divide cell population (Bianco *et al.*, 2001a; Gronthos *et al.*, 1994).

Variation in STRO-1 epitope expression may be due to slight variations in protocols including different serum, patient groups, media, culture plasticware and protocols. It appears that between studies, expression of the STRO-1 antibody in human marrow cells may vary to some extent, with other groups achieving different percentages of isolated nucleated marrow cells and subsequent differences in culture characteristics (Dennis *et al.*, 2002; Stenderup *et al.*, 2001; Stewart *et al.*, 2003).

The lack of STRO-1 binding in histologically prepared sections of total human bone marrow in this study, was a surprising result, however, STRO-1 only identifies 1 in 6000 *nucleated* cells and the sections were essentially 2D which results in a massive reduction in the numbers of cells exposed to IHC. In the histology sections the occasional staining of STRO-1 positive cells could easily match that seen in only minimal background staining of the control sections, thereby masking any result. The staining by STRO-1 observed in cartilage has been corroborated by Bagio Gargulio (Robert Jones and Agnes Hunt orthopaedic hospital, Oswestry) informally, and potentially supports the hypothesis that cartilage stem cells exist in the upper layers of cartilage as proposed by (Archer., 2002) using anti-*msx*1 antibodies. The result also strongly suggests that there was cross reactivity or attachment to cell matrix components at the cell surface.

Normal adult marrow stem cell populations vary little in CFU-F forming potential, however, patients with advanced arthritis have a reduction in the differentiation potential of their stem cells (Murphy *et al.*, 2002; Oreffo *et al.*, 1998a). In a study of

STRO-1$^+$ marrow stromal cells isolated from osteoarthritic patients, a population of constantly maintained stem cells were isolated (Stenderup *et al.*, 2001). These selected cells showed no differences in measured parameters of CFU-F formation, size or alkaline phosphatase on the basis of age, sex, or osteoarthritis condition. The study highlighted the potential to isolate a group of unchanged stem cells from a variety of patients.

Although not performed in this study STRO-1 expression has been manipulated *in vitro* by supplementation of media with FGF-2, which increased the expression of STRO-1 epitope (detected by FACS) (Walsh *et al.*, 2000). The triggered increase in STRO-1 epitope highlights the potential for regimes to increase the STRO-1$^+$ fraction, and if STRO-1 expression is taken as the marker of an undifferentiated stem cell population, then the cultures can be cultured as increasingly undifferentiated cells via the use of FGF-2. In accordance with this theory FGF-2 supplementation has been used to culture expand chondrocytes *in vitro* for the generation of cells for tissue engineering and has been shown to improve the ability of subsequent cultures (Martin *et al.*, 2001). Conversely TGFβ1 limits the expression of STRO-1, and may therefore enhance differentiation cycles shown by a decrease in STRO-1 expression (Gazit *et al.*, 1998; Gazit *et al.*, 1999; Stewart *et al.*, 1999; Walsh *et al.*, 2000; Walsh *et al.*, 2003). If STRO-1 is indeed a marker of the stem cell nature of marrow stromal cells then potential regimes for increasing the STRO-1$^+$ fraction of marrow cells *in vitro,* and therefore potential stem cell populations, may have been achieved by supplementing CFU-F with FGF-2 (Walsh *et al.*, 2000) thereby demonstrating a method of increasing the number of multipotent stem cells for tissue engineering purposes.

So far attempts to isolate *only* the CFU-F stem cells have been unsuccessful, as it appears that only isolation of *groups* of cells containing the stem cells can be achieved.

Therefore, limitations of any isolation procedure may include the need for ancillary cells (Kadiyala *et al.*, 2002), or a single epitope may simply not exist as the stem cells may express only the basic epitopes that a variety of cells express, it has therefore been proposed that true stem cell isolation may need a "cocktail" of antibodies (Stewart *et al.*, 2003).

In summary, isolation by the STRO-1 monoclonal antibody does allow a population of marrow stromal stem cells to be utilised and this group has a higher growth and osteogenic potential than the total CFU-F content, isolated by plastic adherence alone. STRO-1 therefore provides a useful tool to isolate a group of stem cells, with the potential for redressing the imbalance of stem cell differentiation seen in the elderly, the group most likely to need orthopaedic tissue engineering. STRO-1 and any antibody capable of isolating mesenchymal stem cells therefore offers tremendous potential for cell based and gene delivery therapies from a marrow cell isolate.

CHAPTER 4

DELIVERY OF STRO-1$^+$ MARROW STROMAL CELLS AND BMP-2

The author wishes to state that the work was carried out in partnership with Dr Kris Partridge, Dr Xuebin Yang and Dr David Green.

4.1. Introduction

In order to utilise the STRO-1 isolated adult marrow stromal cells it was determined to investigate strategies for *in vivo* use. Marrow stromal cells have previously been shown to generate osteogenic tissue *in vivo* within implanted diffusion chambers (Ashton *et al.*, 1980), therefore it was decided to try to use STRO-1 isolated marrow stromal cells on biodegradable PLA scaffold, for induction of tissue using the diffusion chamber assay. To promote osteogenesis BMP-2 was used, which has been shown to induce the recruitment, proliferation, and differentiation of human mesenchymal stem cells and bone formation *in vivo* (Rickard *et al.*, 1994; Yamaguchi *et al.*, 1991). Combination of STRO-1 selected marrow cells and BMP-2 should therefore make a suitable method of bone tissue induction.

Delivery of BMP-2 can and has been performed by direct injection but the peptide growth factor is expensive, and has a short half-life *in vivo* has a short half-life, a factor which to date has restricted clinical usage. As native BMP-2 is short lived *in vivo,* strategies for modified BMP-2 delivery have been developed, they include the use of controlled release devices like degradable PLA particles with incorporated BMP-2 (Whang *et al.*, 1998) and inserting the BMP-2 gene into human bone osteoprogenitors via an adenoviral delivery system, so cells deliver the growth factor (Partridge *et al.*, 2002).

The combination of using a PLA scaffold to deliver adenovirally transfected STRO-1 isolated marrow stromal cells, expressing BMP-2, to an *in vivo* site within a diffusion chamber should allow generation of an osteoconductive implant material. This approach has already been successfully used with unselected marrow stromal cells and adenoviral BMP-2 on cultured porous biodegradable PLA scaffolds (Partridge *et al.*, 2002).

FDA approved PLA has been extensively used in tissue engineering strategies, combined with the process of supercritical CO_2 formation for structural shapeing means that toxic processes can be minimised and biological factors such as BMP-2 can be incorporated. Another potential candidate was the FDA approved alginate gel which, although not as strong as PLA scaffolds, potentially allows the production of a variety of shapes with encapsulated cells and the possibility of injection strategies. Alginate is similar in composition to proteoglycan side chains of glycos-aminoglycan and is chemically related to hyaluronic acid, keratin and chondroitin sulphate. The matrix of alginate gel has been shown to be able to surround the cells, but still allow solute diffusion, transforming their growth from fibroblastic to rounded (Atala *et al.*, 1994). Alginates can also be combined with other materials such as the minimally antigenic chitosan (VandeVord *et al.*, 2002). Combinations of gels show great promise for biomineralisation by providing a matrix for nucleation of mineral ions thereby increasing the range of applications (Leveque *et al.*, 202). The application of alginate spheres could be useful for orthopaedic tissue engineering purposes and could be used as a controlled breakdown system for BMP-2 and BMP-2 gene delivery.

The aim of this work was therefore to select and culture-expand human osteoprogenitor cells, genetically modify these cells *ex vivo* to express BMP-2 by adenoviral transfer, and to investigate the potential of these cells to be delivered in complexes of alginate and biodegradable PLA polymer scaffold *in vivo,* using the diffusion chamber assay.

4.2. Materials and methods

4.2.1. Infection of cells with adenovirus expressing BMP-2.

Selected human osteoprogenitors were culture expanded *in vitro* for 3 weeks without osteogenic media (without the addition of ascorbate and dexamethasone), and transduced with AxCAOBMP-2, a replication-deficient adenoviral vector carrying the human BMP-2 gene (hereafter AdBMP2). The construction of AdBMP2 has been described previously (Partridge *et al.*, 2002). Briefly adenovirus was culture amplified in packaging HEK 293 cells, purified through a CsCl cushion, and titrated as previously described (Okubo *et al.*, 1999). Adenovirus was added to the cells at a multiplicity of infection (MOI: viral particles per cell) of 20 in media containing 5% FCS. Flasks were rotated every 30 minutes for 1.5 h before addition of the same volume of fresh 5% FCS αMEM.

BMP-2 activity in culture media, obtained from transduced cells, was screened by dilutions of filtered conditioned media, transferred to BMP-2 sensitive promyoblastic C2C12 cells C2C12 cells produce alkaline phosphatase activity in response to BMP-2 which was then compared to activities produced in C2C12 cells in response to known amounts of BMP-2.

4.2.2. Production of alginate capsules

Modified alginate beads were prepared as the method of Leveque *et al* (Leveque *et al.*, 2002). In brief, droplets (~100 μl) of 2 % (w/v) liquid alginate solution in αMEM were added to a polymerising solution of αMEM with mineral ions ($CaCl_2$ 25-30 mM) containing 1% (v/v) acetic acid pH 6.5-7.0 for 60 minutes before placing into the final cell culture media.

4.2.3. Modification of alginate capsules

i) Addition of chitosan shell

As before a 2% (w/v) alginate solution in αMEM was added drop-wise to a polymerising solution, the polymerising solution for this version contained aqueous 1% chitosan (w/v) with 25 mM $CaCl_2$ and 1% acetic acid pH 6.5 for 1 hour, this results in attachment of the chitosan gel at the alginate interface

ii) Addition of a chitosan/mineral shell

As before the 2% alginate solution was added to a polymerising solution. This time in order to incorporate a layer of mineral and chitosan the polymerising solution was used, but the chitosan had 200mM Na_2HPO_4 added prior to droplet formation. The result was formation of a mineral deposition in the outer layers of the polymerising chitosan/alginate this was left for 1 hour to polymerise.

iii) Addition of primary human cells to capsules

Chondrocytes and STRO-1^+ MSC cells were isolated from femoral heads as previously described, the cells were then culture expanded in monolayer and $2x10^7$ cells were gently added to 5 ml of 2 % alginate αMEM media solution. The spheres were produced as before with mineralisation and polymerisation controlled by varying time in the ion polymerisation solution.

iv) Addition of BMP-2

Recombinant human BMP-2 was added to spheres at 100 ng/ml and assayed for BMP-2 activity by addition of 3 spheres to each well of a 6 well tissue culture plate containing a monolayer of MSC which are sensitive to BMP-2 producing alkaline phosphatase activity.

4.2.4. PLA scaffolds.

The specific scaffold used was Poly (lactic acid) (PLA, Alkermes; Mw 12,845) porous (50–200 m). The scaffolds were produced by a supercritical carbon dioxide method as in previous experiments (Partridge *et al.*, 2002), but were of PLA not PGA. Porous scaffolds were sterilised using 70% ethanol for 1 hour and washed in αMEM supplemented with 10% FCS, for 3 hours prior to use.

4.2.5. *In vivo* studies

Cells were recovered from cell expansion monolayers by enzymatic removal by collagenase and trypsin digestion (Clostridium histolyticum, type VII; 25 U/ml, 0.05% trypsin and 0.2% EDTA acid in PBS, pH 7.4).

Adenoviral transduced human bone marrow cells were cultured in αMEM containing 5% (v/v) FCS and transferred to serum free αMEM prior to injection within diffusion chambers (2×10^6 cells/chamber; 130 μl capacity; Millipore, UK). Diffusion chambers were implanted intraperitoneally into athymic mF1-nu/nu mice (20–24 g, 4–5 weeks old, Harlan UK Ltd). Control chambers were prepared for comparison and contained untransduced human bone marrow CFU-F cells on PLA scaffolds alone. After 11 weeks the mice were sacrificed, chambers were removed, examined by X-ray analysis, and fixed in 95% ethanol at 4 °C. Samples were processed un-decalcified, sectioned at 5 μm, and stained for type I collagen protein, mineralisation by von Kossa as well as alcian blue/ sirius red (A/S), toluidine blue and organisation of the matrix detected by polarising light microscopy (birefringence).

4.2.6. Cell viability.

Adenoviral transduced human bone marrow cells were incubated with Cell Tracker green™ (5-chloromethylfluorescein diacetate, CFMDA) (Molecular Probes, Leiden, Netherlands) and ethidium homodimer-1 (EH-I) (Molecular Probes, Leiden, Netherlands) for 45 minutes, to label viable and necrotic cells, respectively. The media was then replaced twice and the cells were incubated in fresh media for a further hour before observation using fluorescence microscopy, with the constructs in PBS in order to reduce background fluorescence.

4.2.7. Histochemistry and immuno histochemistry.

Prior to immuno-cytochemical and histochemical analyses, PLA scaffold samples were fixed with 4% paraformaldehyde or 95% ethanol, dependent on the staining protocol and, as appropriate, processed to paraffin wax and 5 μm sections were prepared. Negative controls (omission of primary antibody) were included in all studies for comparison.

4.2.8. Type I collagen immuno histochemistry.

Reactivity to the type I collagen antibody (LF 67 (anti-1(1) carboxy-telopeptide), Dr. Larry Fisher, NIH, USA) was assessed after fixation in 4% paraformaldehyde. Endogenous peroxidase activity was blocked using 3% H_2O_2 prior to incubation with LF 67 antibody (1:300 in PBS) for 3 h at 4 °C. Samples were incubated with peroxidase-conjugated anti-rabbit IgG (1:30 in PBS) and peroxidase activity was detected using 3-amino-9-ethyl-carbazole (AEC) in acetate buffer, activated immediately before use with 0.015% H_2O_2. Samples were counterstained with Mayer's haematoxylin.

4.3. Results

4.3.1. Adenoviral transfection

STRO-1 marrow stromal cells, plated in monolayer, were transfected with a MOI of 200 by adenoviral BMP-2. After 4, 6 and 8 days the media was removed and filtered to remove viral particles and fresh media added, at 1:1, 1:2 and 1:10 dilutions. Diluted media was then added to a C2C12 cell assay (C2C12 cells are sensitive to BMP-2 and develop alkaline phosphatase activity in response). It can be seen from the results that there was a peak of activity at day 6 and that by day 8 there was a drop to near basal levels, this meant the cells had to be used before day 8 as there was only a transient expression of BMP-2.

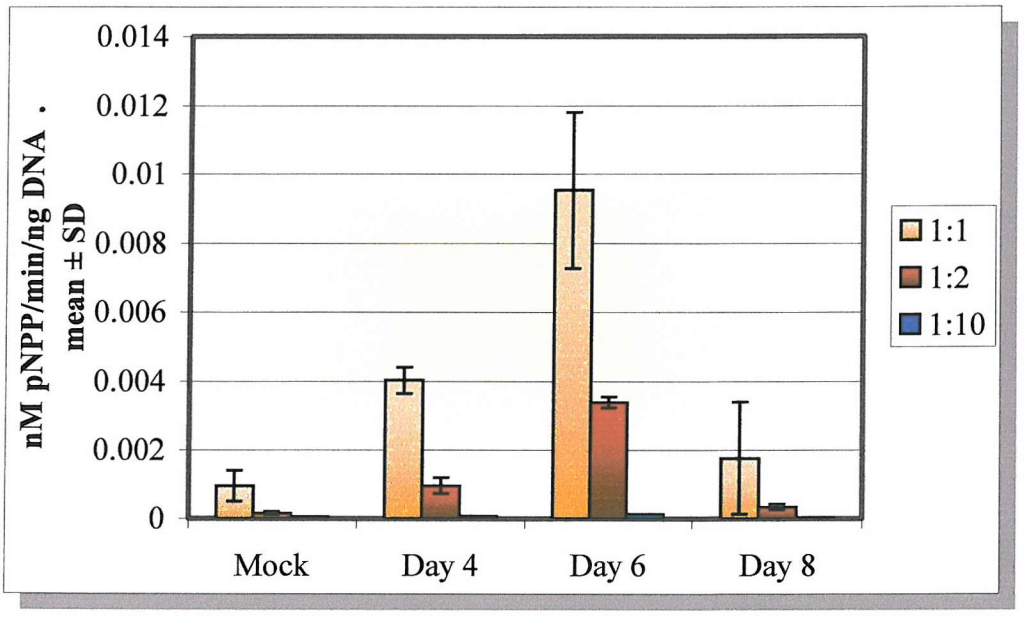

Figure 4.1. Expression of BMP-2 by adenovirally transduced STRO-1^+ marrow stromal cells (values expressed as mean ± standard deviation, courtesy of Dr Kris Partridge).

4.3.2. Growth of adenovirally-transduced osteoprogenitors on PLA scaffolds

Marrow stromal cells transduced with adenoviral vector (AdBMP-2) produced BMP-2 protein as observed by western blotting. Secretion of active BMP-2 by STRO-1^+ bone marrow stromal cells was confirmed in media by C2C12 assay. Resultant alkaline phosphatase activity showed a BMP-2 concentration equivalent to 65 nM of BMP-2 protein in the media 3 days after transduction (control = 0.0 nM).

STRO-1^+ populations of human bone marrow cells transduced with AdBMP2 were seeded onto biodegradable porous PLA (50–200 μm) scaffolds. After 48 h of culture, cell viability was confirmed by the intense green fluorescence observed due to incorporation of the Cell Tracker green™ fluorescent probe into the cell cytoplasm. The absence of significant ethidium homodimer-1 staining emphasised the lack of cell necrosis and indicated that immuno-selected adenovirally transduced osteoprogenitor cells are capable of adhering to, and proliferating, on PLA scaffolds *in vitro*.

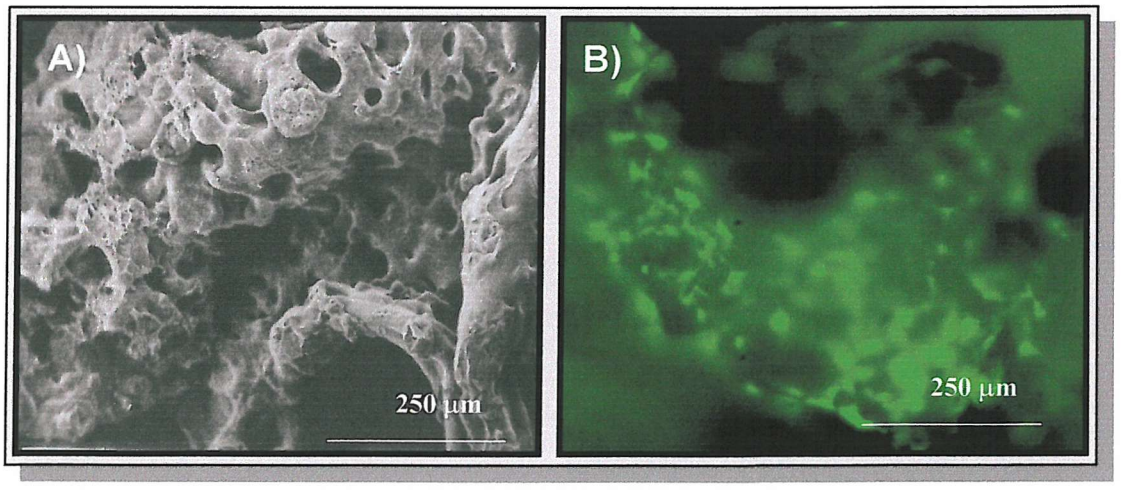

Figure 4.2. PLA scaffold A) With low power scanning electron microscopy and B) with adenovirally transduced Live/dead stained marrow stromal cells.

4.3.3. Encapsulation of STRO-1 cells

Gels could be formed within a mineral/chitosan solution to which the cells were added before polymerisation. Cells were cast into beads and cultured for 1 week in culture media, the results showed that a mineralised bead could be produced and that these beads could be used to contain osteoprogenitors and chondrocytes (figure 4.3). Osteoprogenitors after culture in osteogenic media were positively stained for alkaline phosphatase activity (figure 4.3 C), indicating differentiation of marrow stromal cells within the gel. To answer if the cells were alive within the alginate beads the beads were subject to live/dead staining and observed using fluorescence microscopy. The cells within the alginate showed survival of the polymerisation phase as observed by the presence of green/live cells (figure 4.3 B).

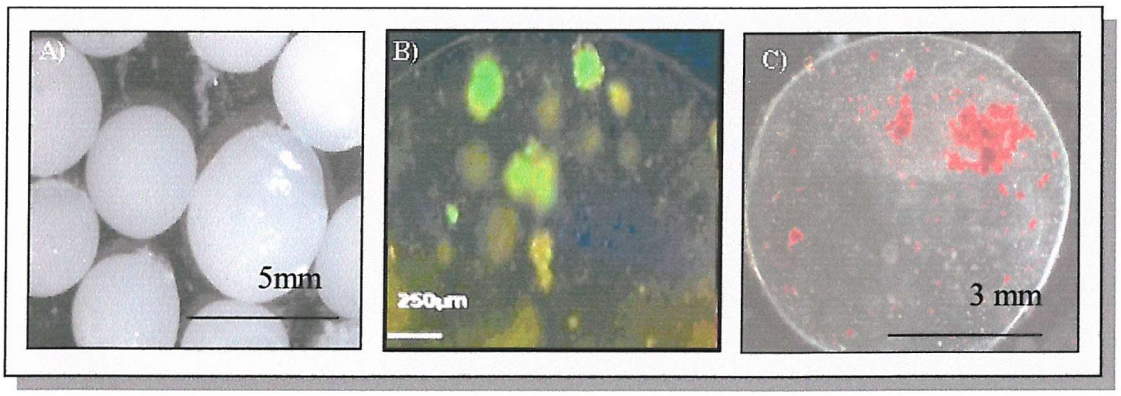

Figure 4.3. A) Alginate beads, B) live/ dead stained STRO-1 selected osteoprogenitor cells within an alginate bead and, C) alkaline phosphatase stained osteoprogenitor cells within an alginate/chitosan/mineral sphere (courtesy of Dr David Green).

4.3.4. BMP-2 encapsulation

BMP-2 was mixed with the un-polymerised alginate solution at 100 ng/ml and then placed into 6 well culture plates with 3x 5mm diameter spheres each. C2C12 cells present within the wells were then measured for alkaline phosphatase activity after 5 days in culture, in comparison to control alginate spheres.

To confirm the ability of the mixed polysaccharide/ mineral beads to be used with human mesenchymal stem cells, $2x10^7$ MSC were added to 5 ml of alginate mixture and 100ng/ml rhBMP-2 added. The groups were then compared for alkaline phosphatase activity induction.

The addition of BMP-2 showed that the beads could be used as a delivery device for active BMP-2 (figure 4.4 A), although time course and effects of gel modification have yet to be performed. Addition of BMP-2 to the spheres also containing osteoprogenitors showed that it also has the potential to initiate the osteogenic process from within the bead (figure 4.4 B), therefore, showing the ability to carry complexes of factors and cells.

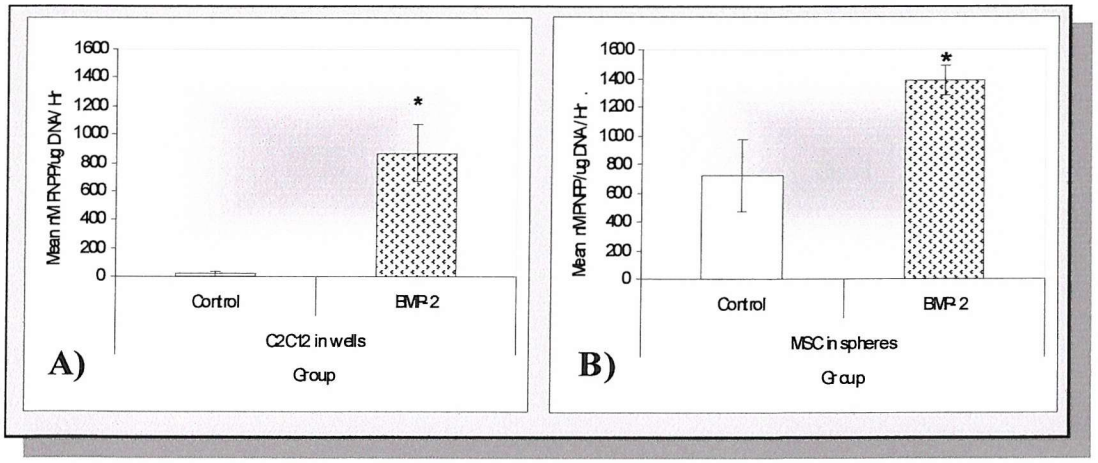

Figure 4.4. Addition of BMP-2 to alginate/chitosan beads with A) release from the beads onto monolayers of C2C12 cells and B) incorporation with mesenchymal cells within the pellet (displayed as mean ± SD, n=3, *P<0.05 by t test) courtesy of Dr David Green).

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

STRO-1^+ adenovirally-transduced marrow stromal cells, seeded onto PLA scaffolds within diffusion chambers, showed evidence of mineralisation as observed by X-ray analysis (figure. 4.5 B) and von Kossa staining (figure.4.5 H) in 3 of 3 diffusion chambers. In addition, formation of organised (polarising light microscopy), alkaline phosphatase positive, type I collagen matrix was confirmed (figure 4.5 G, D, C respectively). No mineralised tissue formation was observed in human bone marrow/ scaffold constructs alone (figure 4.5 A).

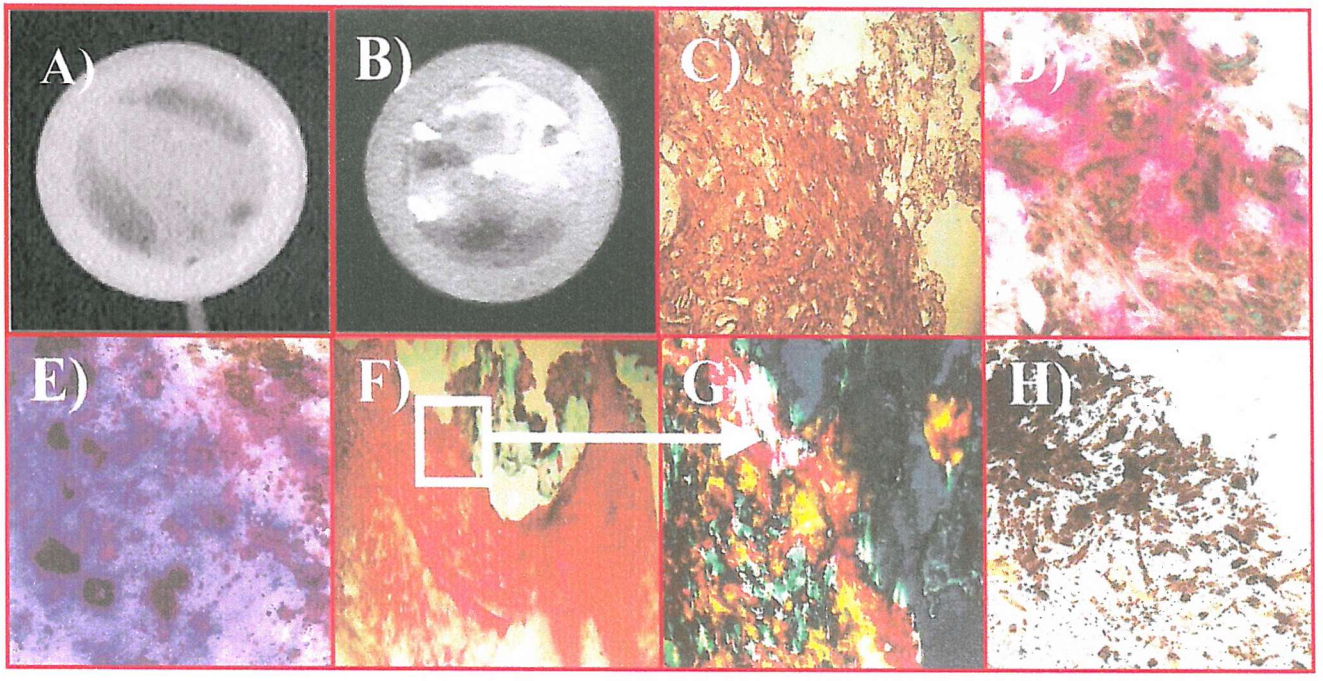

Fig. 4.5. Diffusion chambers after 11 weeks with STRO-1^+ primary human bone marrow cells in basal media, transduced with adBMP-2 seeded onto PLA scaffold, and implanted intraperitoneally into mF1 nu/nu mice. (A) control chamber X-ray (B) X-ray of diffusion chamber containing STRO-1^+ adenoviral transduced cells showing radio-opaque areas. (C–H) Cross-sections of diffusion chamber stained for (C) collagen type I immunostain (original magnification 20×). (D) Alkaline phosphatase activity (original magnification 20×). (E) Toluidine blue stain (original magnification 20×). (F–G) Alcian blue/Sirius red-stained section with polarising birefringence microscopy showing organised collagen structures (original magnification 10–25×). (H) von Kossa stain of mineral (original magnification 20×).

4.4. Discussion

The use of STRO-1 selected cells on PLA *in vivo* showed that STRO-1 cells can be induced into a mineralising tissue and that the general culture scheme was a suitable one for culturing osteogenic tissue implants. The adenoviral system for generating BMP-2 at the immediate site also proved to be efficacious and as the adenoviral transduction process was performed *ex vivo* it should be less immunogenic than viral transduction *in vivo,* although viral immunogens would have to be assayed before any use with patients. Encapsulation of peptide rhBMP-2 in alginate showed that the gel system was able to contain BMP-2 and could potentially be used, like PLA, as a biodegradable BMP-2 delivery device. Chitosan has previously been used for protection and delivery of genes for transfection strategies (MacLaughlin *et al.*, 1998) and could allow incorporation of gene therapies into future tissue engineering schemes. Unfortunately, alginates although flexible, are not as strong (even with extensive polymerisation) as PLA (Weber *et al.*, 2002) and both chitosan and some alginates have been shown to attract macrophages and neutrophils through chemo-attraction, although not necessarily leading to a full inflammatory response (Suh *et al.*, 2000; VandeVord *et al.*, 2002).

PLA scaffold material, unlike the gels, allows spreading growth of cells throughout the matrices and can be made into higher strength architectures. Evidence for the transduced STRO-1^+ cells on PLA scaffolds to generate mineralised tissue was confirmed using the diffusion chamber assay, which allows demonstration of tissue formation by the implanted cells within a closed *in vivo* environment. The histology of the diffusion chamber constructs showed alkaline phosphatase active, type I collagen positive, von Kossa stained, mineralised matrix, which was shown to be composed of organised collagen fibrils by using birefringence microscopy. However, it should be

noted that the tissue produced represents an organised, mineralised tissue and not mature bone material, as birefringence microscopy shows only organisation of collagen fibers and similar patterns can be seen with fibrous repair tissue of cartilage defects. Mineralisation without the correct structure and organisation is merely a mineralised tissue and not bone, which requires a very specific mineralisation by controlled nucleation, using ancillary proteins such as osteocalcin and bone sialoprotein, not examined in this study. The ability to form osteogenic tissue by transduced STRO-1^+ marrow cells could be observed by the occurrence of X-ray positive constructs in 100% of the diffusion chambers, as compared to 60% of diffusion chambers observed in previous studies using unselected marrow cells (Partridge *et al.*, 2002). Control cultures of CFU-F, as expected, produced an undefined fibrous tissue in which no X-ray dense material was observed (Figure 4.5 A-B).

Alginate gels used for distribution of cells and structural support, could allow a complex construct to be produced with a variety of cells/ growth factors and genes incorporated using a bead-in-bead system, the resulting series of cell types and compounds could be tailored to be released at different rates and controlled by a mineralised surface layer for strength and integration. However, although alginate is a versatile material, it has been observed to reduce the proliferation rate of encapsulated cells, an important parameter in tissue engineering protocols, conversely it does appear to promote differentiation and entrapment of matrix in chondroprogenitors (Kisiday *et al.*, 2002; Weber *et al.*, 2002).

In conclusion, it was shown that STRO-1^+ mesenchymal stem cells can be adenovirally modified to express active BMP-2 and following culture on porous biodegradable PLA scaffold subsequently form a mineralised tissue *in vivo*. Alginate

encapsulation is a promising method for cell and growth factor delivery although further *in vivo* work is clearly needed. Ultimately stem cell engraftment, stem cell plasticity, production of mineral nucleating proteins and issues of stem cell fate at the implant site will need to be addressed.

CHAPTER 5

IN VITRO INDUCTION OF THE CHONDROGENIC PHENOTYPE

5.1. Introduction

Tissues rely upon the cells present to produce the set of matrix molecules and enzymes required for function of that tissue. Loss of these molecules results in dysfunction or degradation of the organ. Therefore, to have the correct molecules produced for the maintenance and functioning of the tissue is imperative. The production of tissue engineered cartilage would rely upon the generation of the main features of cartilage, notably, type II collagen and aggrecan. However, chondrocytes cultured in monolayers cease to express the phenotypic markers that they do in native cartilage and switch to a primarily undifferentiated type I collagen producing cell. To circumvent the change of the chondrocyte phenotype, observed by the loss of expression of type II collagen and aggrecan, a technique was developed by Holtzer *et al* 1960 (Holtzer *et al.*, 1960) to revert the cells back to a more chondrogenic phenotype. The method devised, reformed some of the three dimensional shape and resulted in the re-expression of collagen type II and aggrecan, probably through changing the surface molecule interactions. Thus chondrocytes can be re-differentiated by a culture method, for the purpose of reversing the de-differentiation seen in monolayer cultures (Holtzer *et al.*, 1960). The re-induction of type II collagen production was achieved by culturing chondrocytes at a high cell density in a tight centrifuged pellet of cells, and culture continued in this format, enabling chondrocytes to attach to each other, and not to tissue culture plasticware (figure 5.1).

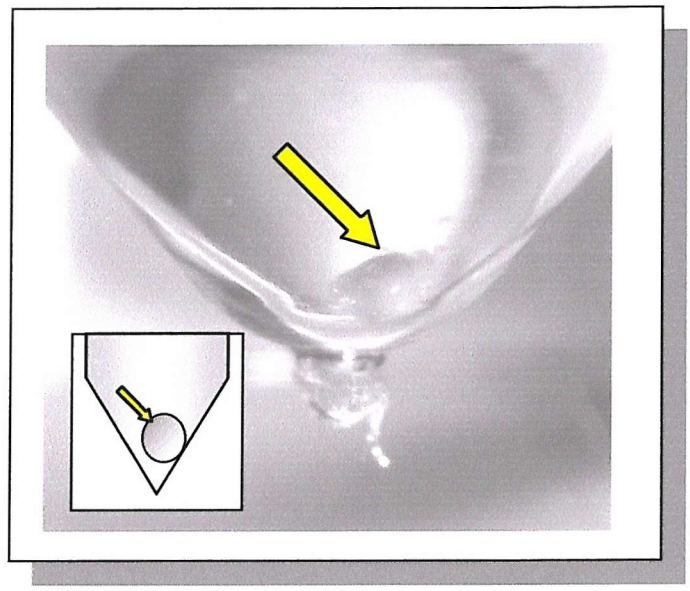

Figure 5.1. Pellet culture within a universal tube

(pellet was approximately 1mm in diameter).

The micromass pellet technique was further adapted for the differentiation of not only monolayer chondrocytes, but for mesenchymal stem cells to express the markers of hypertrophic chondrogenesis - namely type X collagen and type II collagen (Johnstone *et al.*, 1998; Mackay *et al.*, 1998; Yoo *et al.*, 1998). This method could therefore be used as a model system to observe the potential of cell groups to be transformed into cartilage-like material, including STRO-1 selected mesenchymal stem cells and a chondrogenic cell line ATDC5. The differentiation seen in pellet culture would allow a method of verifying the ability of the cells to produce a matrix suitable for tissue engineering of a cartilage substitute, and the cell line would allow a model system to be set up for continued experimentation. The cell line ATDC5, are a clonal murine cell line that would be ideal for testing further tissue engineering systems, as they have the ability to be induced from a general fibroblast like cell to a hypertrophic chondrocyte phenotype, even in monolayer (Atsumi *et al.*, 1990).

The aim of this study was therefore to differentiate STRO-1 selected marrow stromal cells to the chondrocyte phenotype, using the 3D "pellet culture" method. To provide an appropriate comparison for the marrow stromal cells, primary human chondrocytes were isolated from articular cartilage, cultured in monolayer and then in pellet form to show the retrieval of the chondrocyte phenotype. Pellet cultures could also be used to validate a model cell line for chondrogenesis. Any results could then be interpreted for use in tissue engineering strategies.

5.2. Method

5.2.1. Pellet culture. (Figure 5.2) ATDC5, human articular chondrocytes, marrow stromal cells or STRO-1^+ selected marrow stromal cells were trypsinised from monolayer cultures and $1x10^5$ cells in 1000 µl of culture media were added to a sterile 25ml polycarbonate universal and centrifuged at 400g for 10 minutes. The resultant pellet was not dispersed but incubated in a humidified incubator at 37°C 5% CO_2, after 1-2 days the pellets formed 0.5 - 2 mm diameter sized clusters of cells and could be swirled, to allow media access to all sides and prevent adhesion. After 2 days the media was replaced with fresh media and this was repeated every two to three days thereafter. The media used for pellet culture was a defined media composed of a modification of the media used by MacKay *et al* (Mackay *et al.*, 1998) with 10ng/ml TGFβ3, 10nM Dexamethasone 100uM Ascorabate-2-phospate and 1µl per ml linoleic acid, but based on αMEM media, not DMEM.

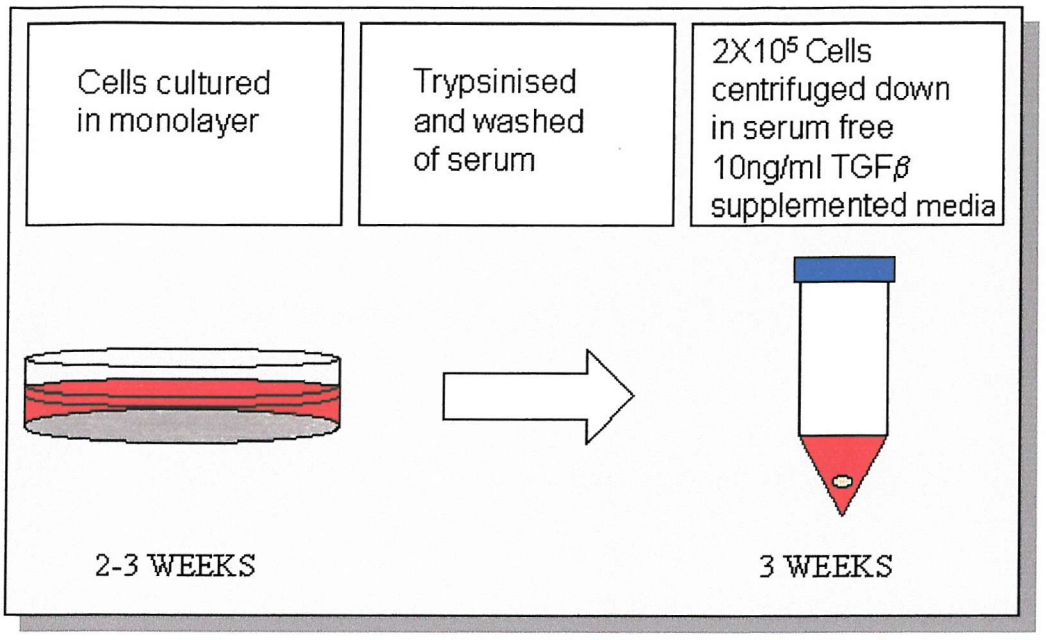

Figure 5.2. Scheme of pellet culture, cells were cultured in monolayer to increase cell numbers and then trypsinised and $1x10^5$ cells further cultured in a centrifuge tube.

5.2.2. Histochemistry. Prior to histochemical analyses, PGA scaffold samples were fixed with Formol calcium (4% (v/v) formaldehyde, 1% (w/v) calcium chloride), processed to paraffin wax and 7 μm sections prepared.

5.2.3. Proteoglycan extraction. Proteoglycans were extracted from the pellets using 1ml of solution for each pellet, and for control cartilage 10mls per gram of sample of cold (4°C) 4 M Guanidine HCl containing 0.05 M sodium acetate, 0.01 M di-sodium EDTA, 0.1 M 6-aminohexanoic acid, and enzyme inhibitors 0.005 M benzamidine HCl, 0.5 mM PMSF (phenylmethyanesulfonyl fluoride) and 0.01 M n-ethylmaleimide added just before use. Extraction was performed for 24 hrs at 4 °C with agitation on a sample roller. After extraction, tissue was removed by centrifugation and the supernatant dialysed against 3X 5 l of stirred distilled water using 12-14,000 dalton pore size dialysis tubing before assays.

5.2.4. Sulphated glycosaminoglycan (GAG) assay. GAG content was measured as in the method of Farndale *et al* (Farndale *et al.*, 1986). Briefly, GAG was extracted as previously described and sulphated GAG was measured by dye binding to di-methyl-methylene-blue (DMMB SERVA via AMS biotechnology, Oxon, UK) in 37mM NaOH and 4.5mM formic acid (BDH, Poole, UK) by comparison to shark cartilage chondroitin sulphate-C standards (Sigma, Poole, UK) over 0-40 μg/ml range (0, 10, 20, 30, 40 μg/ml). 200 μl of dye solution was added to triplicates of 40 μl of sample. The dye changes colour with binding and was measured immediately at an absorbance of 575 nm on a Titertek multiscan.

5.2.5. GAG western blot. Extracted proteoglycans were prepared for western blotting by first exposing the epitopes using an enzymatic treatment of chondroitinase ABC (0.01Unit per 10μg GAG), keratinase (0.01Unit per 10μg GAG), and keratinase II (0.0001Unit per 10μg GAG) at 37°C for 2 hours in 100mM tris acetate pH6.5. After digestion, the samples were dialysed (3X 5 l of stirred distilled water using 12-14,000 dalton pore size dialysis tubing) to remove salts and concentrated by rotary evaporation. Samples (10 μg GAG as measured by DMMB dye binding) were added to sample buffer containing 10% β-mercaptoethanol. Samples were then run on 4 to 12% Novex™ Tris glycine precast gels (Invitrogen, Paisley, UK) and transferred to nitrocellulose membranes using a submerged wet blotting system. Once the samples were transferred to the nitrocellulose membrane blots, they were blocked with 5% fraction V BSA, then incubated with antibodies to differing parts of the aggrecan molecule (figure 5.3; antibodies 2-B-6 to chondrotin-4-sulphate, 3-B-3 chondrotin-6-sulphate, 1-B-5 to chondrotin-0-sulphate, A-IGD6-B-4 to interglobular domain, 7-D-1 to globular domain 1 and 8-A-4 to link protein), provided courtesy of Professor Bruce Caterson, University of Cardiff.

Detection of the primary antibodies was via alkaline phosphatase conjugated anti mouse IgG developed enzymatically using BCIP/NBT (5-Bromo-4-chloro-3-indolyl phosphate/ nitro blue tetrazolium chloride) solution.

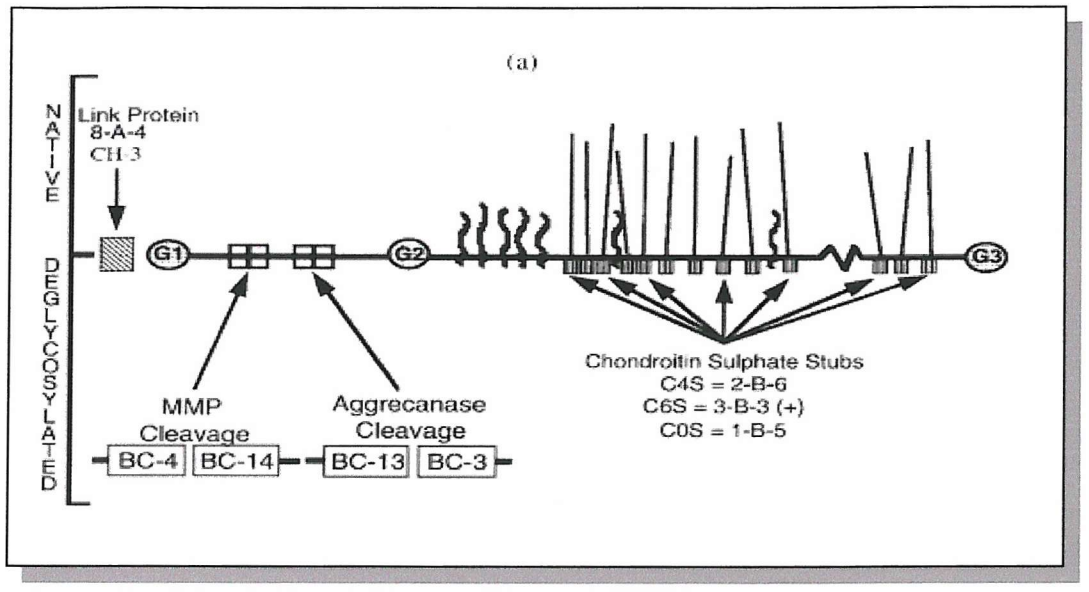

Figure 5.3. Aggrecan molecule with specific antibody epitopes marked. (modified (Caterson *et al.*, 2000)).

5.2.6. Type II Collagen Western blot. In order to disrupt pellets and dissolve some of the collagen molecules present with minimum loss of small samples, pellet culture samples were prepared by 5 s sonication in 100 µl loading mix.

Loading mix containing the samples was heating briefly at 75°C before loading, in order to denature protein for correct running. A standard 10 µl sample was run on 10% PAGE gels with a 5% stacking gel. The samples were run through the gel at 75V until through the stacking gel (approximately 1hr) and at 150V through the resolving gel (approximately 2hrs). Gels were blotted onto nitrocellulose membrane in 20% methanol based transfer buffer, using a semi-dry system at 45mA for 1hr.

Nitrocellulose membranes with adsorbed proteins were added to 5% marvel blocking solution in tween tris buffered saline (TTBS) for 1hr, rinsed 3 times in TTBS (5 min each) prior to incubation with 1:1000 primary antibody anti type α1(II) (Calbiochem, Human molecular weight 134.5kDa) in TTBS overnight at 4°C. Detection was performed using biotinylated anti IgG and extra-avidin horseradish peroxidase. Bands were detected using BCIP/NBT colour solution for no longer than 15 minutes.

5.3. Results

5.3.1. Chondrocyte pellet culture.

Chondrocytes were used as a control cell type, they were able to form pellets and the pellets at 21 days displayed mixed areas of alcian blue/ sirius red staining (figure 5.4 B). With alcian blue binding primarily to glycosylated proteins and picro-sirius red binding primarily to fibrous collagen, it indicates a mixed extracellular matrix, but one that potentially contains glycosylated proteins such as glycosaminoglycan (GAG). Chondrocytes also developed lacunae (figure 5.4 C) and displayed type II collagen by immuno histochemistry (figure 5.4 D), features indicative of chondrocytes.

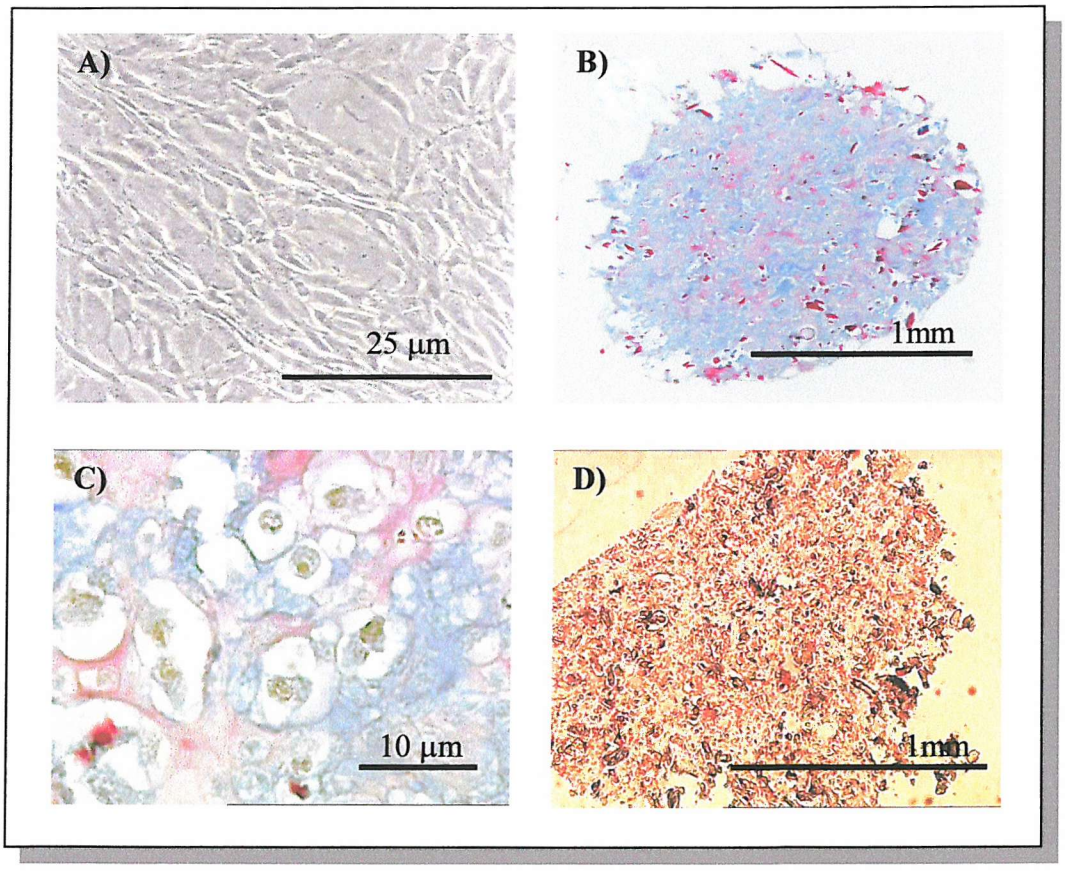

Figure 5.4. Pellet cultures of chondrocytes showing A) initial expansion of cells in monolayer, B) Histology of the whole pellet at low power, C) High power histology showing lacunae features and matrix accumulation and, D) Type II collagen immuno histochemistry.

5.3.2. ATDC5 Pellet culture.

The continuous cell line ATDC5 has previously been used only in monolayer systems to mimic chondrocyte hypertrophy. In micromass culture the ATDC5 cells were able to form pellets. The pellets at 21 days displayed alcian blue/ sirius red staining, with the presence of lacunae and strong type II immuno histochemistry (figure 5.5.).

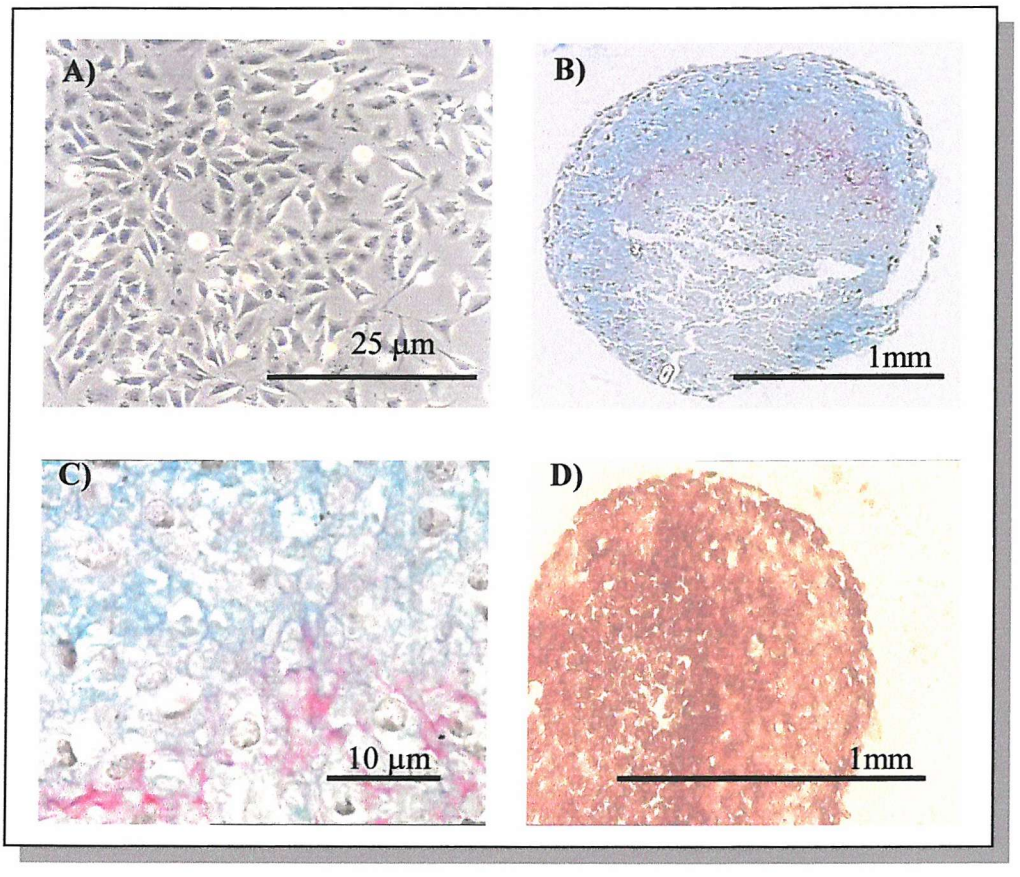

Figure 5.5. Pellet cultures of ATDC5 cells show A) initial expansion of cells in monolayer, B) Histology of the whole pellet at low power (bar represents 1mm), C) High power histology showing lacunae features and matrix accumulation and, D) Type II collagen immuno histochemistry.

5.3.3. Marrow CFU-F pellet culture.

Human marrow CFU-F from total hip replacement patients were able to form pellets, which, at day 21, displayed few lacunae and limited type II collagen by IHC (figure 5.6). Alcian blue sirius red staining of CFU-F pellet cultures showed mixed features of bone, cartilage and occasionally features of mixed marrow cells. Human marrow CFU-F, although varying in morphology between patients, consistently produced pellets with matrix material in a coherent pellet of tissue, thereby allowing some hope for producing a tissue in subsequent tissue engineering protocols.

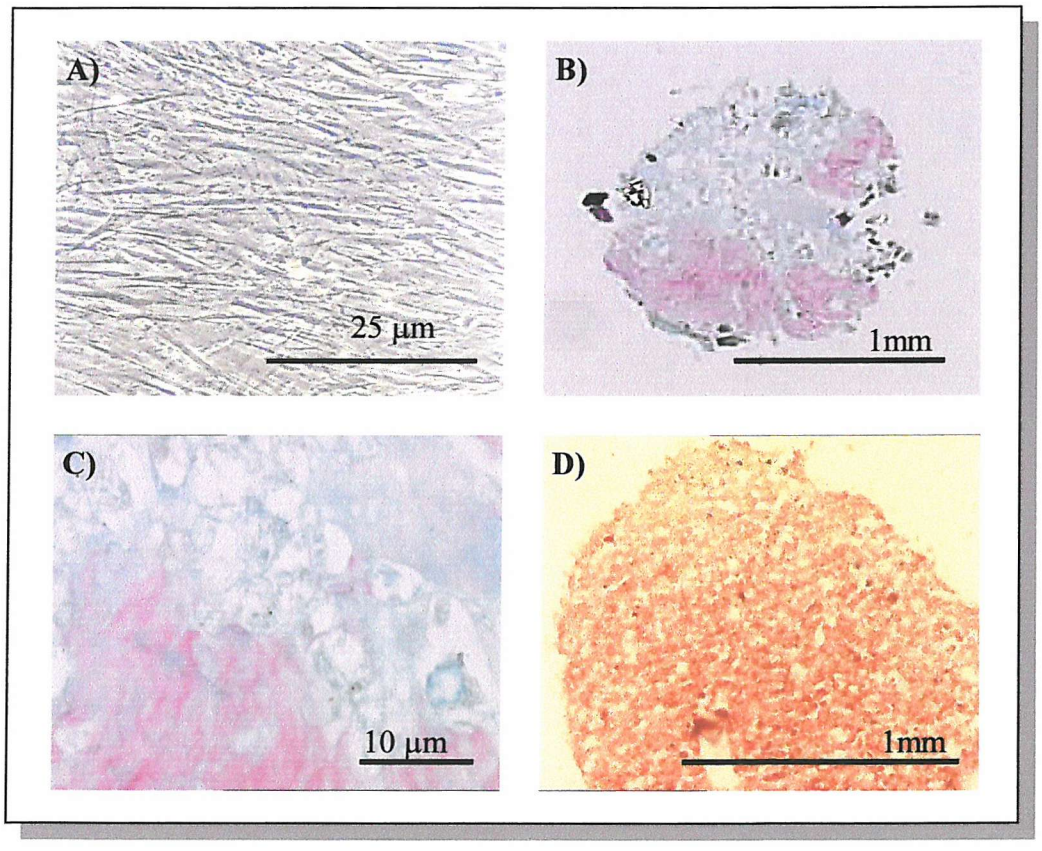

Figure 5.6. Pellet cultures of CFU-F show A) initial expansion of cells in monolayer, B) Histology of the whole pellet at low power, C) High power histology showing few lacunae features and matrix accumulation and, D) Type II collagen immuno histochemistry.

5.3.4. STRO-1 selected CFU-F pellet culture.

Human marrow cells were immuno-selected using STRO-1, as it was unclear if STRO-1^+ selected cells were capable of differentiation into the chondrogenic phenotype as well as the osteoblast phenotype.

The results showed that STRO-1^+ CFU-F were able to form pellets with matrix, which, at 21 days displayed alcian blue / sirius red staining and some of the characteristics of the cartilage phenotype, with mixed bone-like areas. Pellets also displayed type II collagen by IHC.

A noticeable difference was observed in the features of pellet formation in STRO-1^+ selected cells to form pellets as compared to control CFU-F alone, which was repeated throughout the experiments. STRO-1^+ selected cells displayed lacunae in comparison to CFU-F cultured as pellets. Type II collagen immuno histochemistry appeared stronger in the STRO-1 selected pellet cultures (figure 5.7, 5.6 D).

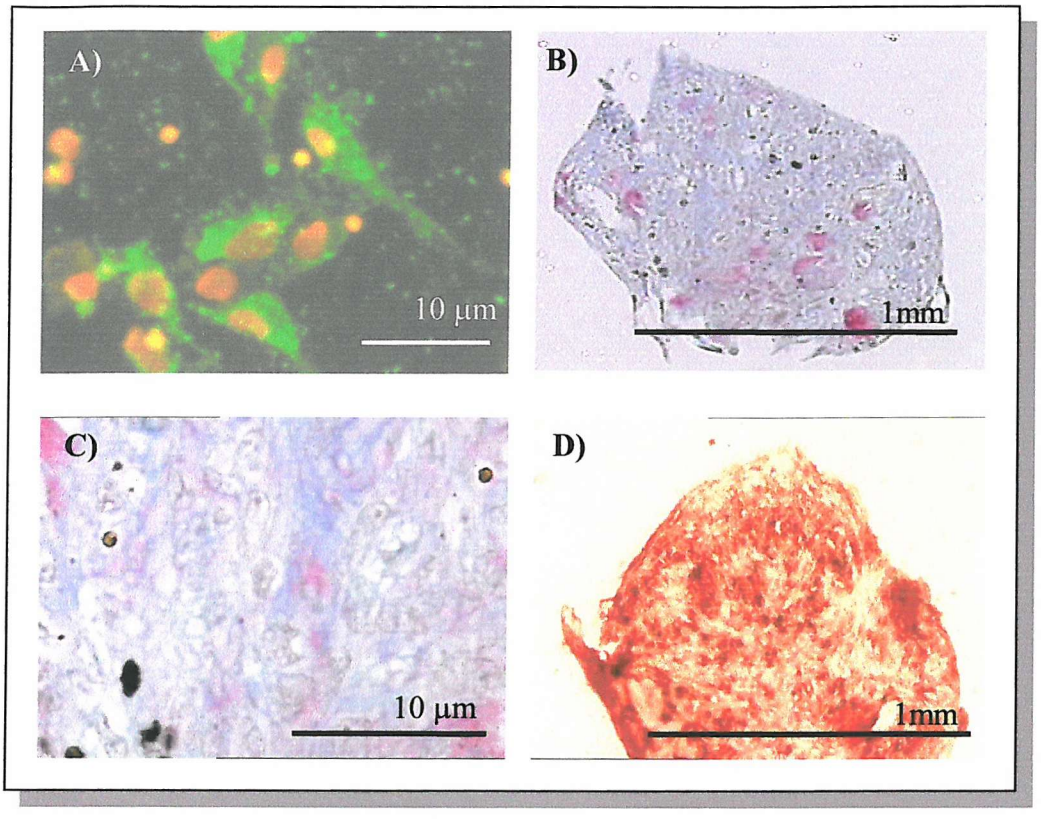

Figure 5.7. Pellet cultures of STRO-1^{+} cells show A) initial expansion of cells in monolayer stained for STRO-1 using a fluorescently labelled STRO-1 antibody, B) histology of the whole pellet, C) high power histology showing lacunae features and matrix accumulation stained with alcian blue/ picro-sirius red and, D) type II collagen immuno histochemistry.

5.3.5. Cell activity within cell pellets

Culture of marrow stromal cells in pellets allows development of some of the features of cartilage morphology and some of the biochemical markers of cartilage, however, there remain questions as to the viability of the cells in a tightly packed pellet. High cell density would serve to reduce oxygen and nutrient supply in the central cells with the potential to cause necrosis and effect the surrounding cells. Histology has shown that in the centre of many pellet cultures in this study there was a marked reduction in cell numbers, although many cells were seen within the centre as indicated by the intact nuclei stained using the A/S protocol (particularly visible in the ATDC5 cultures, figure 5.5 B). Staining using the "live/dead" method allowed observation of the metabolic activity of the cells in pellet cultures and not the level of growth/division. As expected cells of pellet cultures grew mainly on the outer surfaces of the pellet culture (figure 5.8 C), however, the internal surfaces also showed some activity and that only a low distribution of dead cells occurred in the centre of the pellets (figure 5.8). Live/dead stained cells on the outer side of the pellet culture not only showed more metabolically active cells but also contained more dead (red) cells (figure 5.8). These results show that the cells in the middle of pellet cultures were not necrotic at day 21 but appear as "maintained" cells. Studies on cartilage have previously shown that although cells were clearly present in healthy cartilage, the chondrocytes did not label strongly with live/dead stain, whereas in damaged cartilage the cells appear more active and therefore more stained with green, live/dead stain (Smith *et al.*, 2003) Live/dead staining may be subject to entrapment of dye throughout the micromass samples, although similar protocols have worked for articular cartilage.

Pellet cultures have been shown only to grow minimally once established, probably because the main growing zone only represents a small volume of the pellet culture, the remaining cells appear to exist as low activity cells in the centre of the pellet although earlier time points would have to be performed as the majority of cell death may occur at earlier time points.

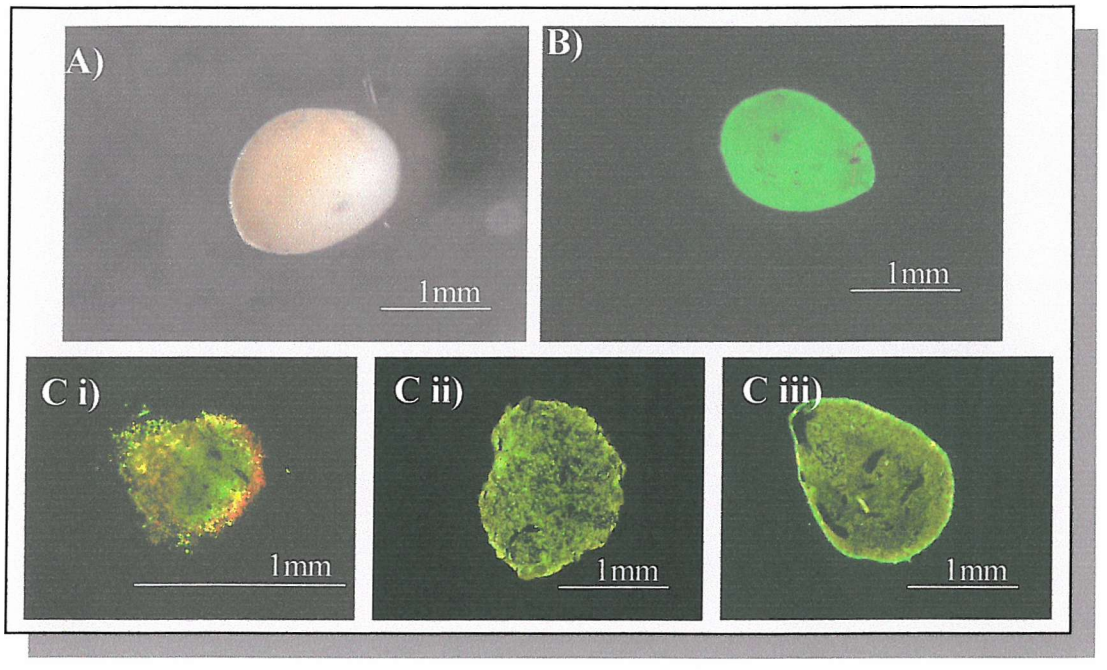

Figure 5.8. Pellet culture of chondrocytes at day 21, showing A) gross morphology, B) gross morphology with live dead stain and, C) i-iii) histology of 7 μm histology section of 3 pellet cultures showing live and dead cells

5.3.6. Type II collagen in pellet cultures.

In order to analyse the production of type II collagen in pellet cultures, in an alternative method to IHC, a western blot of extracted protein from pellet cultures was performed. The samples were run on a 10 % PAGE gel, transferred to nitrocellulose and probed with an antibody to α1[II] collagen, with comparisons to extracted type II collagen and articular cartilage (figure 5.9). The gels of controls and different cell types in pellet cultures were stained with coomasie blue to determine the relative levels of expression of protein in the samples. The antibody to type II collagen binds strongly to proteins of a variety of different molecular weights in an extract of articular cartilage, but a common molecular weight band could be seen in all the samples, highlighting the production of type II collagen in all the pellet cultures.

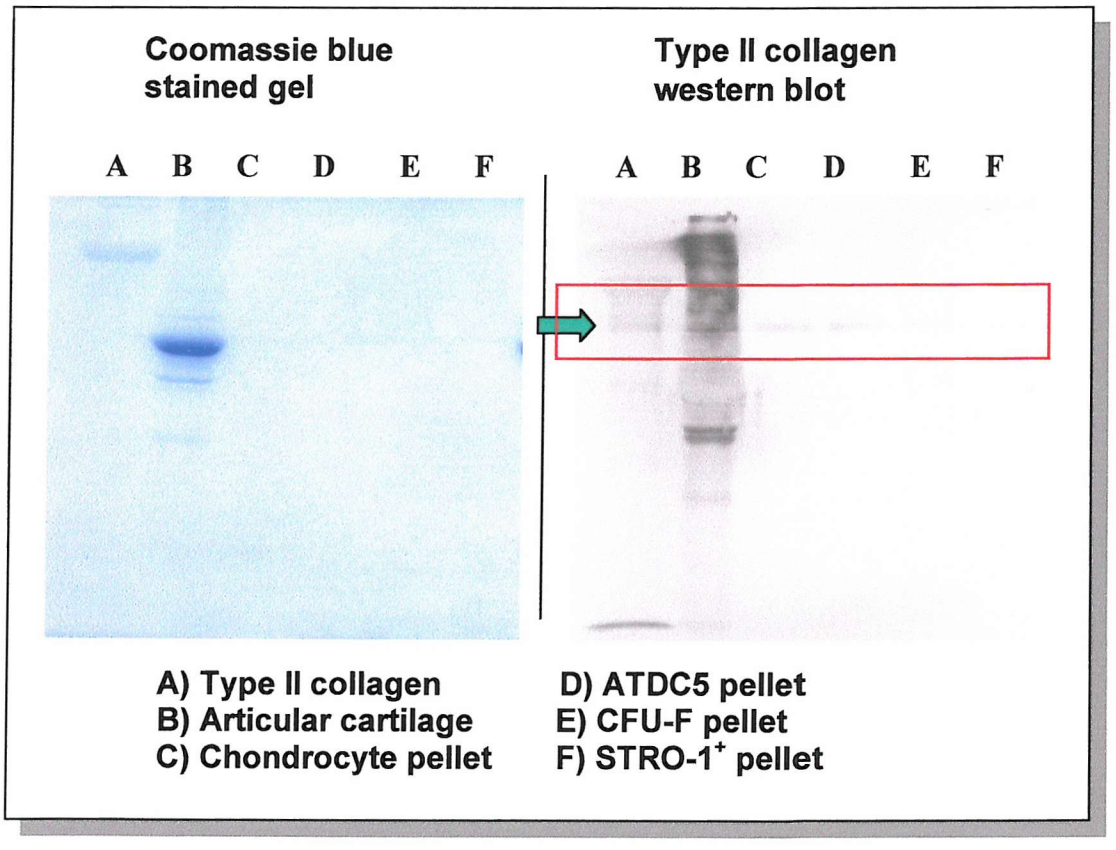

Figure 5.9. Coomasie blue protein stain and the corresponding western blot for type II collagen.

5.3.7. Glycosaminoglycan (GAG) in pellet cultures.

The DMMB (di-methyl-methylene-blue) dye binding assay for GAG was used to examine one of the important phenotypic markers of the cartilage phenotype. Production of GAG was evidenced (figure 5.10), with no significant difference in glycosaminoglycan levels between pellet cultures of chondrocyte (13.8±2.7mg GAG/g tissue), ATDC5 cells (14.1±2.3mg GAG/g tissue), marrow CFU-F and STRO-1^{+} selected CFU-F (15.8±3.3mg GAG/g tissue). Monolayers of chondrocytes expressed significantly less levels of GAG (0.9±0.4mg GAG/g tissue), although ATDC-5 cells did express GAG when cultured in monolayer (9.4±1.6mg GAG/g tissue).

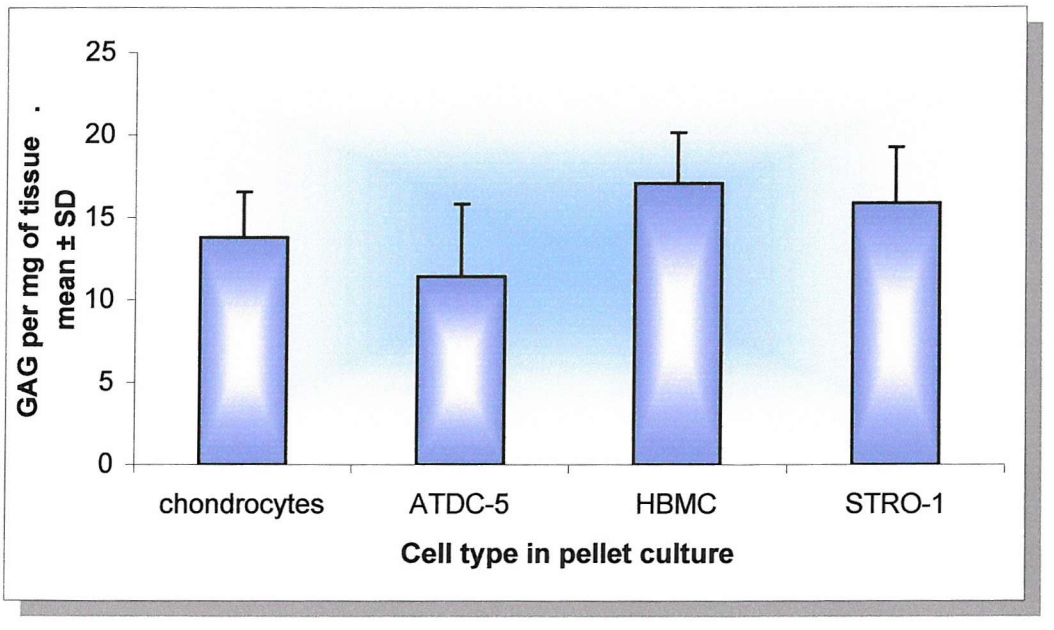

Figure 5.10. DMMB dye binding assay of GAG content in pellet cultures of chondrocytes, ATDC5 cells, Human bone marrow cells (HBMC) and STRO-1 selected CFU-F. No significant differences were observed (displayed as mean ± SD, n=4, statistical analysis by unpaired t test).

5.3.8. Comparison of GAG with culture type.

Although there was no significant difference between the pellet cultures examined, monolayers of chondrocytes expressed minimal levels of GAG compared to ATDC5 cells. Culture conditions and the effects of GAG production were further examined in chondrocytes using monolayer and pellet cultures. ATDC5 cells did express some GAG when cultured in monolayer (9.4±1.6mg GAG/g tissue). Pellet cultures were found to express significantly (15.8±3.3mg GAG/g tissue: p=**<0.005) more GAG than monolayers of chondrocytes, however it was significantly lower than articular cartilage (51.1±12.5mg GAG/g tissue), as shown by figure 5.11 below, although this was a measure of the final accumulation of GAG and not production rate.

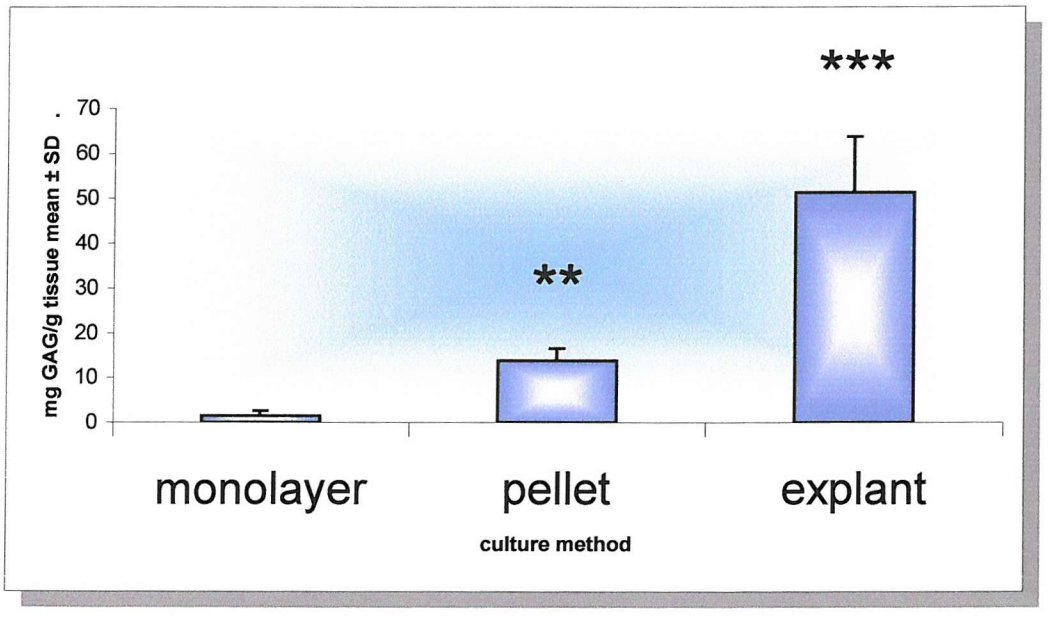

Figure 5.11. GAG measured by DMMB dye binding assay showing the relative levels of proteoglycan in monolayers of chondrocytes, pellet cultures of chondrocytes and of control articular cartilage explant (n=4 tested using by unpaired t test **p<0.005, *** p<0.001 as compared to monolayer).

5.3.9. Examination of GAG epitopes in pellet cultures

In order to ascertain if aggrecan was present within the pellet cultures, a series of antibodies were used, they recognise different epitopes along the molecule and the main associated link protein (antibodies courtesy of Professor Bruce Caterson).

5.3.9.1. Globular domain 1 in pellet cultures (7-D-1 antibody)

Western blotting was used to verify the presence of specific GAG molecules of aggrecan and to further highlight the distribution of different epitopes of aggrecan in the pellet culture models. Both media and tissue samples were compared in order to examine the loss of aggrecan and elements from the cartilaginous matrix into the surrounding media. A proteolytic susceptible region, globular domain 1, was observed in control cartilage, ATDC5 and CFU-F pellets with surprisingly little expression observed in chondrocyte pellet cultures (figure 5.12 chondrocytes for both media and sample). Articular cartilage showed a low level in media highlighting degradation and secretion into the media of the cartilage explants.

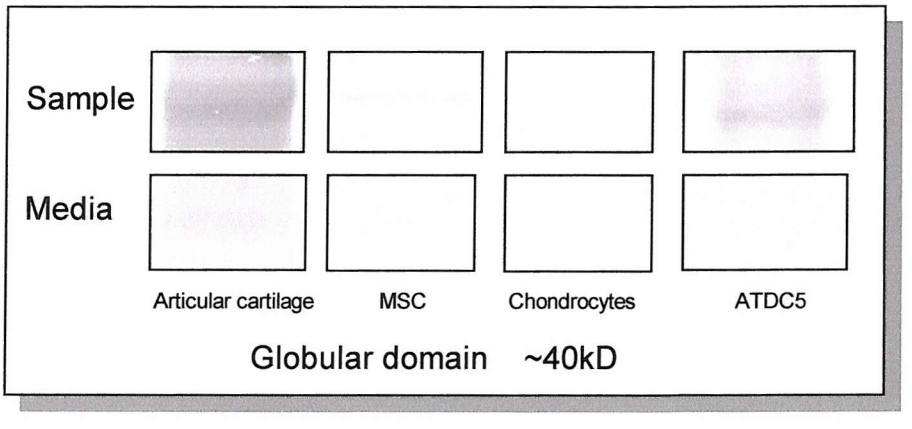

Figure 5.12. Western blots of GAG globular domain 1 epitope.

5.3.9.2. Chondroitin -0- Sulphate epitopes in pellet cultures (antibody 1-B-5)

Detection of the epitope for 0-sulphated GAG stubs on the core gag protein, showed that there was release into the media of GAG. This marker also shows that the molecule was present in all pellet cultures, as well as articular cartilage.

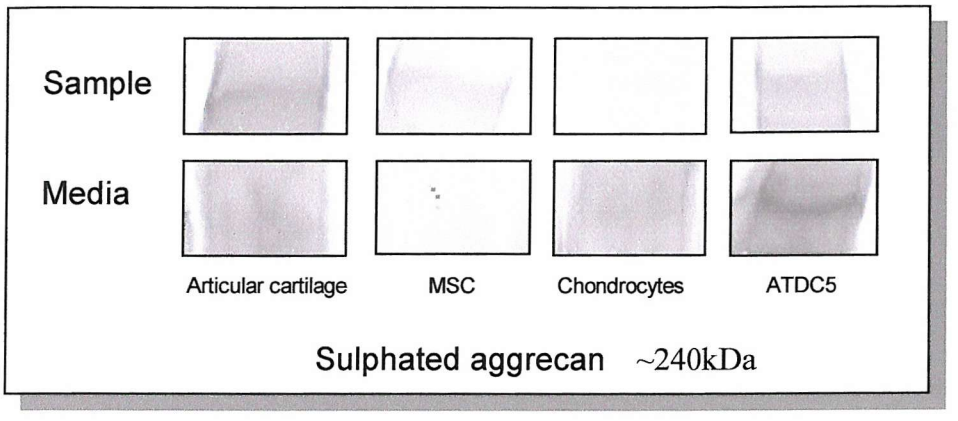

Figure 5.13. Western blots of sulphated aggrecan epitope.

5.3.9.3. Chondroitin-4-sulphate expression in pellet cultures (2-B-6 antibody)

The differently sulphated aggrecan epitope showed that there were different chondroitin arrangements attached along the core protein, which were expressed strongly in articular cartilage control as well as in the continuous cell line ATDC5. Limited expression was observed in cultures of marrow CFU-F and chondrocytes (figure 5.14).

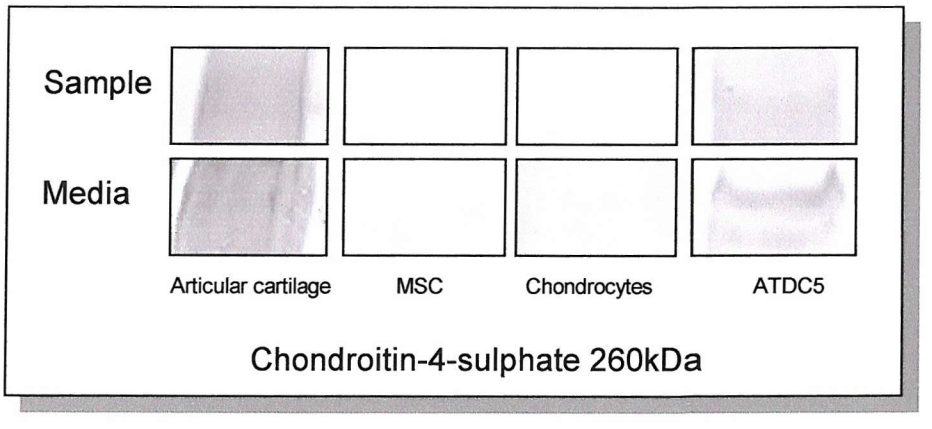

Figure 5.14. Western blots of GAG Chondrotin-4-sulphate epitope.

5.3.9.4. Chondroitin-6-sulphate in pellet cultures (antibody 3-B-3)

The epitope for the 6-sulphated regions was expressed in the cartilage control and in the sample of the chondrocytes in the media (figure 5.15). This represents a difference between pellets and between sample and media (with more in the media). This suggested a release into the media, which was accentuated by the methodology that was able to concentrate large media volumes during processing. ATDC5 pellet cells appear to be relatively strong in expression, with chondroitin-6-sulphate detectable in sample and media. The articular cartilage showed release into the media, as did the ATDC5 cells, although it was unclear if this represented a disease process or a feature of the pellet culture model.

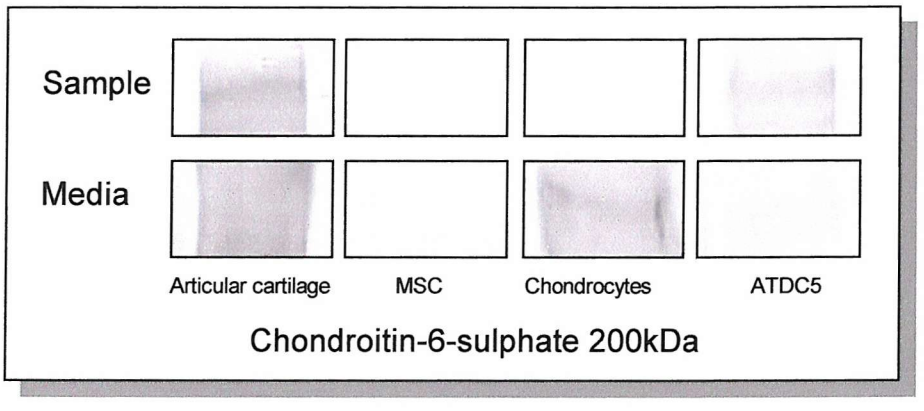

Figure 5.15. Western blots of GAG chondrotin-6-sulphate epitope.

5.3.9.5. Link protein expression in pellet cultures (antibody 8-A-5)

Link protein allows the attachment of aggrecan to larger molecules of hyaluronan and has been shown to be important for the development of cartilage in knockout mice, which developed deformities following removal of the link gene (Watanabe *et al.*, 1999). Articular cartilage was seen to express link protein and to have strongly retained it within the matrix of the sample. Of all the groups examined, only ATDC5 cells appear to express link protein. Link protein was undetectable in pellet cultures of marrow CFU-F and, surprisingly, pellet cultures of human articular chondrocytes.

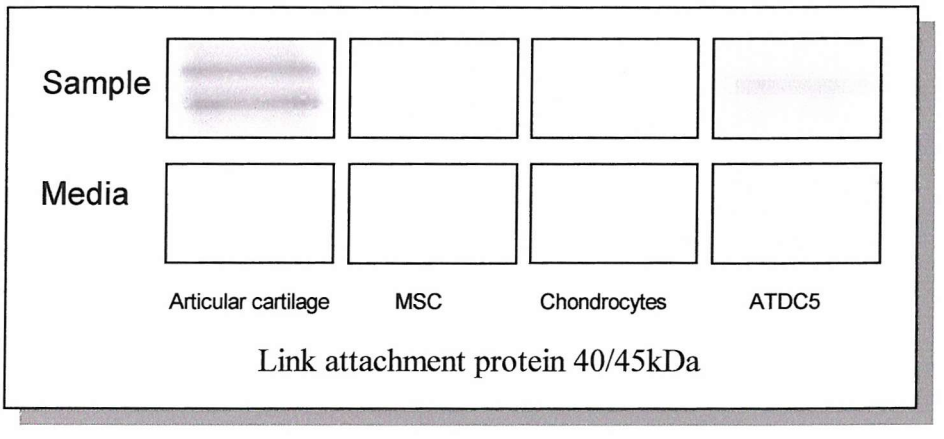

Figure 5.16. Western blots of link attachment protein.

5.4. Discussion.

Matrix production is an essential part of the functioning of chondrocyte and of cartilage. The production of GAG and type II collagen are distinctive markers of cartilage matrix, as are the morphological features of matrix and lacunae. Encouragingly, many of these features were found in almost all pellet cultures of the cells used (table 5.1). Pellet cultures of chondrocytes were able to produce type II collagen and aggrecan, as did mesenchymal stem cells, STRO-1^+ selected marrow stromal cells and ATDC5 cells in pellet culture. Chondrocytes, ATDC5 and STRO-1 MSC showed features of cartilage morphology when cultured in pellet culture systems, including GAG and type II collagen. ATDC5 cells provide a continuous cell monolayer differentiation culture system for the examination of chondrocytes (Shukunami *et al.*, 1998) and in pellet culture systems displayed potential to produce lacunae and excess matrix, containing type II collagen and GAG epitopes.

Table 5.1. Summary of markers in comparison to articular cartilage

Assay/ cell	Cartilage	Chondrocyte monolayer	Chondrocyte pellet	STRO-1 MSC pellet	MSC pellet	ATDC5 pellet
Type II collagen (Immuno histochemistry)	*****	/	***	***	**	****
Type II collagen (western blotting)	*****	/	*	*	*	*
GAG (by DMMB)	*****	/	***	***	***	***
Link	*****	/	/	N/A	/	**
Globular domain 1	*****	/	*	N/A	*	****
Interglobular domain	*****	/	/	N/A	/	*
Chondroitin 0 sulphate	*****	/	****	N/A	****	*****
Chondroitin 4 sulphate	*****	/	*	N/A	*	**
Chondroitin 6 sulphate	*****	/	/	N/A	**	**
Cell lacunae	*****	/	**	**	*	****
Excess extracellular matrix	*****	/	**	**	*	****

*detectable, ** expressed, *** increased expression, **** strong expression, ***** very strong expression, compared to native cartilage, / not present, N/A not performed.

Chondrocytes when cultured as pellets are able to produce type II collagen, as observed using methods of immuno histochemistry and western blotting. In addition, aggrecan was detected by western blotting and by DMMB dye binding assay. The series of antibodies to different epitopes on aggrecan was able to further determine that the aggrecan was in both the sample and the media, a distribution pattern indicative of lack of binding material, to hold the proteoglycan content within the pellet culture and also of enzymatic cleavage releasing degraded GAG into the media. However, and interestingly, this had changed in cultured pellets of chondrocytes from the same articular cartilage tissue. The antibodies showed that there was some loss into the media as well as some degradation products of GAG but that biochemically, not histologically, the MSC were indistinguishable from the chondrocytes. The reason for degradation products is uncertain, although not unexpected, as the cells at the surface may excrete molecules into the media during growth and the cells were derived from total hip replacement patients where disease processes have occurred, which could lead to degradation and loss of proteoglycan when cultured in pellets. Research by Kafienah *et al* (Kafienah., 2002; Kafienah *et al.*, 2003) has shown that chondrocytes cultured as tissue engineered constructs are still susceptible to the same disease processes as the original material and hence pellet cultures may still display disease features. There were apparent differences in histology between pellets, and all the pellet cultures including the chondrocytes did not express detectable levels of link protein by western blotting, an absolutely essential part of the cartilage phenotype (Watanabe *et al.*, 1999). Morphology of the pellets, although type II collagen and GAG containing, were not histologically comparable to native articular cartilage and could be seen to produce a much more

defined band in the collagen type II western blots compared to articular cartilage (figure 5.9), which displayed a range of bands.

These results are encouraging for the production of tissue engineered constructs as all the cell types including marrow stromal cells and STRO-1^+ selected marrow cells produce matrix containing GAG and type II collagen, although the lack of an observable amount of link protein may cause problems with the correct accumulation and maturation of matrix, in tissue engineered constructs.

CHAPTER 6

In vitro tissue engineering strategy

6.1. Introduction

The body's complex tissues are supplied with oxygen and nutrients via an exchange fluid with close tissue vasculature, even cartilage still requires exchange with synovial fluid and an underlying vascular bed in the sub-chondral bone. In tissue-engineered constructs, previous studies have shown the need for enhanced exchange of media supplied by a variety of bioreactor designs, although the size of the constructs still appears to be limited to the depth of only a few mm (Glowacki *et al.*, 1998).

High growth rates of cells growing in a monolayer require large volumes of nutrients, removal of toxic waste and access to gas exchange equivalent to the composition of the new cell and the energy expended. When cells are cultured three dimensionally, the cells not only loose the access to nutrients but also growth space and the flat high exchange area. Bioreactors can be used to enhance the exchange of nutrients over the cell's decreased exchange area. However, detrimental factors such as shear stress on delicate outer cells must be taken into account. The methods by which the cells, scaffolds and growth factors are combined also have been shown to be as important as the scaffolds, cells and growth factors used.

In this study two bioreactors were utilised, a novel perfusion chamber designed at the University of Bath by Dr Julian Chaudhuri and Dr Marcus White. The system is a sealed chamber, with media circulated through where the constructs are cultured, the exchange of gasses is enhanced by the addition of silicone tubing, which is permeable to gas but not liquid. Media flow was controlled to flow rates of approximately 1ml per hour. The second system studied was a rotating vessel, the vessel was a high aspect ratio vessel (HARV) produced by Synthecon, the bioreactor allows 50mls of media to be constantly rotated over the tissue construct, gas exchange is via one end of

the vessel through a silicone membrane, both bioreactors were compared against static cultures.

In order to test a range of tissue engineering options, a series of experiments examined the effects of seeding and of different cultures. Static seeding was via drip seeding, where a concentrated solution of cells was dripped over a scaffold material and left for approximately an hour to attach, the constructs were then cultured. Glowacki *et al* (Glowacki *et al.*, 1998) used another method, which was to circulate cells in solution over the scaffold material until the cells attached to the scaffold material, designated as "dynamic" seeding.

In tissue engineering it is common practice to generate an excess of cells by culture in monolayer, to expand the cell numbers, then to seed these cells onto a matrix scaffold material. Increases in cell number could be used to decrease the time of culture and increase the chance of creating a more viable tissue construct. It has been observed that the pre-culture can have a strong effect in the development of the construct, for example, pre-culture of cells in FGF-2 is able to modulate subsequent tissue construct morphology (Martin *et al.*, 2001).

Different scaffold materials were also used, as each material may behave differently with different cells and with different culture conditions. In this study PLA foam produced by supercritical CO_2 expansion was used, as was *Porifera* sea sponge, a material which is naturally designed, from a collagen analogue (Exposito *et al.*, 1991), to support cells and allow diffusion. The other scaffold material used was PGA fleece, a biodegradable material with good diffusion characteristics, which had previously been used to generate tissue engineered constructs (Freed *et al.*, 1993).

The aims were therefore to investigate the potential of bioreactors, seeding strategies and growth factors on production of suitable tissue constructs.

6.2. Method

6.2.1. Effect of perfused culture

Initially to observe the effects of culture vessels alone, the scaffolds were seeded in a standard fashion before incubating in the chosen bioreactor, all of which were compared to controls. Controls were cultured in normal "static" wells of a 24 well tissue culture plate. To improve the saturation and increase the cell adhesion $3mm^3$ PLA scaffolds were soaked overnight in 10% FCS supplemented αMEM, to provide a media saturated FCS adsorbed scaffold. Individual scaffolds were placed into 24 well tissue culture plates and $1x10^6$ trypsinised MSC were placed into each well. The material was left overnight before removing to either a fresh well of a 6 well plate containing 5mls of 10% αMEM, or the culture chamber of the "Bath" bioreactor with a flow rate of 1ml per hour. The cells were then observed using "live dead" staining.

6.2.2. Effect of static, perfused and rotating culture.

Sponge *Porifera* fleece was cut into $5mm^3$ cubes and sterilised by exposure to UV light for 1 hour. Sterilisation was continued by placing the porifera into 75% Ethanol for 5 minutes followed by rinsing this off with 2 washes of sterile PBS and then into αMEM culture media supplemented with 10% FCS (note; cells have been shown to attach in serum free media). The sponge scaffolds were placed into one well of a 24 well tissue culture plate, and $1x10^6$ cells were attached to the scaffolds by culturing with the scaffolds overnight, in order to provide a standard starting point for culture comparison. Scaffolds were then further placed into, i) static culture (1 scaffold per well of a 6 well TC plate with 5mls of αMEM), ii) the Bath bioreactor with a flow rate of 1ml per hour or iii) into a 50ml, disposable, high aspect ratio vessel (HARV) rotating bioreactor (Synthecon) for 3 weeks for live/dead staining and histological

examination, and 4 weeks to determine the effects on growth parameters of total DNA representing cell number and total protein, as an estimate of extra cellular matrix.

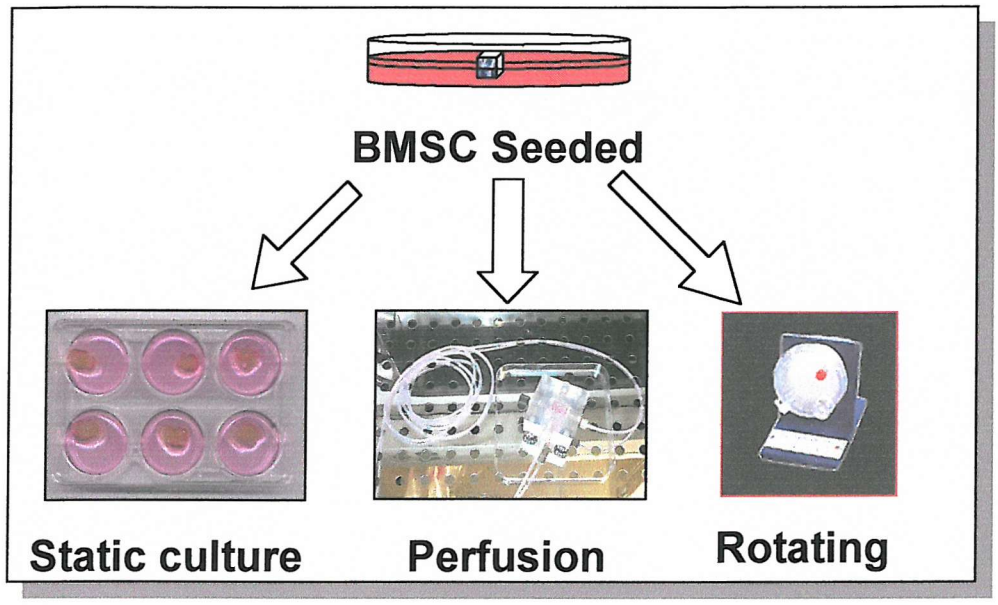

Figure 6.1. Work scheme for the comparison of bioreactor effect.

6.2.3. Comparison of seeding protocols

i) Sponge

To provide a comparison for different methods of adding cells to scaffolds, *Porifera* sponge was used to test different seeding methods to distribute cells throughout the scaffold material and to grow, in order to determine if the rotating culture has a similar effect to the dynamic seeding of spinner flasks (as used by (Glowacki *et al.*, 1998)). One group was seeded in static culture as before, however a second group was seeded by addition of the cells directly to the rotating (HARV) culture vessel with a circulating unseeded sponge, as a "dynamic" seeding method. Thereby providing the difference between static seeding and culture, and dynamic seeding and dynamic culture.

ii) PGA fleece

The seeding experiment was repeated with a variation of the inclusion of perfused culture prepared using the same number of MSC cells ($1x10^6$). The scaffolds were seeded statically for static culture and perfused culture and placed into the rotating culture vessel with the PGA fleece for dynamic seeding and culture, thereby providing the current maximal strategy for each culture method.

iii) Cell distribution and state throughout the PGA - MSC constructs

In order to see the distribution of living active cells throughout the scaffold, a PGA fleece dynamically seeded with MSC was incubated with CMFDA-1 and Ethidium homodimer-1 (live dead stain) and then subject to histology and fluorescence microscopy.

6.2.4. Effect of TGFβ3

In order to test the effects of TGFβ on tissue engineered constructs, the cells were seeded into rotating culture. Cells were seeded onto the scaffold using the same media which was used for pellet culture, producing the collagen type II and GAG matrix in MSC both serum free and serum free with 10 ng/ml TGFβ3. These were the same conditions as for the pellet culture method, that reproduces the features of chondrogenesis in MSC. The second culture was performed using 10% FCS supplemented culture in order to support growth.

6.2.5. Monolayer culture attachment and expansion with FCS

To test the effects of FCS on seeding and growth of CFU-F colonies, marrow aspirates were taken from 4 patients (81M,79F,78F,86F), and then prepared in a standard manner. Cells were resuspended in 4mls of media containing FCS from 0 to 100%, in 10% steps. Cells were incubated for 14 days to observe cell attachment and growth.

6.2.6. Monolayer growth of passaged MSC with increasing FCS

To provide the growth rates of CFU-F in increasing concentrations of FCS (without the factor of attachment of colonies from marrow aspirates), passaged MSC were cultured in αMEM containing 0-100% FCS, in 10% increments. Marrow cells were cultured for 7 days before staining with toluidine blue. Duplicates were used for total DNA and total protein level assay.

6.3. Results

6.3.1. Attachment and growth of MSC cells with FCS

Normal serum addition is typically in the range of 10% of the volume, or occasionally 20% in other labs for CFU-F isolation (Walsh *et al.*, 2001). In order to test the ability of serum to enhance CFU-F attachment, a series of serum concentrations were prepared and 4 fresh marrow isolates were resuspended in serum and αMEM from 0 to 100% in 10% steps. After 2 weeks, one group of cells were stained using toluidine blue, in order to highlight the cells, and the other groups were subject to protein and DNA assay. The results showed that the CFU-F cells can be cultured from 10% FCS to 100% serum, 0% serum had some colonies but they appeared not to grow in subsequent culture. These results highlight that the CFU-F can survive and grow in 100% serum and that more serum results in more cell attachment and growth.

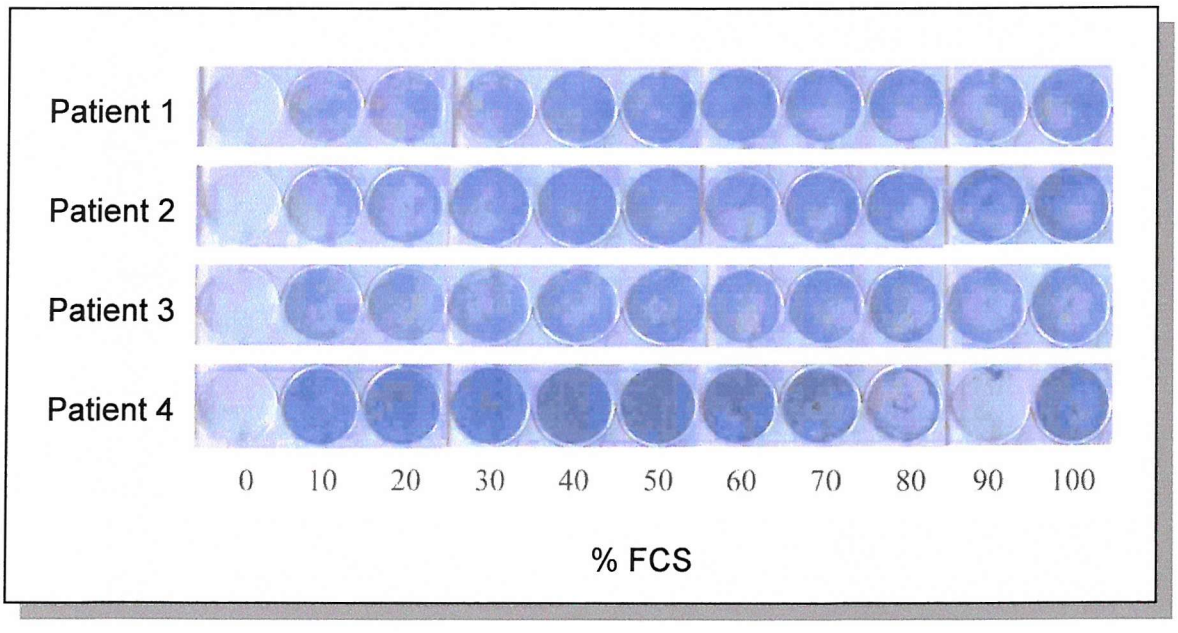

Figure 6.2. Effect of FCS concentration on seeding of CFU-F marrow cells stained with toluidine blue.

6.3.2. Marrow cell attachment biochemistry

Quadruplicate marrow samples were cultured in 0 to 100% FCS in 10% steps and cultured for 2 weeks for observation of attachment and growth. Cells could be observed to be increased in density using staining and gross observation. The staining observed was confirmed by quantifying protein and DNA. A clear increase in DNA and protein was observed with a significant trend in all graphs, which was still significant when the 0% FCS point was discounted.

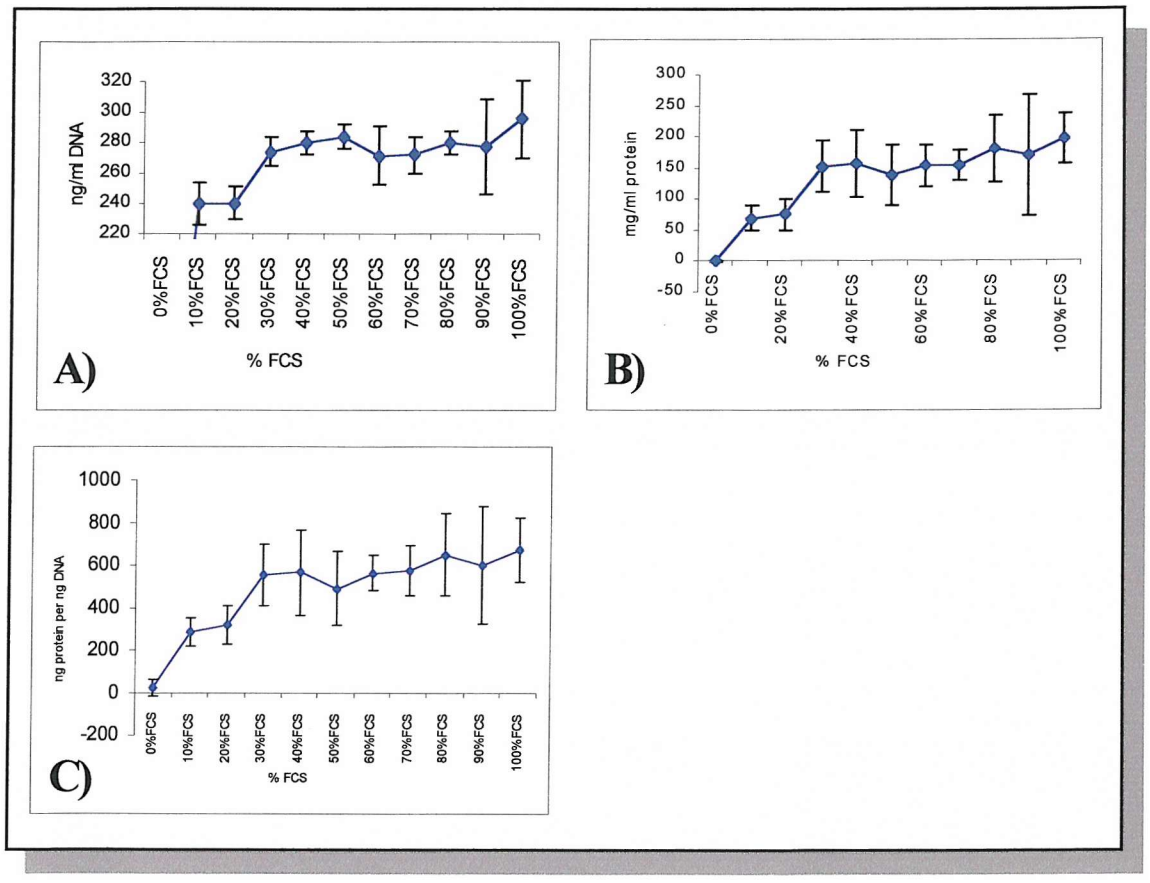

Figure 6.3. Effects of increasing concentrations of FCS on cell attachment A) DNA levels, B) protein levels and, C) relative protein levels. (values expressed as mean ± standard deviation, n=4, significant trend by one-way anova with post test, in DNA $p<0.05$, Protein $p<0.05$ and protein per DNA $p<0.05$ (with 0% FCS removed, also 10% FCS point was significantly different from 50% FCS by welches corrected t test)

6.3.3. FCS effects on passaged MSC cells

The previous experiment with the change in CFU-F attachment showed that more FCS resulted in more attachment and growth up to 100%. This did not necessarily represent the best culture conditions for growth of passaged CFU-F. Therefore, passaged CFU-F cells were added to culture in equal numbers and cultured in 0 to 100% serum supplemented αMEM media for 1 week. The cells were stained with toluidine blue in order to observe the cell growth and the remaining groups subject to DNA and protein quantitation. The stained cells clearly showed that although the original cultures were able to survive in up to 100% serum, the passaged marrow CFU-F cells grew best at a peak of approximately 40-50% FCS. This was different to the initial attachment cultures but highlights the potential to easily and significantly increase the growth rate of marrow CFU-F cells in culture. With 50% in attachment cultures not being significantly different to 100%, the use of 50% FCS serum for attachment and growth could be used to enhance the growth of marrow stem cells.

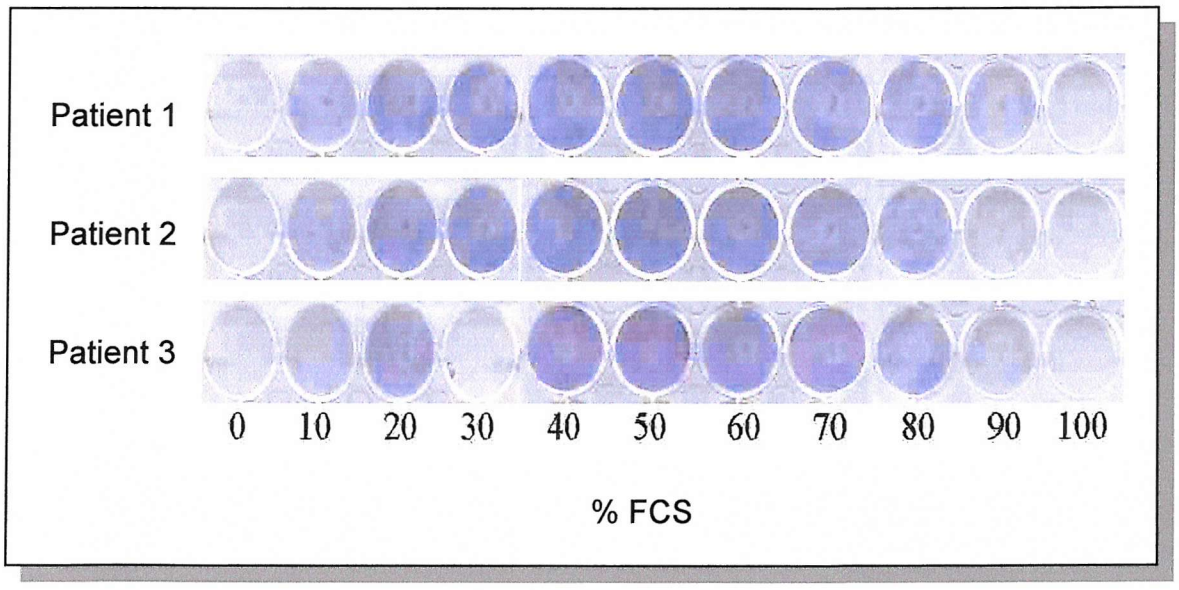

Figure 6.4. Passaged CFU-F cultured in 0-100% FCS stained using toluidine blue.

6.3.4. Passaged CFU-F cell biochemistry

FCS added to passaged CFU-F marrow cells shows that the DNA and protein peak at around 40% FCS supplemented media, verifying the results seen using gross staining of cultures. The attachment and passaged marrow cells vary in the final outcome of culture, with the attachment cultures not being as affected by over 50% FCS as the passaged cells. This suggests that the cell attachment was a significant factor in the culture of CFU-F and that growth of passaged cells was less dependant on attachment or FCS factors.

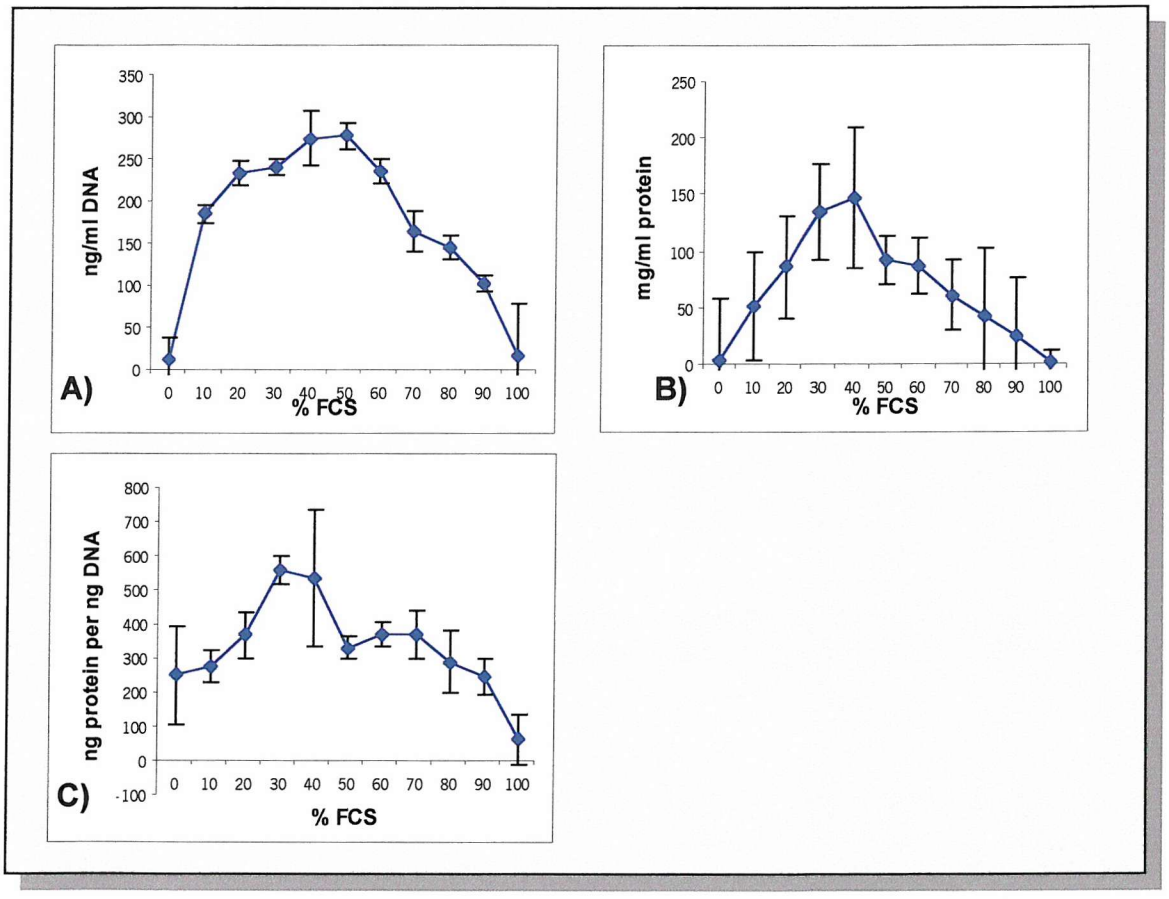

Figure 6.5. Effect of FCS concentration on passaged CFU-F, A) total DNA, B) total protein and, C) protein per unit of DNA. (values are expressed as mean ± standard deviation, n=4, significantly ($p<0.005$) difference from 10% to 50% FCS by welches corrected t test in DNA only)

6.3.5. Effect of perfused culture on MSC seeded PLA scaffolds

PLA scaffolds were simultaneously seeded in static culture. The seeded scaffolds were then placed into 1 of 4 bioreactors, which were fed in parallel with fresh 10% αMEM at 1ml per hour. A bioreactor was then removed from the system at 7, 14, 21 and 28 days of culture and the constructs analysed by live/dead staining and compared to static controls (figure 6.6). Marrow stromal cells seeded PLA in static and perfused bioreactors both showed good cell adhesion and growth, with few dead cells visible. There was no observable difference between the two groups.

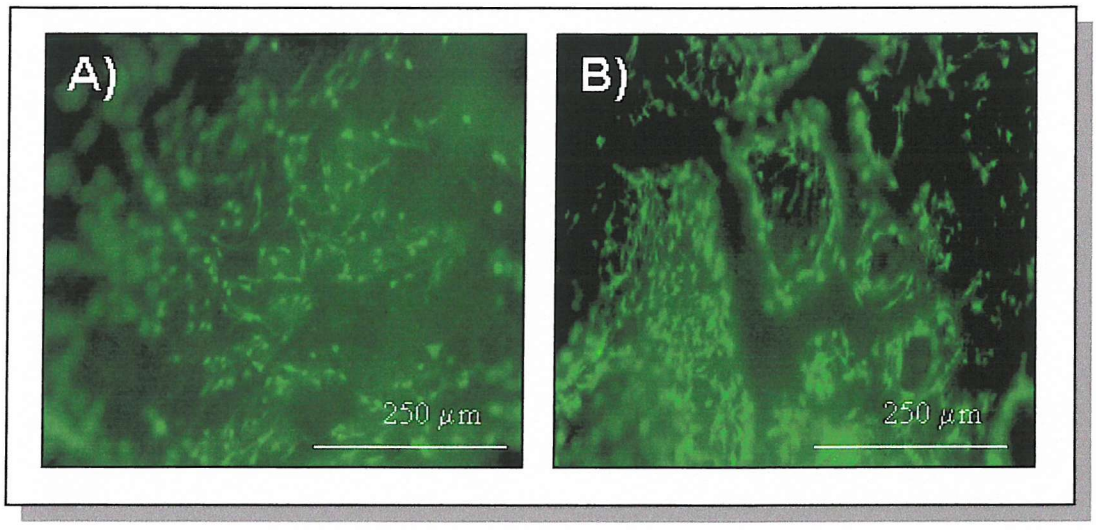

Figure 6.6. Live/dead stain of cells cultured on PLA scaffold in A) static culture and, B) perfused bioreactor culture after 3 weeks (Magnification originally 50X, field of view approximately 500 μm).

6.3.6. Bioreactor effect on *Porifera.*

Standard static seeded sponges were observed using live/dead stain after 3 weeks in culture. The statically seeded scaffolds and all other cultures had a covering of human marrow stromal cells throughout, which shows the ability of the collagen like material of *Porifera* to support cell growth in a three dimensional culture system (Figure 6.7). The difference in culture systems shows that static culture, as expected, produces an environment sufficient for cells to cover the scaffold. In perfused culture, cells proliferated by branching over all but the largest of gaps and finally, in the rotating bioreactor, the cells spread throughout the entire matrix and covered most gaps (figure 6.7 B and C respectively). All groups show that the porifera scaffold provides extensive ingrowth of cells throughout, which was enhanced in perfused and rotating bioreactor systems (figure 6.8).

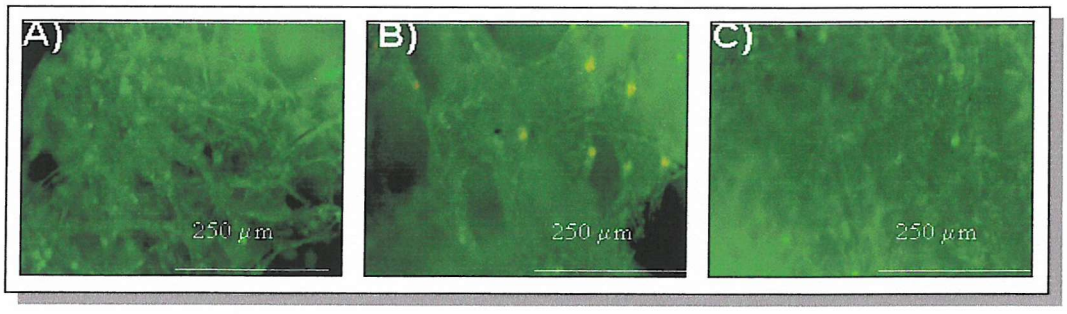

Figure 6.7. Live dead stained MSC cells cultured on sponge in A) static, B) perfused and C) rotating culture (Original magnification 50X).

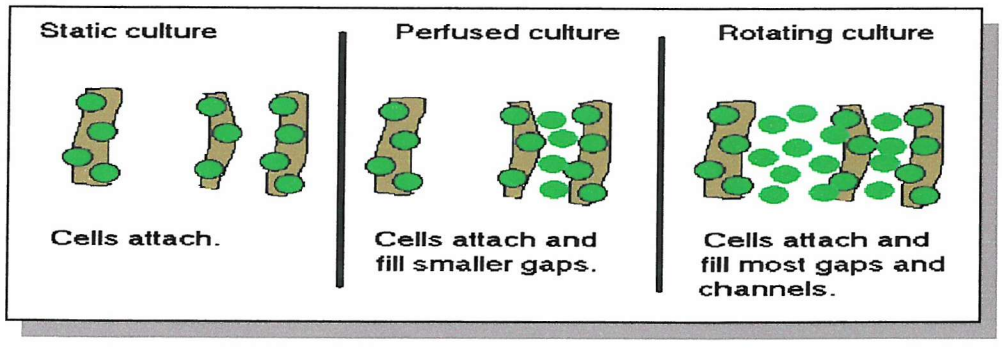

Figure 6.8. Explanation of live/dead stained marrow stromal cells cultured on sponge in static, perfused and rotating culture.

6.3.7. Growth on scaffold in static perfused and rotating culture

As the previous experiment showed by histology that it was possible to increase the growth of the cells on scaffolds by culture in perfused and rotating culture systems, the biochemistry was examined. In order to test the effect on cell protein and DNA expression of the systems, quadruplicate samples were statically seeded to standardise the start point and then placed in static, perfused or rotating culture systems. Samples were then taken at 7, 14, 21 and 28 days and the DNA and protein analysed (figure 6.9), each series of cultures were run at the same time in order to minimise any external influences on the outcome.

Static culture produced cell covered constructs, as did the previous experiments, however in rotating culture, DNA and protein synthesis were significantly increased at week 4 in comparison to both static and perfused culture systems.

In perfused culture, protein was significantly reduced at week 4, but, interestingly, the production of protein per unit of DNA was significantly increased throughout culture in the perfused bioreactor compared to both the static control and rotating culture.

It appears that the data shows the rotating culture was best for cell growth, but that the perfused system was probably the best system for protein production.

The most favourable culture system, however, is not easy to pick as the systems have positive and negative feature combinations. Therefore systems may have to be used for the optimal construct production dependant on requirement, or in combination, as seen in other groups where seeding is performed in spinner flasks and continued culture is in orbiting shakers or in rotating microgravity bioreactors (Freed *et al.*, 1998; Kafienah *et al.*, 2002).

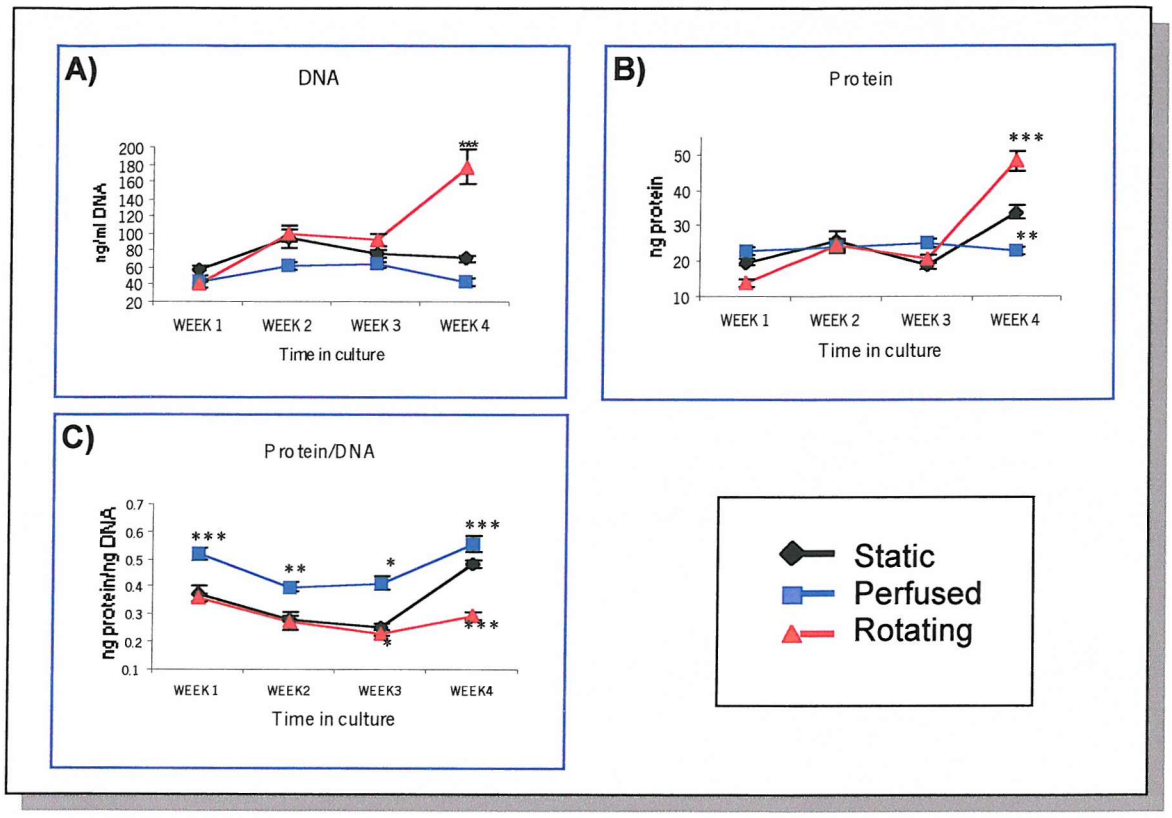

Figure 6.9. Effect of static, perfused and rotating culture on A) DNA, B) protein and, C) the protein per unit of DNA in constructs from standard seeding status over time. (expressed as mean ± standard deviation, n=4 *p<0.05, **p<0.01, ***p<0.001)

6.3.8. Effect of different culture conditions.

After standard seeding in static culture, the constructs were sectioned and matrix formation observed using alcian blue/sirius red staining (figure 6.10). The marrow stromal cells cultured in static culture displayed a low dispersion throughout the PGA fleece material, with cells dispersed sparsely on the outer surface with some degree of loss of PGA fleece fibres. In perfused cultures a few cells were observed dispersed in the centre of the fleece with a thick layer of cells on the outer surface of the construct. Rotating cultures showed cells dispersed throughout the construct.

Combination of histology with DNA and protein analysis highlights that although static culture was able to support some cell growth in the scaffold material the perfused culture system promoted greater support for protein production per cell than static or rotating systems. However, the rotating bioreactor was able to culture cells throughout the fleece and allow the expansion of more cells as observed by increased total DNA and total protein. Interestingly the rotating bioreactor does produce more cells but less protein per cell, which was apparent in the histology (figure 6.10 C).

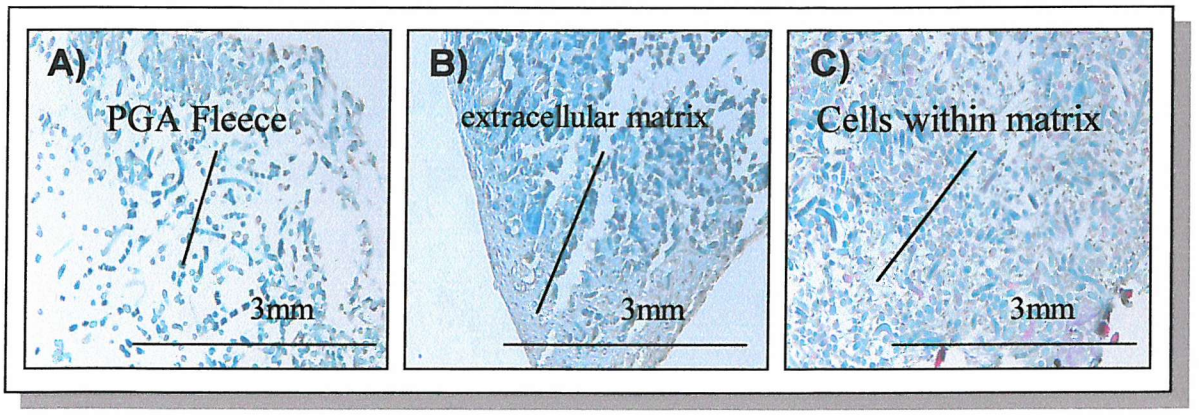

Figure 6.10. Culture on PGA fleece in A) static, B) perfused and, C) rotating culture. (note, the PGA fleece can be seen as bright blue dots most clearly observable in static culture (A) with cells observed distributed throughout, as seen in comparison to rotating culture (C)).

6.3.9. Seeding strategy

Initial observations of cultures before this experiment were performed using static seeding, in a standard fashion, to ensure that the results were due to the bioreactor conditions and not differences in method. However, more efficient seeding of the scaffolds may help to produce a better construct morphology or distribute cells more thoroughly. In this experiment one group of marrow stromal cells were seeded and cultured in rotating culture and one group seeded by direct addition of the cells to the rotating vessel as a "dynamic seeding" method. The construct's gross morphology showed the fibrous nature of the sponge scaffold in the statically seeded group whereas the dynamically seeded culture group has clearly filled in with cells, even though the same numbers of cells were added (figure 7.11). This simple study highlights the necessity to have an efficient method of seeding the scaffolds to maximise the use of cells and increase the coherence of the final construct.

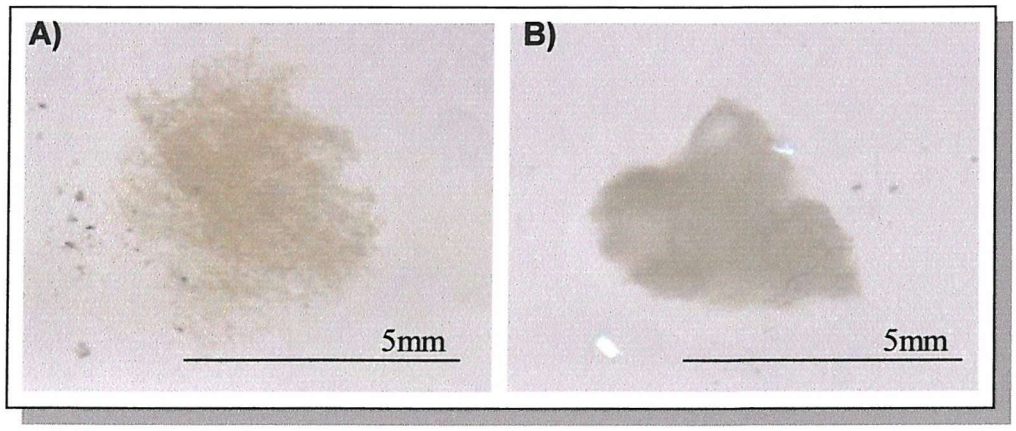

Figure 6.11. Sponge scaffolding that was seeded with the same number of cells in A) static culture and, B) dynamic seeding in rotating culture after 21 days (original magnification 10X).

6.3.10. Seeding and culture effect using PGA fleece and chondrocytes

Previous experiments, by necessity, have had to standardise the culture method or seeding method. However, to compare the best method of each system, PGA fleece was seeded statically for perfused cultures and dynamically in the rotating bioreactors and compared to static cultures. The scaffolds showed that static seeding and culture only produced a small construct. The statically seeded and further perfused cultured group showed large areas of solid tissues but the fleece had probably dispersed. Rotating culture with dynamic seeding resulted in a fully filled construct with cells distributed throughout the construct, although perfused culture still produced constructs with more matrix.

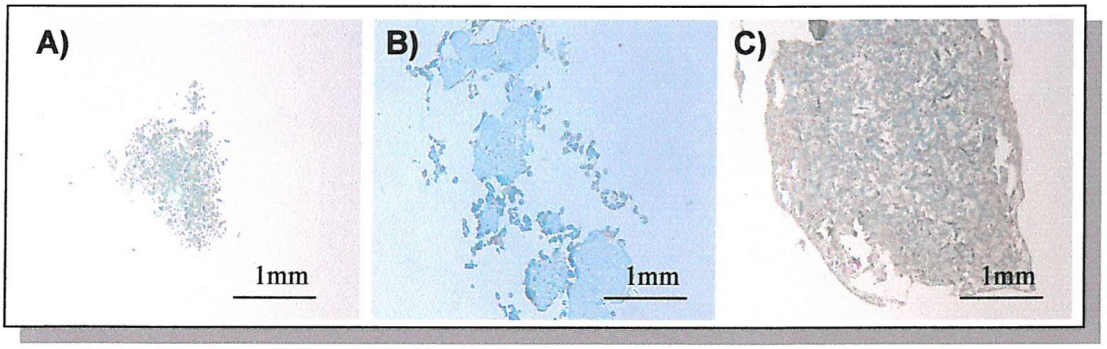

Figure 6.12. PGA fleece seeded with the same number of cells which were A) seeded statically and cultured statically, B) seeded statically and cultured in perfusion and C) seeded dynamically and cultured in rotating culture.

6.3.11. Cell viability through rotating culture constructs

Marrow CFU-F cells were culture expanded in monolayer and added dynamically to the PGA fleece in rotating culture. The resulting gross morphology of the culture showed a solid construct of approximately 5mm in diameter. The histology and live/ dead stain showed that the cells were distributed throughout the scaffold material (figure 6.13). Surprisingly, even the cells in the centre, although less in number, were still active and not dead. Cells stained as dead typically occurred toward the more active outer perimeter of the construct, where the cells were more numerous. This may in part be due to reacquisition of the dead cells from the circulating media or occasional impacts with the sides of the vessel.

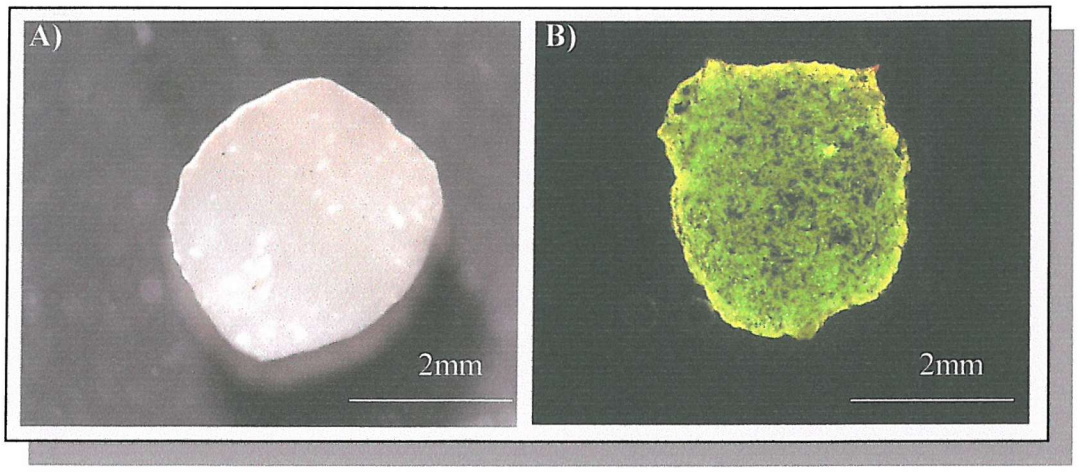

Figure 6.13. PGA fleece MSC cell construct made using dynamic seeding and rotational culture at 3 weeks seen in A) gross morphology and B) in section with cells stained using live/dead.

6.3.12. ATDC5 tissue engineering.

ATDC5 cells were able to reproduce some of the biochemical markers of chondrocytes in monolayer and were successfully cultured in "pellet" cultures displaying the features of cartilage as observed by type II collagen IHC, type II collagen western blotting, DMMB GAG assay, and western blotting for aggrecan. ATDC5 were therefore used in the devised tissue engineering strategy of dynamic cell seeding and culture in rotating culture on PGA fleece (figure 6.14). ATDC5 cells were able to proliferate over the fleece material and after 21 days in a rotating culture system produced a solid coherent cell mass, alcian blue/ sirius red staining showed a cartilage like matrix with lacunae features.

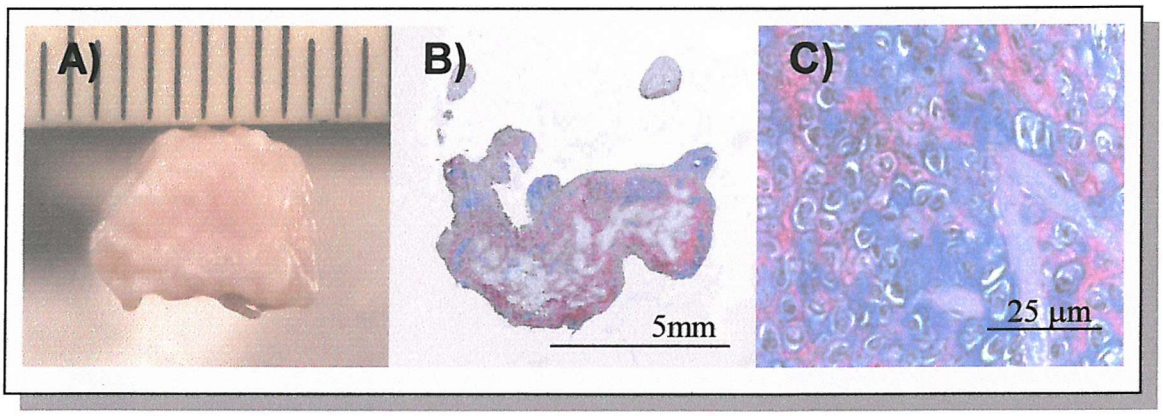

Figure 6.14. ATDC5 cells cultured on biodegradable PGA fleece in a rotating bioreactor system, A) overview (scale in mm), B) low power (10x) histology at (approximately the same size as (A)), C) high power histology showing lacunae.

6.3.13. Transformed marrow stromal cell line pZIP

The previous result obtained with a continuous cell line showed that a coherent tissue mass can be produced, however this may have been an extraneous result with the cell line being able to grow in such a manner. The cell line pZIP is a line derived from transformation of a marrow stromal cell line, the cells were seeded onto the scaffold using dynamic seeding and cultured as before (figure 6.15). The cell line pZIP formed dense cell layers over the scaffold. The deposited stained matrix appeared as a mixed cartilage/bone like tissue. The ability to culture both a chondroprogeitor and a marrow stromal cell line confirms the ability of the culture system to support tissue growth.

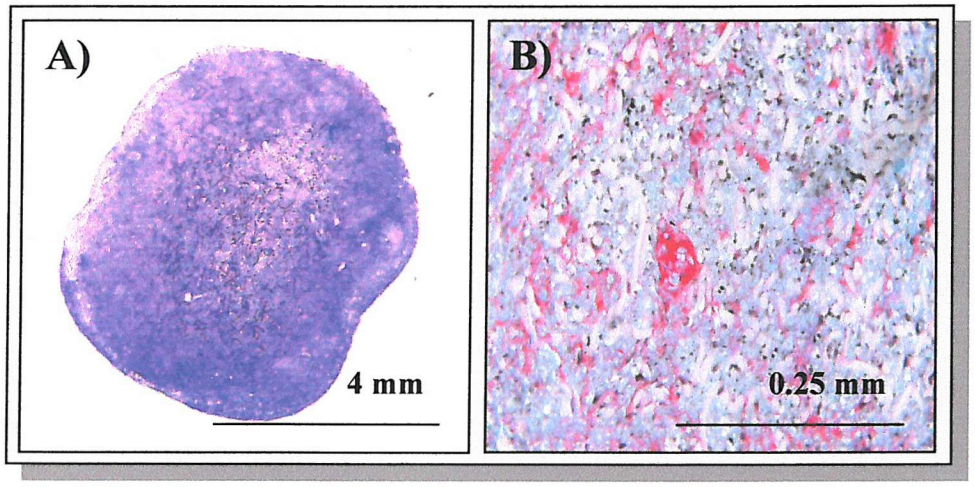

Figure 6.15. Transformed marrow stromal cell line pZIP cultured on PGA fleece in rotating bioreactor A) toluidine blue stained histology overview and B) A/S stained histology showing presence of red and blue stained tissue.

6.3.14. STRO-1$^+$ marrow stromal cells in tissue constructs

As STRO-1$^+$ selected cells appear to have an increased differentiation ability and grow more rapidly than unselected MSC, they were used to tissue engineer a construct to see if they produce a matrix where unselected cells did not. Whereas ATDC5 cells produced a filled construct, unfortunately the STRO-1$^+$ constructs appeared to produce the same effect seen in all the primary human cell sources. As with all the other primary cell sources the cells appeared throughout the scaffold, however the cells also appeared as elongated fibroblastic cells and apparently not cells which secrete matrix.

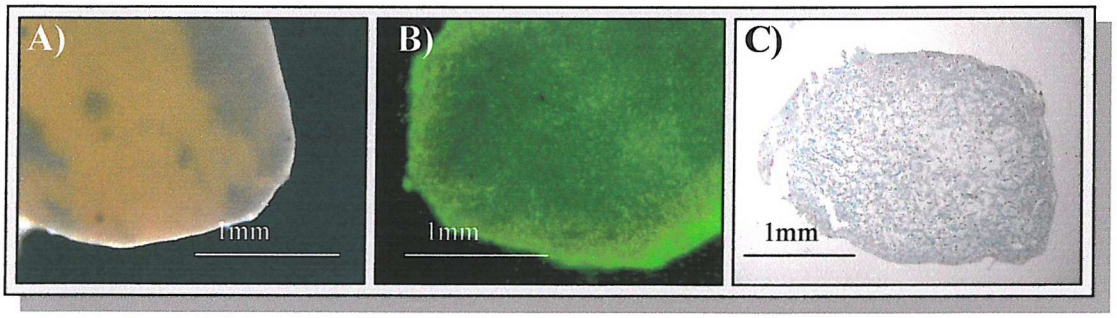

Figure 6.16. STRO-1+ selected MSC on biodegradable PGA fleece cultured in a rotating bioreactor system A) gross morphology, B) live/dead stained, C) sectioned and stained with A/S.

6.3.15. Culture of constructs with TGFβ3

The experiments using ATDC5 cells demonstrated that the tissue engineering system was able to support a cartilage-like construct. However, the ATDC5 cells are a continuous cell line and will grow and produce matrix more strongly than primary cell cultures. Primary human CFU-F from total hip replacement patients therefore need to be modified to increase cell number and matrix production. As TGFβ has been used in pellet cultures to induce the chondrogenic phenotype, using high cell density and serum free conditions, it may have been possible to use the same conditions to induce the chondrogenic phenotype on cell scaffolds for tissue engineering cartilage constructs.

In order to test this, two groups were seeded using the method of dynamic seeding in rotating culture using the same number of cells; with serum free media, serum free containing 10ng/ml TGFβ3 (as for the media used in pellet culture) and as a comparison 10% FCS supplemented αMEM. In FCS supplemented culture the cells were present throughout the construct and filled most of the scaffold. The cells in serum free TGFβ3 supplemented media showed limited areas of cell clusters throughout the cell scaffold (figure 6.17) and the serum free cultures did not continue to grow. The outcome of this study highlighted the need for continued cell growth as well as matrix production and the limitations of serum free culture for tissue engineering protocols.

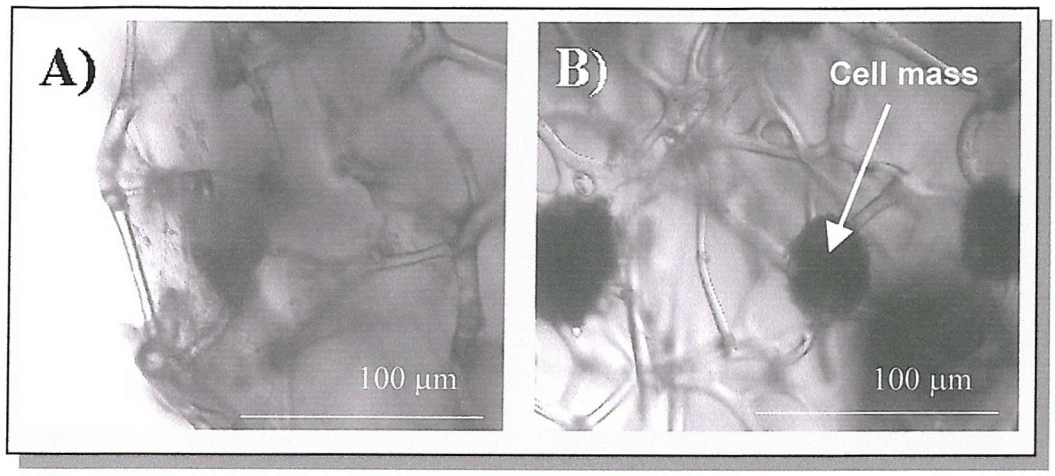

Figure 6.17. Effect of TGFβ3 in culture of sponge and human CFU-F in A) serum supplemented TGF free control media and B) serum free TGFβ3 supplemented media. (Note; serum free cultures are not shown here as they seeded well, but died after a week in culture)

6.3.16. Culture of constructs with TGFβ3 and serum.

The culture of scaffolds with TGF supplemented serum free culture showed limited growth as cell nodules throughout the cell scaffold, so in order to increase growth, serum was added to the culture media. Modification of cell constructs appears to be of paramount importance as are the phases of tissue engineering, with the system currently split into phases of expansion of cell numbers and subsequent culture, followed by modification of the 3D scaffold morphology. In order to produce an excess of matrix of the correct molecular composition, growth factors were used to supplement serum free conditions used in pellet cultures and some cultures had serum to increase the potential growth rate of the cells. We have previously seen that even chondrocytes do not strongly produce matrix in rotating cultures compared to perfused cultures (figure 6.12), so modulation of the final construct is key. Addition of TGF into serum free media did not allow suitable growth, although higher cell numbers may serve to improve the construct, however, these would probably constitute an unfeasible amount of cells to culture expand.

The experiment showed that addition of 10% serum to the TGF supplemented media did work to increase the matrix accumulation over 10% FCS supplementation alone (figure 6.18), with the constructs being significantly larger, having an average diameter of 5.45 ± 0.08 mm compared to controls which were 4.34 ± 0.28 mm in diameter (***$p<0.001$ unpaired t test).

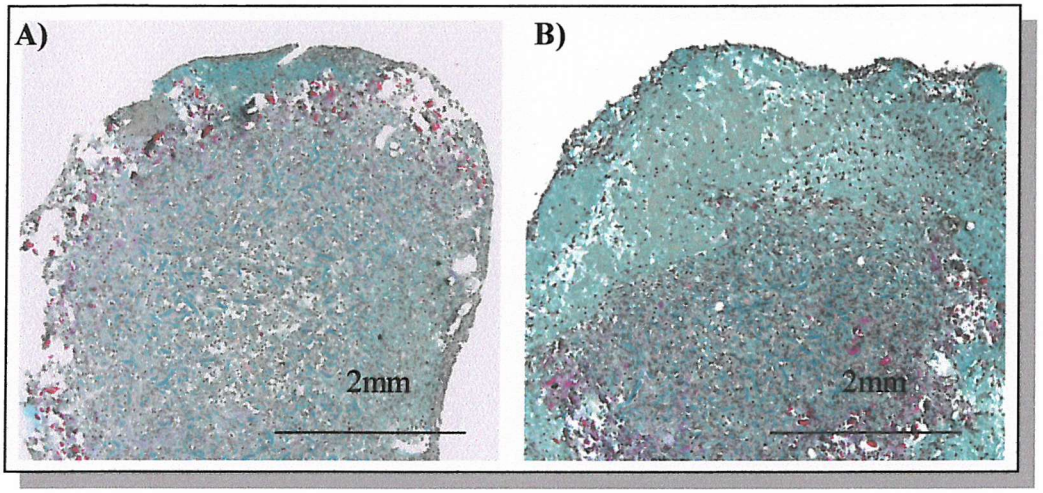

Figure 6.18. A) PGA fleece dynamically seeded with human CFU-F from total hip replacement patients and B) with 10ng/ml TGFβ3 supplement, stained with A/S.

6.4. Discussion

With this series of experiments it was clear that the manner in which the materials were combined was critical. Static culture was able to support human marrow CFU-F and chondrocytes on PLA, *Porifera* and PGA scaffolds, and the number of cells were limited when compared to perfused and rotating culture. Bioreactor culture appears to be necessary to generate constructs with good cell distribution but highlights a new set of problems in the form of cell behaviour within the different systems.

Rotating culture initially appears to be better than perfused culture, with the increased total cell numbers and total protein, however, the rate per cell of matrix production was actually higher in perfused culture and the histology appeared to confirm this. The smaller constructs seen in perfused culture were probably due to the lower starting cell number, since perfused cultures were seeded in static culture. Perfused culture was seeded separately as the media flow would simply wash the cells out of any reticulated scaffold material, highlighting the need for both a cell seeding and cell construct culture regime. Glowacki *et al* (1998) (Glowacki *et al.*, 1998) used a standard cell seeding regime of a spinner flask before further culture due to the high (nearly 100%) seeding efficiency of the spinner flask and then cultured in different systems.

Cell growth appeared to predominantly occur on the external part of the tissue culture construct and there have been reports on the reduction of the proliferating cell nuclear antigen (PCNA) in the centre of tissue engineered constructs (Glowacki *et al.*, 1998), however, the live/dead staining shows that there were still many activated cells even in the centre of a $5mm^3$ PGA MSC construct (Figure 6.13). As with pellet cultures the cells may not have been as active/ proliferative as in surface layer, hence the loss of PCNA seen in other studies.

In growing tissue constructs the cells require a high level of nutrition, but as the continuous cell lines ATDC5 and pZIP produced matrix in a construct of over 5 mm in diameter, the system clearly provided the majority of the factors within the media and culture system (figure 6.14, 6.15). The experiments with the continuous cell lines showed that the nutrients were not a limiting factor therefore the small constructs seen with primary human chondrocytes and marrow stromal cells were most probably due to the cells used and not the system, although the system would have to be adapted to the primary cells requirements.

The experiments using FCS to increase the growth rate of human marrow CFU-F showed that addition of 50% FCS can significantly increase the attachment and growth rate compared to 10% FCS supplement, this leads to a simple method of greatly increasing the numbers of cells for use in tissue engineering which is currently a major requirement. Addition of FCS makes media more expensive but provides a simple and effective way of increasing cell numbers for subsequent tissue engineering, although this could be replaced by a synthetic substitute. Human serum is used for *in vitro* adipogenic cultures of human marrow cells, however, using the individual patients own serum has been shown to vary from patient to patient, therefore to guarantee the growth of cells, a tested batch of FCS from a safe source would probably be the best method for further tissue engineering, although some antigenicity and potential pathogens may still be present.

In summary, these results show that the rotating system was able to supply the conditions needed for tissue growth (in a continuous cell line), that the rotating system distributed cells throughout the scaffolds when used as a dynamic seeding system and that perfused culture was able to enhance the cells ability to produce protein (matrix) in a construct. Addition of growth factors may be necessary to induce a similar growth

to that seen in the continuous cell lines and TGFβ3, when used with serum, can increase the size of the constructs produced. The starting seed population could also be increased to reduce the need for growth modulation in later tissue engineering phases. Marrow stromal cells were increased here by simply using higher serum concentrations, although the effects of increased serum on ensuing constructs has yet to be assessed. The use of PGA however has previously been shown to induce a granulocytic response in the final degradation stages due to acid end products (Bostman., 1998), although cartilage may be less susceptible than most tissue to this effect. Future work will have to focus on matrix production and modulation by primary cells, as the cells can grow on the scaffold but not produce matrix.

CHAPTER 7

DISCUSSION

The skeletal system is a load adaptive and articulated system which most of us take for granted, its operational lifetime is often over 70 years and can quite easily outlast many of the modern alloy joint replacement systems intended for repair. There are a multitude of repair options which have been generated over the years, but none of them are able to replace the full function of the living bone and cartilage, with the exception of the more recent cartilage repair strategies which use autologous culture expanded cells to fill defects. Unfortunately, even these cartilage repair strategies suffer from unreliability, as only 25% of operations result in full repair with articular cartilage, 50% repairs with fibrocartilage, and 25% do not repair (Ashton *et al.*, 2001). Although the autologous cartilage implantation (ACI) operation has the advantage of not effecting any subsequent operations, a 25% failure rate for any operation (even if the harm done in this case is limited) is still a relatively unacceptable rate of failure compared to total joint replacement.

As neither mechanical replacement or cell based treatments currently produce an acceptable level of repair, replacement with autologous tissue could conceivably result in a full repair and restore functioning to the tissue. The current primary surgical options for autologous tissue replacement is material sourced from the same patient, this is commonly procured by using non-load bearing material from elsewhere in the skeleton i.e. iliac crest. These procedures are the first choice, but problems still occur with the suitability of the material, especially if the tissue needed is greater than can be sourced, resulting in weakening and donor site morbidity (Goldberg *et al.*, 1993).

An alternative is development of a tissue replacement from the patients' own cells, that could be specifically tailored to integrate and repair the damaged tissue, with possible inclusion of resistance to any disease process, which may have caused the process. Therefore, it was considered that marrow stromal cells would be suitable,

with their ability to be isolated from patients in a relatively low risk procedure, with subsequent frozen storage until usage, when the cells can be culture expanded and prompted to form both bone and cartilage material.

Cells of marrow with the ability to form bone and cartilage have been isolated by attachment to plastic cultureware as fibroblastic colonies (CFU-F), however a direct isolation of a more defined cell population can be achieved using an antibody selection system.

In these experiments, the STRO-1 isolated group, in the population examined, appeared to contain a lower percentage (7%) of marrow nucleated cells, whereas many studies show twenty plus percent. This discrepancy may have come from a difference in isolation between labs, which in itself highlights different results obtained from the same antibody and different patient groups used for each study (Dennis *et al.*, 2002; Gronthos *et al.*, 1994; Simmons *et al.*, 1991; Stenderup *et al.*, 2001; Stewart *et al.*, 1999). This current study showed that STRO-1 isolated marrow nucleated cells included an increased proportion of CFU-F forming cells, and that potentially these cells could be used to provide structural tissues for engineering strategies of both bone (as observed using the *in vivo* diffusion chamber (Ashton *et al.*, 1980)) and cartilage (as seen with the pellet cultures (Johnstone *et al.*, 1998; Mackay *et al.*, 1998)). Futhermore, STRO-1 CFU-F had an increased size of colony and possibly an increased differentiation ability, this suggests that the parent group of CFU-F from which the STRO-1^{+} cell were derived, may consist of a range of minor cell divisions. This was consistent with other reports suggesting that there are different alkaline phosphatase activities (Aubin., 1998) and potential outcomes of varying CFU-F colonies with continued cell divisions (Muraglla *et al.*, 2000).

In order to prove the potential of STRO-1 selected stem cells, they were subject to tissue engineering strategies that had previously been shown to produce mineralised tissue in unselected stem cells (Partridge *et al.*, 2002). STRO-1^+ CFU-F cells were seeded onto PLA foamed scaffolds and placed *in vivo* within diffusion chambers, which allowed separation of host and introduced cells whilst maintaining an *in vivo* environment. STRO-1^+ marrow stromal cells were also prompted along the osteogenic line by the inclusion of a replication deficient adenovirus stage containing the sequence for bone morphogeneic protein –2 (BMP-2), to ensure differentiation. Release of BMP-2 with this system was transient but it was proposed that the BMP-2 could cause a cascade of events ensuring production of mineralised tissue. Therefore, transduced STRO-1^+ cells on PLA scaffolds could be strongly considered for the production of tissue engineered bone substitute.

Furthermore, the experiments described throughout, show that a solid tissue engineered construct can be produced *in vitro,* by using the culture system of cells, PGA fleece and rotating culture with dynamic cell seeding, as shown by chondrogenic ATDC5 and pZIP continuous cell lines. The comparison between continuous and primary cells, showed that the primary cells; chondrocytes, marrow stromal cells and STRO-1^+ selected MSC had a limited accumulation of matrix when cultured on PGA fleece in rotating culture. If this result is compared to micromass, where primary cells were able to produce a morphology and some molecular markers reminiscent of cartilage, it would appear to be a failure of one of the factors of the *in vitro* tissue engineering culture system for aged primary human cells. The growth of the continuous cell lines showed that the culture system, culture expansion, media, growth factors and scaffold, need to be optimised with respect to the use of primary human cells isolated from aged patients. With this in mind, constructs were optimised by

using similar conditions to the pellet cultures, which had shown suitable matrix accumulation in primary human cells. Constructs generated using serum free media supplemented with 10ng/ml TGFβ3, in a method designed to reproduce the micromass culture matrix accumulation, showed that the cells did not attach evenly but formed clumps of cells (figure 6.17). The culture differences between serum supplemented and defined serum free TGFβ3 supplemented media highlighted the need for growth and careful modulation to produce the final morphology. Therefore, media supplemented with both serum and TGFβ3 was used and this showed an increase in the accumulation of the matrix, although it appeared that the matrix occurred predominantly around the outside of the scaffold.

To ensure a high number of cells during the initial seeding phase (a major restriction in tissue engineering), the CFU-F were observed to be increased in attachment and growth rate by the addition of 50% serum, however the effects of culture expansion in this manner have not been tested as the monolayer culture expansion phase has been shown to alter the performance in subsequent tissue engineering stages (Martin *et al.*, 2001).

Assessment of seeding methods shows clearly that seeding of the scaffolds was an important part of the procedure and that dynamic seeding using the closed environment of the rotating culture vessels was far superior to static cell seeding. Alginate gels circumvent the problem of seeding as the cells can be incorporated within the material in any shape desired. However, larger constructs would have to be made with consideration of the nutrient diffusion rate as well as structural integrity, this is compounded by the need to incubate cells longer in the non-ideal environment of the polymerisation solution. The lessons learned with alginates may be applicable to designer synthetic peptides (Kisiday *et al.*, 2002), which could have features of

degradation, adhesion and strength easily modified. The alginate cultures were performed in static culture, future work would need to focus upon upregulation of constructs using the perfused culture systems.

Assessment of the different bioreactor systems appeared to show that rotating culture was optimal for growth, but may not be better for all factors, as matrix accumulation may have been compromised. This rotating system produced well seeded, but poorly matured, constructs. Perfused culture showed poor cell growth (total DNA) but appeared to increase the level of protein and cell matrix accumulation, therefore bioreactors, like scaffolds, should be considered on an individual basis to balance a variety of construct features. In tissue engineered constructs the factor of maximal cell matrix accumulation would probably be at the expense of the cells ability to grow, therefore it may be necessary to separate the phases of tissue production into, i) monolayer expansion of the cells, ii) optimal seeding of the cells, iii) optimal distribution and growth of the cells in the construct and, iv) final accumulation of matrix and maturation of the construct. Alternative methods have been used by other groups to seed and mature constructs utilising combinations of monolayer, spinner flask, perfused systems and orbital shakers (Glowacki *et al.*, 1998; Kafienah *et al.*, 2002; Martin *et al.*, 2000; Sittinger *et al.*, 1997). Increasing the starting cell population could allow bigger constructs to be produced, although the strategy for developing larger, useable constructs may need revision, as producing a smaller construct with less cells and allowing it to mature, will need to be investigated. The reason for the lack of production of matrix components by primary human cells may have been hinted at by other groups, who have shown that bovine chondrocytes were positive for smooth muscle actin (SMA) when cultured on PGA fleece, making them act as contractile fibroblasts and inducing shrinkage in constructs, an effect that was negated

by culture expansion in the presence of FGF-2 (Martin *et al.*, 2000). The decrease in primary cells ability to produce matrix may be due, at least in part, to the reduction of differentiation and growth potential observed in aged patients (Murphy *et al.*, 2002; Oreffo *et al.*, 1998a) and the reduction of differentiation potential seen with extensive cell subculture (Muraglia *et al.*, 2000).

Even though the primary cell constructs did not strongly produce matrix as shown by histology, constructs produced from aged primary human chondrocytes and marrow stromal cells still showed good cell viability and cell distribution. The constructs produced may therefore still be viable as implants, as the cells may not necessarily need to be an exact copy of skeletal tissue, but merely a stable device for introducing a three dimensional cell distribution. The basic constructs produced from aged primary human cells may still integrate, grow and mature *in vivo,* as has been suggested by Wakitani (Wakitani *et al.*, 2002b). In this respect the device may be suitable already as an implant, however, an implant already triggered to react correctly, with a more durable structure, resistant to the harsh mechanical environment of bone or an articulating joint, would clearly be more advantageous.

These results taken together show that it was possible to isolate a group of stem cells, culture the cells on biodegradable scaffolds as a coherent material and that the material had the potential to be modulated to produce more matrix and express the defining characteristics of bone and cartilage tissue. The constructs made from primary cell sources, although not as definitive as continuous cell lines, could still be used as tissue constructs working with the tissue environment to complete the transformation (Wakitani *et al.*, 2002a). Ultimately, the constructs could deliver cells to a site for repair, however, there are still questions of integration, disease resistance and practicality to be addressed. Caution should also be taken with cell phenotype markers

as it has been observed that cells expressing osteopontin, osteonectin, bone sialoprotein and alkaline phosphatase may equally be hypertrophic chondrocytes or osteoblasts (Aubin., 1998).

The use of *Porifera* sea sponge as a scaffold appeared to mimic a bonded fleece network, and the use of the ATDC5 and pZIP cells showed that it was the primary cells that needed focused attention. *Porifera* is fortuitously made from a collagen analogue, *spongin,* which is closely related to type XIII collagen (Exposito *et al.*, 1991) and shows great promise as a scaffold, however degradability, immunogenicity and the sponges ability to uptake toxic chemicals during growth may present serious problems for future development. The observation of ATDC5 cell growth showed production of a cartilage-like morphology in pellet culture and thus provided a useful cell line for culture in micromass as well as interpretation of tissue engineering protocols. A large scale chondrogenesis model achieved with a continuous cell line could allow a simple reproducible system on which to elucidate chondrogenic events and to test factors which change the course of chondrogenesis such as BMP's and other factors able to modulate matrix composition in tissue engineered constructs.

The study as a whole was limited primarily by minimal *in vivo* observations, which were restricted to only PLA scaffolds in diffusion chambers, whilst other limitations were the need for the full characterisation of the constructs for definitive markers of lineage. Pellet culture techniques, although useful in determining that primary human cells can produce matrix with some of the expected markers of cartilage, highlighted that the same conditions cannot be viably applied to the production of tissue engineered constructs.

STRO-1 antibody has not yet been characterised fully and although it selects CFU-F it also clearly selects for other cells and was seen to cross-react with cartilage matrix.

Limitations of STRO-1 includes the characterising assay, whereby using the CFU-F formation as an assay for stem cells may not account for other ancillary cells. Using *only one* antibody to isolate stem cells may be an insurmountable task as other groups have suggested that due to the apparent nature of stem cells, "cocktails" of antibodies may be needed for isolations (Stewart *et al.*, 2003).

Although the scope of this thesis did not extend to the final production of a tissue construct from primary cells *in vitro* which could be described as bone or cartilage, these series of experiments still showed production of a coherent tissue mass which could be suitable for implantation and that these cells were capable of producing both bone and cartilage.

The immuno-selected mesenchymal stem cells in this study were shown to be increased in growth and differentiation potential when cultured *in vitro,* with the potential to increase the viability and reliability of implants within diffusion chambers *in vivo.* As well as the increased growth, differentiation and reliability of the STRO-1 isolates, STRO-1 selected cells have been demonstrated here to be able to be transformed into both bone and cartilage, implying that STRO-1^+ MSC constructs may be used for both bone and cartilage repair.

The tissue produced from the rotating bioreactor did not contain extracellular matrix to the same extent as was observed in pellet cultures, or with continuous cell lines, however, the addition of TGFβ3 and serum was able to significantly increase the accumulation of matrix.

Furthermore the tissue engineering strategy devised here with addition of STRO-1 selected marrow stromal cells to biodegradable PGA fleece, with dynamic seeding and culture in rotating vessels, was a novel approach with respect to the use of STRO-1^+

mesenchymal stem cells and the dynamic seeding strategy, which was able to produce a coherent tissue mass with the potential for implantation.

In conclusion, the results presented here show that a defined group of cells can be isolated from elderly patients, culture expanded, and further cultured using a tissue engineering strategy into a solid tissue mass suitable for implantation as either bone or cartilage.

In summary, these experiments show that adenoviral modulation of selected MSC combined with PLA scaffolds, as well as the rotating culture system, with PGA fleece and dynamic seeding, are viable candidates for tissue engineering orthopaedic implants. The insight into the potential of the systems, given by the transformed cell lines ATDC5 and pZIP, gives great hope that in the near future a more mature implant will be achieved with isolates of marrow.

8. FUTURE WORK

The tissue engineered construct produced in this thesis could potentially be used as a simple cell delivery device, therefore, development of a suitable *in vivo* model would be key in deducing the fitness of the implant already described.

Furthermore, the system devised was able to produce a cartilage material, albeit with continuous cells, which suggests that the primary cells, or at least the culture conditions effecting only primary cells, needs to be addressed. Therefore, defining the matrix produced by the primary cells and the influence of factors upon the composition of the final construct needs to be investigated. As the tissue created using primary cells did not produce a tissue with the amounts of matrix seen in pellet culture, inclusion of a maturation phase may be necessary. The maturation phase could be performed by perfused culture, which has already been shown to produce an increase

in protein production in tissue engineered constructs, observable by both biochemistry and histology.

Further manipulation of culture factors also needs to be investigated, as this would be a complex process, it could be simplified by dividing the culture scheme into different stages and observing the effects on the outcome. The procedure can be divided into the phases of 1) isolation of the cells, 2) culture expansion, 3) seeding, 4) primary phase of tissue engineering and 5) maturation phase. Addition of FCS to each or combination of these phases would allow observation of the effects of a complex of growth factors which have already been shown to increase the CFU-F growth rate. Then, dependant upon the results seen with FCS, an artificial serum mixture could be tested in the different phases of tissue engineering, for modulation of the final outcome.

Modification of the construct using defined growth factors would require extensive empirical investigation, for example; TGF addition at only one concentration would need from five to twenty six experiments, increase this by the use of another growth factor and two concentrations and the system would require many hundreds of experiments, therefore a quick, simplified, three dimensional, model culture system needs to be devised to aid further analysis.

In summary, future work would have to include; the assessment of the current construct in a suitable *in vivo* model and maturation of the construct using a modified culture regime.

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List of publications and abstracts

Journal articles

1. Howard, D., Partridge, K.A., Yang, X., Clarke, N.M.P., Okubo, Y., Bessho, K., Howdle, S.M., Shakesheff, K.M., and Oreffo, R.O.C., Immunoselection and adenoviral genetic modulation of human osteoprogenitors: *in vivo* bone formation on PLA scaffold. Biochemical Biophysical Research Communications, Vol 299, October 2002.

2. Green, D.W., Howard, D., Yang, X.B., Kelly, M., Oreffo, R.O.C., Natural marine sponge fibre skeleton: a biomimetic scaffold for human osteoprogenitor cell attachment, growth and differentiation. Tissue Engineering (In press; vol 9, no 6, 2003).

3. Green, D.W., Leveque, I., Walsh, D., Howard, D., Partridge K.A., Mann, S., and Oreffo R.O.C., Mineralised polysaccharide capsules for encapsulation, organisation and delivery of human cells and growth factors. Nature biotechnology, submitted.

Presentations

1. Howard, D., Partridge, K.A., Green, D.W., Yang, X., Roach, H.I., Clarke N.M.P., and Oreffo, R.O.C., The use of Immuno-selected bone marrow cells for cartilage and bone tissue engineering. Health and Biological Sciences Postgraduate Conference. Southampton General Hospital. 26th June 2003.

2. Howard, D., Partridge, K.A., Yang, X., Okubo, Y., Bessho, K., and Oreffo, R.O.C., Adenoviral BMP-2 gene transfer into STRO-1 selected human osteoprogenitors. British Orthopeadic Research Society, Cardiff Inernational arena, Cardiff. September 16-17, 2002. (Awarded best science presentation)

3. Howard, D., Partridge, K.A., Yang, X., Okubo, Y., Bessho, K., Howdle, S.M., Shakesheff K.M., and Oreffo, R.O.C., Adenoviral BMP-2 gene transfer in STRO-1 immunoselected human osteoprogenitor cells for bone formation. 4th annual conference of the Tissue and Cell Engineering Society TCES, University of Glasgow 19th and 20th of September 2002.

4. Howard, D., Roach, H.I., Clarke N.M.P., and Oreffo, R.O.C., An *in vitro* model of chondrogenesis using human mesenchymal stem cells. Faculty of Medicine, Health and Biological Sciences Postgraduate Conference. 27th June 2002.

5. Howard, D., Partridge, K.A., Yang, X., Clarke, N.M.P., Okubo, Y., Bessho, K., Howdle, S.M., Shakesheff, K.M., and Oreffo R.O.C., Adenoviral BMP-2 Gene Transfer in STRO-1 Immunoselected Human Osteoprogenitor Cells - *In Vivo* Bone Formation on Biodegradable Scaffolds. 3RD Smith and Nephew international Symposium - Translating tissue engineering into products. Georgia Institute of Technology, Atlanta, GA. 13-16th October 2002.

6. Green, D.W., Walsh, D., Partridge, K.A., Howard, D., Mann S., and Oreffo, R.O.C., Cell therapeutics- Mesenchymal stem cells, growth factor and gene delivery using innovative biomineralised polysaccharide templates.

7. Green, D.W., Walsh, D., Partridge, K.A., Howard, D., Mann S., and Oreffo, R.O.C., Calcium phosphate-polysaccharide composite scaffolds for tissue regeneration. B.O.R.S. Nottingham, April 2003.

8. Green, D.W., Howard, D., Yang, X., Partridge, K.A., Clarke, N.M.P., Oreffo, R.O.C., Marine Invertebrate Skeletons: Promising Tissue Engineering Scaffolds Particularly for Orthopaedic Applications. 3rd Smith and Nephew International Symposium, Atlanta, October 2002.

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