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Analysis of osteogenesis on a novel collagen glycosaminoglycan scaffold- *in vitro* application for bone tissue engineering

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BSc. MSc.

A dissertation submitted to the Royal College of Surgeons Ireland
for the degree of

Doctor of Philosophy

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Declaration

I hereby declare that this thesis has not been submitted to any other university as an exercise for a degree and that the work contained within it is entirely my own.

I grant the Royal College of Surgeons in Ireland full permission to lend or copy this thesis on request.

Signed,

Michael Keogh

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30 June 2010

Abstract

Currently, there exists a need to develop new bone graft substitutes as an alternative to conventional autografting and allografting treatments due to disadvantages such as cost, scarcity of tissue, multiple surgical procedures and the risk of infection. Tissue engineering provides an alternative solution and relies extensively on the use of porous scaffolds to provide the appropriate environment for the regeneration of tissues and organs. These scaffolds are typically seeded with cells and occasionally growth factors or subjected to biophysical stimuli in the form of a bioreactor and are either cultured *in vitro* to synthesise tissues which can then be implanted into an injured site or are implanted directly into the injured site and through the body's own systems, regeneration of tissues or organs is induced *in vivo*. In our laboratory, we use a type 1 collagen glycosaminoglycan (CG) scaffold for tissue engineering applications. These scaffolds have been successfully used to clinically treat burn patients and received FDA approval in the mid-1990's. This thesis examines the ability of the CG scaffold towards bone repair and regeneration.

Chapter 2 of this thesis examined the ability of the CG scaffold to support attachment, growth and differentiation of human pre-osteoblastic cells hFOB 1.19. Following optimisation of hFOB cells on CG scaffold, an alamar blue viability assay was adapted for use with the hFOB cell line on CG scaffolds. However, it was shown to be a more useful as a tool to identify general cell viability rather than to accurately calculate cell numbers. Long term culture of hFOB cells on CG scaffold demonstrated that cells migrated to the centre of the scaffold by 14 days, resulting in a homogenous, confluent construct by 35 days which displayed both osteoconductive and osteoinductive qualities.

Chapter 3 examined the potential of the CG scaffold to support osteogenesis of human cells under long term culture conditions up to 49 days. The effect of TGF-β_1 was examined and found to enhance osteogenesis with optimal results occurring when using

an initially high exposure of 10ng/ml TGF for 7 days and reducing this to 0.2ng/ml thereafter. Cell-seeded CG constructs remained viable with fully infiltrated homogenous cell distribution; high levels of cell-mediated contraction and increased in compressive modulus reported over time. A cell capsule along the scaffold periphery and core degradation developed at 49 days; however, mineralisation was shown to be uninhibited with highest levels late stage osteogenic gene expression and mineralisation detected in the highly confluent cellular outer region of the scaffold.

The effects of scaffold mechanical properties on cell behaviour were assessed in Chapter 4 which demonstrated that different crosslinking techniques can be used to produce scaffolds with varying stiffness. Results showed that CG scaffold stiffness and its ability to contract displayed an opposite effect on cell proliferation in comparison to differentiation; where the less stiff and contractible DHT-crosslinked CG constructs displayed greater osteogenic maturation while the stiffer, non contractible EDAC and GLUT-crosslinked scaffolds resulted in increased proliferation but reduced osteogenic differentiation.

Chapter 5 examined the effects of exposing cell-seeded CG scaffolds to fluid flow using a flow perfusion bioreactor. This study showed that bioreactor culture improved cell distribution and osteogenic priming of hFOB pre-osteoblasts. No difference was observed in levels of cell number or metabolic activity between bioreactor and static culture. However, cell distribution improved following bioreactor culture becoming more homogenous throughout the construct and avoiding the formation of a peripheral cell capsule along the scaffold edges which is a notable problem with long term static culture (as found in Chapter 2). While bioreactor cultured scaffolds displayed lower levels of mineralisation than static cultures, the mineral was more homogeneously distributed and gene expression analysis showed that bioreactor-cultured constructs gave higher cellular expression levels of bone formation markers than static culture alone and that there was no difference in mechanical stiffness between groups. This

suggests that the bioreactor can be beneficial for improving cell distribution and osteogenic priming of cells seeded onto CG scaffolds.

In conclusion this thesis shows that the CG scaffold can support attachment, growth, viability and osteogenesis of human cells during long term culture; that CG scaffold stiffness can influence osteoblast maturation and that a flow perfusion bioreactor can be used as a tool in bone tissue engineering to improve cell distribution as well as providing osteogenic stimulation of human cells on CG scaffold. This thesis thus demonstrates excellent capabilities of the CG scaffold as a bone graft substitute.

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Nomenclature

°C	Degrees Celsius
μm	Micrometre ($x10^{-6}$)
2D	2 dimensional
3D	3 dimensional
ALP	Alkaline Phosphatase
ANOVA	Analysis of Variance
BMP	Bone Morphogenic Protein
CO_2	Carbon Dioxide
COL-1	Type 1 Collagen
CG	Collagen glycosaminoglycan
CP	Calcium Phosphate
DHT	Dehydrothermal
DMEM	Dulbecco's Modified Eagles Medium
DNA	Deoxyribonucleic Acid
DPBS	Dulbecco's Phosphate Buffered Saline
DPX	Di-N-Butyl Phthalate in Xylene
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic acid
EtOH	Ethanol
FBS	Foetal Bovine Serum
FDA	Food and Drug Administration
FGF	Fibroblast Growth Factor
GAG	Glycosaminoglycan
H_2O	Water
HA	Hydroxyapatite
H&E	Hematoxylin and Eosin
HAMS F12	Hams F12 modified medium
HCl	Hydrochloric Acid
hr	Hour
hFOB 1.19	Human foetal osteoblastic cell line
IGF	Insulin like Growth Factor
l	Length
M	Molar
MC3T3-E1	Mouse Calvarial Osteoblast Cell Line

mg	Milligrams
min	Minute
MIT	Massachusetts Institute of Technology
ml	Millilitre ($x10^{-3}$)
mm	Millimetre ($x10^{-3}$)
mM	Milimolar ($x10^{-3}$)
MSC	Mesenchymal Stem Cell
mTorr	Millitorr ($x10^{-3}$)
nM	Nanomolar ($x10^{-9}$)
Oc	Osteocalcin
Op	Osteopontin
On	Osteonectin
PBS	Phosphate buffered saline
PGA	Polyglycolide acid
PLA	Polylactide acid
PLGA	Poly-DL-lactic-co-glycolic acid
PLLA	Poly- (L-lactic) Acid
RCSI	Royal College of Surgeons in Ireland
RLT	Cell lysis buffer for protein extraction
rpm	Revolutions per Minute
SD	Standard Deviation
T	Temperature
TGF	Transforming Growth Factor
™	Trade Mark
UV	Ultra-Violet
V	Volume
X	Magnification
α-MEM	Alpha-Minimum Essential Medium
µg	Micrograms ($x10^{-6}$)
µl	Microlitre ($x10^{-6}$)
®	Registered Trade Mark

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Ba mhaith liom cúlpla focal a rá as gaeilge anois! Gabhaim fíor-bhuíchas le mo chlann le mo chlann go léir: mo dheartháir mór Níoclás, an deartháir beag Caoimhín,mo dheirfiúr Dearbhla agus mo thuismitheoirí Nioclás agus Damhnait. Gan iad, is cinnte nach mbeadh an leabhar seo ar an saol! (ná mise ach an oiread!)

Go raibh maith agaibh go léir!

"Dóchas a ghineann misneach , Misneach a ghineann gníomh."

"Hope creates courage, courage creates action"

Publications, awards, conference presentations and courses attended during course of PhD

Published papers:

Keogh, M. B., O' Brien F.J., Daly, J. S. (2010) *"A novel collagen scaffold supports human osteogenesis-applications for bone tissue engineering"* Cell Tissue Res **340** (1) 169-77

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Awards:

Third prize oral presentation (new research). Annual Bioengineering conference of Ireland (BINI). *Molecular analysis of osteogenesis by human osteoblasts on a three dimensional collagen-GAG scaffold.* Co. Fermanagh, NI. Jan. 2007.

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International:

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DMMC course "Techniques & Strategies in Molecular Medicine" Conway Institute of Bimolecular & Biomedical Sciences, University College Dublin; Dublin Molecular

Medicine Centre, March 2006

Certificate of attendance DMMC "Immunobiology & Inflammation" Conway Institute of Bimolecular & Biomedical Sciences, University College Dublin; Dublin Molecular Medicine Centre, February 2006

CHAPTER 1

Introduction and Literature review

1.1 Overview

Currently, there exists a need to develop new bone graft substitutes as an alternative to conventional autografting and allografting treatments due to disadvantages such as cost, scarcity of tissue, multiple surgical procedures and the risk of infection. Within bone tissue engineering, many scaffolds have been used to promote extracellular matrix formation and mineralisation including bioactive glass, ceramics, titanium and polymers in an effort to substitute autologous bone. None of these have replaced the autograft gold standard.

One potential substitute is the type 1 collagen glycosaminoglycan (CG) scaffolds which has been successfully used in a clinical setting as a viable treatment for conjunctiva and has received FDA approval in 1996 for epithelial regeneration. These scaffolds are typically fabricated by lyophilising collagen based slurries to form a highly porous sterile scaffold. The CG scaffold has been successfully shown to support attachment and proliferation of various animal cell types including fibroblasts, chondrocytes, and neurons.

This PhD thesis focuses on the potential of the CG scaffold to support osteoblast growth, development and osteogenesis *in vitro*. Using the CG scaffold conditions were initially optimised to study long term osteogenesis of human cells; this included cell seeding, real time cell viability and growth factor osteoblast stimulation. Following optimisation, the ability of the CG scaffold to support human osteogenesis *in vitro* over long term culture was assessed. This included an examination of the effects of

transforming growth factor (TGF) on human cell differentiation within the scaffold. At this point an assessment was conducted into how scaffold stiffness and contraction properties may affect osteogenesis; by focusing on the relationship of crosslinking the CG scaffold to improve mechanical properties. Finally, this lead to an assessment using a bioreactor; to both improve cellular homogeneity within the scaffold and as a method to stimulate an osteogenic response in order to develop an improved bone graft substitute.

1.2 Regenerative medicine & tissue engineering

Historically, damaged tissue was surgically treated via forms of mechanical closure to reduce bleeding and infections like septicaemia. Less attention was given to tissue regeneration or the recovery of organ function (Yannas, 1992). Only within the last 50 years due to technological advances has this trend altered. Now more innovative approaches such as transplantations, cloning techniques, prosthetic limb replacements and tissue grafting have been successfully examined. Tissue engineering (TE) is a recent term for a discipline of science which tries to encompass these approaches. TE has been applied to almost all organs in the body particularly those of connective tissues (bone, cartilage, ligaments, muscle and skin).

1.2.1 Clinical success and need for tissue engineering

The term tissue engineering (TE) was initially defined in 1988 by the National Science Foundation as "the application of principles and methods of engineering and life sciences toward fundamental understanding of structure function relationships in normal and pathological mammalian tissue and the development of biological substitutes to restore, maintain or improve tissue function" (Nerem, 1992) .

Bladder repair was shown to be possible in dogs using fascia as early as the early 1900's. Further clinical success has been achieved more recently in treating humans; including bladder regeneration using autologous bladder cells seeded onto a collagen-

polyglycolic acid scaffold and inserted to the damaged region resulting in organ repair (Atala *et al.*, 2006). The Lancet similarly reported successful TE of a tracheal graft by Macchiarini *et al.* (2008) (Fig 1.1). The patient was a 30 year old woman with severe dyspnoea caused by tuberculosis damage to the trachea. Following unsuccessful treatments with a Duman stent an autologous tracheal graft was attempted. A 7cm section of trachea was removed from a 51 year old donor. Loose connective tissue was removed and the tissue completely decellularised. Cells were taken from the patient via bronchoscopic biopsy. Chondrocytes and epithelial cells were separately isolated, expanded in culture and reseeded onto the donor graft (chondrocytes seeded on the outside and epithelial cells inside the tubular graft). The tissue was cultured in a bioreactor prior to implantation into the patient. Results were successful with authors reporting angiogenesis at 30 days and lack of rejection as the donor graft had HLA antibodies removed and the cells were autologous (from the patient themselves) (Macchiarini *et al.*, 2008; Zhang *et al.*, 2009).

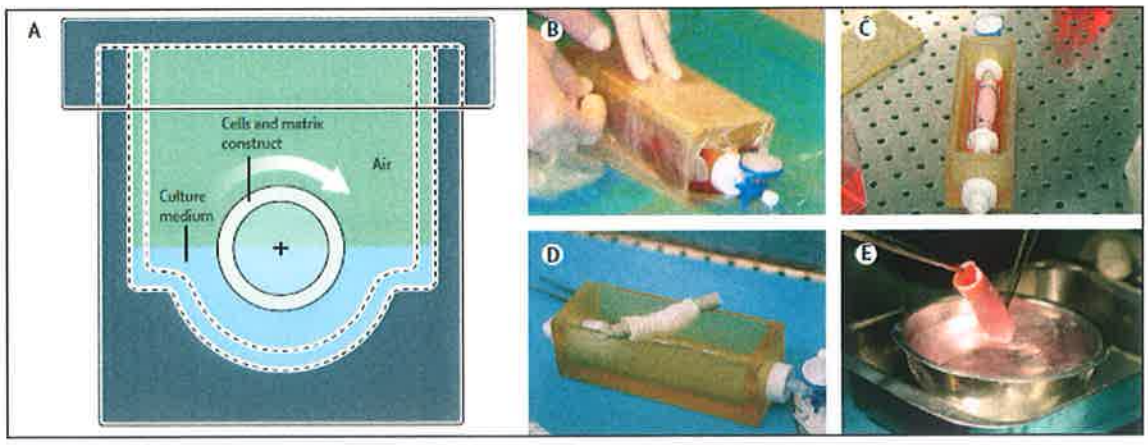

Fig 1.1 Bioreactor developed for airway tissue engineering (Macchiarini *et al.*, 2008)

1.2.2 Tissue engineering triad principle

The most common concept of tissue engineering is to combine a scaffold or matrix, living cells and/or bioactive molecules to form a "tissue engineered construct (TEC)"

(Hutmacher and Garcia, 2005). Once prepared, this triad construct may be transplanted to the effected injury site to initiate healing. Eventually, the aim is that the injured site will produce healthy tissue and thus become healed (Fig 1.2). This guided bone regeneration provides a 'passageway' for cells to regenerate without the production of fibrotic scar tissue.

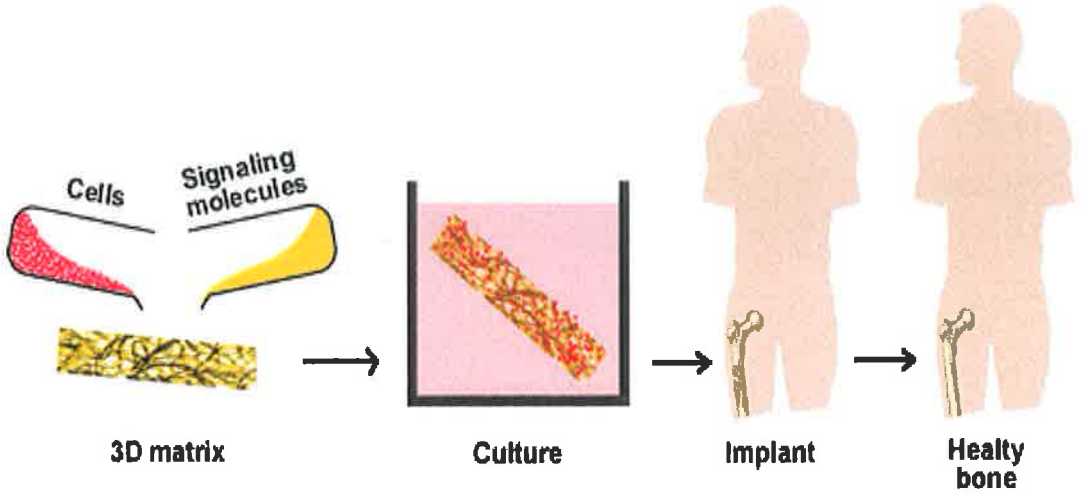

Fig 1.2 Tissue engineering triad principle consists of cells and signalling molecules on a scaffold which can be cultured in vitro with the intention of being implanted into an area of damaged tissue to initiate healing of a critical defect. Figure obtained online from http://www.btec.cmu.edu/tutorial/bone_tissue_engineering/bone_tissue_engineering.htm [Accessed 05 June 2009].

1.2.3 Bone tissue engineering

The importance of bone TE has risen recently due to the obvious clinical therapeutic need for repairing bone fractures, osteosarcoma and spinal fusions (partially due to an ageing population) coupled with the major economic requirement. It has been estimated that in 1999 alone as many as 500,000 bone graft procedures were performed in the United States with an estimated cost of $2.5 billion (Giannoudis *et al.*, 2005; Ilan and Ladd, 2002; Ladd *et al.*, 2010). It should also be considered that with an aging population these figures are set to rise.

Meijer *et al.* (2007) summaries the historical landmarks of bone TE by highlighting five key papers; beginning with Urist *et al.* (1965) who showed that bone tissue contains specific growth factors that can stimulate bone formation if transferred to ectopic sites; Friedenstein *et al.* (1987) used mesenchymal stem cells (MSCs) which formed colonies (also known as colony forming unit-fibroblasts) to show that bone marrow cells could differentiate into different cell types including osteoblasts; Caplan in 1991 wrote about how MSCs could be isolated, expanded *in vitro* and implanted to a region of damaged tissue to act as an autologous repair. More recently, Quarto *et al.* (2001) was the first clinical paper to report on a successful large bone defect repaired using autologous marrow stromal cells. Finally, Schimming and Schmelzeisen (2004) reported that human periostum derived osteoblasts could form lamellar bone within three months of transplantation.

1.3 Characteristics of normal bone biology

1.3.1 Structure, form & function

Bone is a hard connective tissue that forms the skeleton within most vertebrates. It comprises of calcium phosphate wherein bone cells are embedded in a collagenous fibre matrix. The primary functions of bone include structural support, calcium and phosphate storage and production of immuno progenitor cells. There are two main types of bone; compact and trabecular bone. Compact bone is always located at the surface of a bone, is quite dense and makes up about 80% of bone mass. It consists of concentric layers called *lamellae* surrounding *Haversian* canals and is covered with a protective sheath called the periosteum (Martini, 1993). Trabecular bone forms open networks of struts and plates known as trabeculae, within which is the marrow cavity and endosteum (Fig. 1.3).

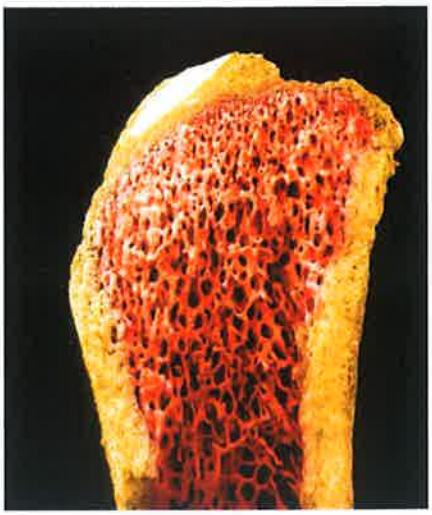

Fig 1.3 Sectioned human femoral head demonstrating the variation in bone structure with trabecular bone in red and compact bone in yellow. Image obtained from http://visualsunlimited.photoshelter.com/gallery [accessed 28 Oct 2009]

1.3.2 Bone cell types

The major cell types important in bone formation and development include marrow stromal stem cells, bone-forming osteoblasts, regulating osteocytes, and resorbing osteoclasts. Undifferentiated MSCs are multipotent, therefore they possess the ability to differentiate into chondrocytes, fibrocytes and adipocytes as well as osteoblasts. Osteoblasts are bone forming cells located on the surface of bone and are responsible for bone formation by secreting collagen, growth factors, cytokines and several proteins to support mineralization. They occur *in vivo* during various stages of maturity from progenitor pre-osteoblasts to bone forming mature osteoblasts. Osteoblasts secrete factors which regulate osteoclast differentiation and activity (Teti *et al.*, 1991).

As osteoblasts lay down matrix they can become isolated within the newly formed bone and are termed osteocytes. Osteocytes are the most common cell type in bone comprising of 80% of bone; however, less is known about this cell type in comparison to other types of cells (Soloman, 2005). Their primary function appears to be in bone regulation; communicating with neighbouring cells via cell processes which channel

through small openings called canaliculi.

Osteoclasts are large, multinucleated cells involved in bone resorption and remodelling. They are derived from haematopoietic stem cells present in bone marrow that have differentiated via the monocyte/macrophage lineage (Sabokbar *et al.*, 2005). They are capable of lacunar bone resorption due to their ability to secrete acid and specific degrading enzymes into specialised resorption pits to dissolve bone minerals (Kartsogiannis and Ng, 2004).

1.3.3 Bone matrix

Bone matrix is a combination of organic and inorganic materials. The organic osteoid part is comprised of collagens, proteoglycans and glycoproteins. It represents one third of the bone matrix and is responsible for the limited flexibility and great tensile strength of bone. The predominant component is collagen type I the most abundant protein in the body; a structural connective protein comprised of a triple helix. There are over 20 types of collagen differing in their polypeptide sequence. Collagen has two alpha-1 chains and one alpha-2 which are supercoiled in a right handed manner to form a triple helix. At each end of the protein there is a carboxyl (C-terminal) and an amino (N-terminal). Once the triple helical structure forms collagen proteins align themselves in fibrils which may be identified by a distinctive banded appearance due to a staggered structural alignment (Fig. 1.4).

Inorganic calcium phosphate is an important constituent in bone and exists primarily as calcium hydroxyapatite (HA) $(Ca_{10})(PO_4)6(OH_2)$. It may occur in different ratios of calcium to phosphate depending on the location in the body. For example dentin has a molar ratio of 1.64 whereas in bone it is closer to 1.71. HA is resorbable in the body as it occurs in nano sized particles.

There are several other important matrix proteins which comprise of non collagenous proteins such as osteopontin, osteonectin, bone sialo protein and

osteocalcin. These can regulate bone structure and function and often play a close role in the mineralisation process and will be discussed further in section 1.3.4.

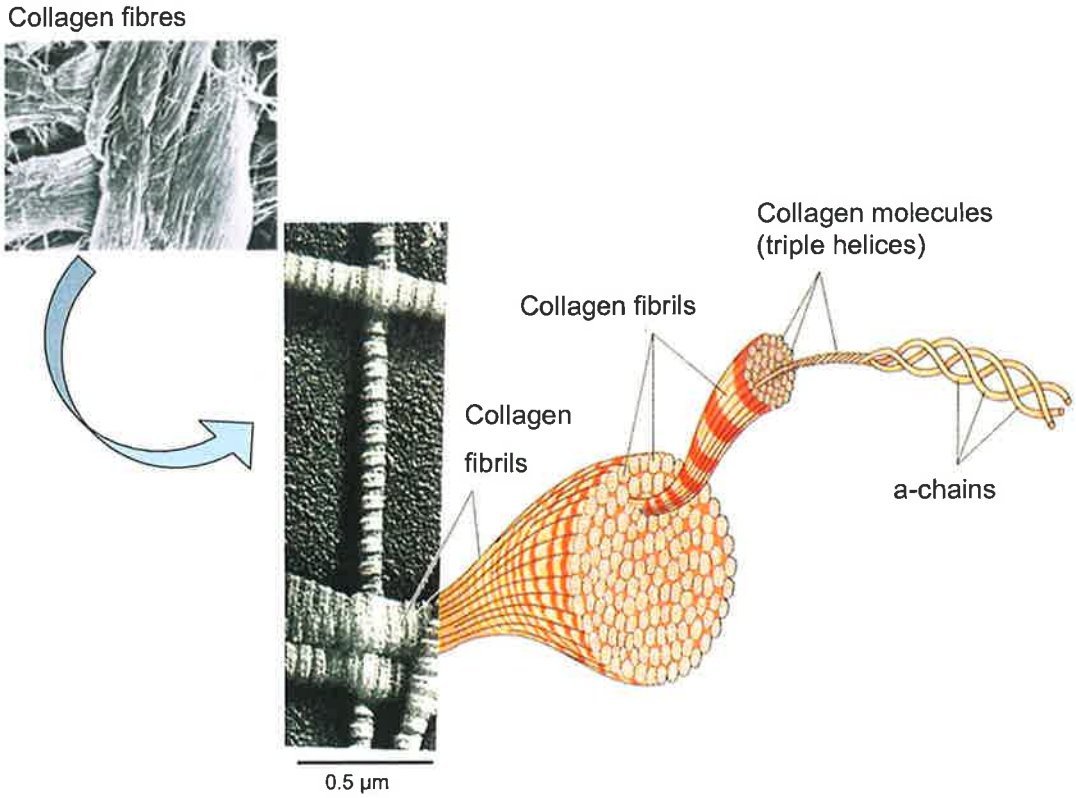

Fig 1.4 Composition of collagen- [Online Image] illustrating striations formed by staggered structural alignment. Adapted from http://www.bio.miami.edu/~cmallery/150/physiol/40x2collagen.gif [accessed 26 March 2007].

1.3.4 Osteogenesis and bone formation markers

During normal bone formation many specific proteins are expressed which are essential for maintaining growth and function of bone. These proteins may exist in various forms; cell surface bound, matrix bound or in the surrounding pericellular fluid. Some of these markers are expressed in a staged temporal manner allowing us to identify early, mid and late stage osteoblast maturity; the identification of which is known as osteogenesis. These markers can be expressed during different phases of osteogenesis although the exact timing and level of their expression may have cellular variability (Hayami *et al.*, 2006). Figure 1.5 below, illustrates the phases over time in rat

osteoblasts exposed to osteogenic conditions.

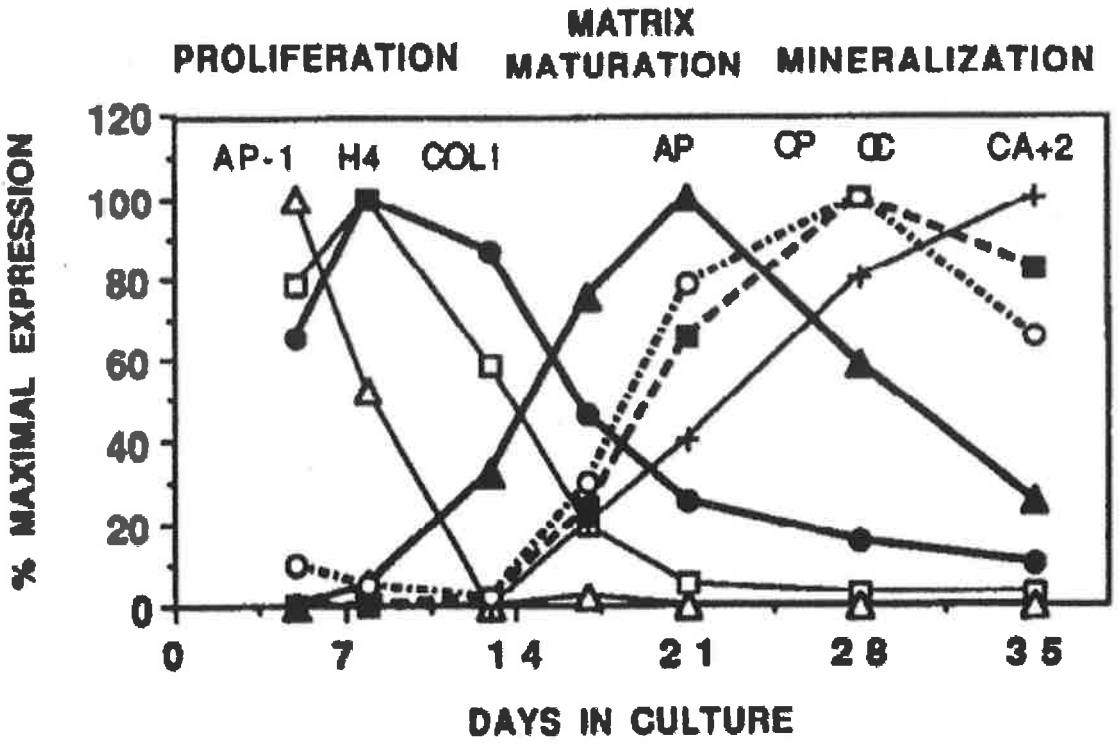

Fig 1.5 Expression of bone formation markers over time. AP-1, H4 and COL1 are measures of proliferation. AP-1 represents c-fos and c-jun which are cell growth regulated genes. H4 histone is a cell cycle gene which reflects DNA synthesis. COL1 is type 1 collagen. AP (alkaline phosphatase) is expressed during matrix maturation. OPN (osteopontin), OCN (osteocalcin) and Ca2+ are expressed during mineralisation (Stein and Lian, 1993).

Once proliferation concludes cells can begin to differentiate when stimulated appropriately. For bone early stage osteogenesis can be identified by looking at osteogenic transcription markers like Cbfa1/RUNX2 (a cytosolic transcription factor for osteocalcin), which has been shown to increase in differentiating MSC cultures up to about 12 days (Montjovent *et al.*, 2004). More commonly expression levels of osteogenic proteins are examined. These may be membrane bound, part of the ECM or secreted into the circulatory system. One can examine the up regulation of receptors on a cells

membrane such as vitamin D_3 receptor (VDR) and parathoiroid hormone receptor (PTHR); however, the most common early bone formation membrane bound protein is alkaline phosphatase (ALP). ALP is a membrane bound phosphoprotein which releases phosphates prior to mineralisation. Rat osteoblasts have been shown to begin to express ALP postproliferatively with a gradual rise until day 21 (Stein and Lian, 1993). In 2D studies expression levels of ALP tend to peak with the onset of osteogenesis and decline once mid to late stage osteogenesis begins (Donahue *et al.*, 2000). Another common early stage marker is collagen type 1 where the carboxyl terminal (CTX) and amino terminal (P1PP) of the protein are typically detected in serum and are routinely used during osteoporotic analysis.

Osteonectin (On) is a 32kD phosphoprotein which unlike many other bone markers is bone specific. It is secreted by osteoblasts where it binds to surrounding HA and collagen. Its regulation is less temporal but may be considered a mid stage marker of bone formation as it is responsible for binding collagen and HA. On is an interesting marker to study during osteogenesis as it is a bone specific marker (Shiba *et al.*, 1998).

Bone sialo-protein (BSP), a phosphor protein which tends to be expressed in a bimodal fashion; expressed during active proliferation, followed by a decrease and is re - induced at late stage differentiation where it peaks (Kartsogiannis and Ng, 2004). The function of BSP is not fully known; however, it appears to be involved with calcium binding and nucleation; therefore is important in mid to late stage osteogenesis. Osteopontin (Op) is a non-collagenous protein found in bone and plasma serum as well as many other tissues. It has roles in ECM adhesion binding to fibronectin. (Bilezikian, 2001) much of its binding ability may be due to being highly negatively charged (it contains a run of 10-12 aspartic acids). Although linked with calcification of urinary stones its role is yet unknown. OP expression typically occurs in a bimodal fashion

where expression is at a low level initially* during cell proliferation and then peaks as mineralisation proceeds. Op is not bone specific, it affects many cells such as fibroblasts. Therefore the role of Op although not fully defined appears to be involved with cellular adhesion to surrounding ECM. This is further supported by the fact it contains Arg-Gly-Asp sequences in its structure which aid cell attachment.

Osteocalcin (Oc) is a small highly specific osteoblastic calcium binding protein; Oc also known as bone GLA protein (gamma-carboxyglutamic acid) is a small (6kD) protein that makes up 10-20% of all non collagenous proteins in bone matrix (Watrous and Andrews, 1989). Oc is an indicator of ossification and therefore is a late stage marker for osteogenesis; however, it may also retard mineralisation if prolonged high levels present (Mundy, 1995). Thus Oc plays a role in preventing excessive mineralization as well as initiating it. This vitamin K dependent protein[1] is detected in blood serum as well as bone matrix bound to HA. Oc has been shown to be synthesised by osteoblasts when incubated with 1,25-dihydroxyvitamin D_3 (Mundy, 1995). Levels of Oc in rat osteoblasts were found to increase up to 28 days of culture with expression being detected as early as at 5 days culture (Stein and Lian, 1993). Yoshikawa *et al.* (1998) found that osteoblasts cultured on HA could yield similar levels of Oc to cancellous bone at 8 weeks of culture.

There are several other bone formation markers that may also be chosen to identify osteogenesis, many of which are matrix proteoglycans like decorin and biglycan, important in maintaining bone ECM. Biglycan and decorin tend to be located pericellularly and are thought to regulate osteoblast response to TGF.

As well as identifying the range of osteogenic markers it is also useful to test for markers of similar lineages particularly because MSCs can differentiate into several cell

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* Approximately 25% of maximal expression during this stage

[1] Vitamin K is required to synthesise glutamic acid

types and are capable of transdifferentiation between phenotypic groups. The embryonic stem cell marker Oct-4 is downregulated once stem cells begin to differentiate. As MSCs may differentiate into chondroblasts, adipocytes as well as osteoblasts/osteocytes it can be beneficial to analyse these markers during osteogenesis. Collagen type II, X and aggrecan are typical markers for chondrogenesis; lipo poly lipase (LPL), peroxisome proliferator-activated receptor-Y2 (PPARY2) and leptin are markers for adipogenesis and E11 and matrix extracellular phosphoprotein (MEPE) for osteocytes.

1.3.5 Bone formation and growth

There are two types of bone formation; endochondral ossification and intramembranous ossification (Fig. 1.6). Endochondral ossification occurs where bone replaces preformed cartilage. Specifically, chondrocytes near the centre of shaft increase in size. As they expand the lacunae get bigger and the struts begin to calcify. Blood vessels grow into the perichondrium. Cells at the perichondrium differentiate into osteoblasts which calcify the shaft of cartilage. The perichondrium becomes periosteum. The blood supply increases; cartilagenous matrix breaks down, bone spicules form at primary ossification site (in the diaphysis) spreading to both epiphyses (there is no marrow cavity yet, just spongy bone). The bone enlarges; osteoclasts erode trabeculae at the centre of the diaphysis forming a marrow cavity. Capillaries and osteoblasts migrate to the epiphysis and a secondary site of calcification occurs. The epiphysis ossifies and becomes separated from the diaphysis by an epiphysial plate (cartilage plate in the metaphysis) (Bilezikian, 2001).

During intramembraneous ossification, bone develops directly from mesenchyme or fibrous connective tissue. Bones formed by this method are known as dermal bones and include the flat bones of the skull, the mandible and the pectoral girdle. Here,

undifferentiated stem cells form clusters which lay down collagen and osteoid matrix as the cells undergo osteogenesis; calcification then proceeds with the development of bony struts or spicules. Osteoblasts that become embedded in the matrix become osteocytes. Blood vessels also become trapped between fusing spicules (Soloman, 2005).

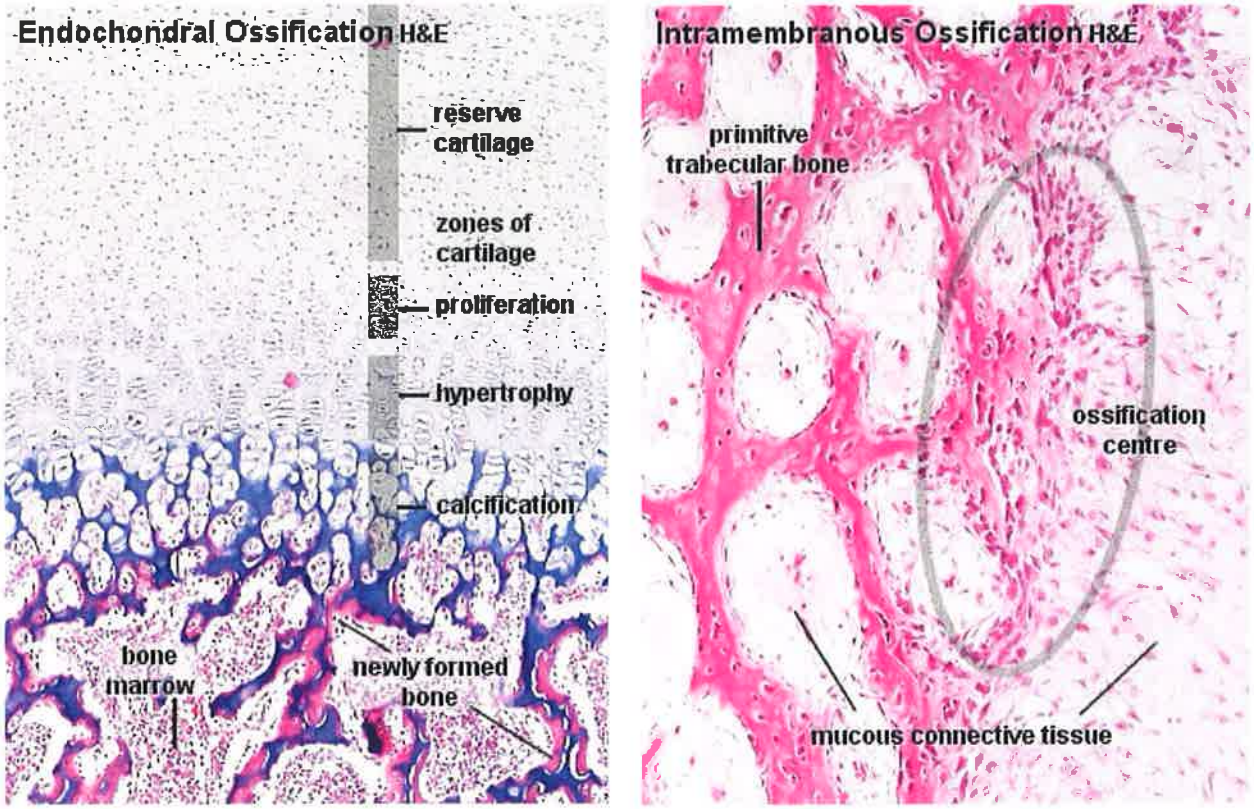

Fig 1.6 Histological analysis of endochondral and intramembranous ossification; online images were obtained from http://www.lab.anhb.uwa.edu.au/mb140/CorePages/Bone/Bone.htm [accessed 04 July 2010]

1.3.6 Bone remodelling

Bone is continually being eroded and deposited through a process known as bone turnover. It is estimated that osteoblasts and osteoclasts can shape and replace the entire skeleton every 10 years. The human skeleton accumulates bone up to the age of thirty but continues to remodel itself throughout ones lifetime in response to chemical, physical and mechanical stresses. Remodelling in vivo takes place in discrete regions

throughout the skeleton taking about three to four months to complete (Mundy, 1995). Normally bone remodelling occurs in cycles where osteoclasts adhere to bone matrix. Here they digest the surrounding bone matrix using proteolytic enzymes and acidification. The result of which is an absorption site which can be invaded by osteoblasts whose role it is to lay down matrix in the form of osteoid and initiate mineralisation to form osseous tissue. In cases where the osteoclast resorption exceeds osteoblast formation a person increases their risk of developing osteoporosis; a bone disease characterised by enhanced skeletal fragility (Fig. 1.7).

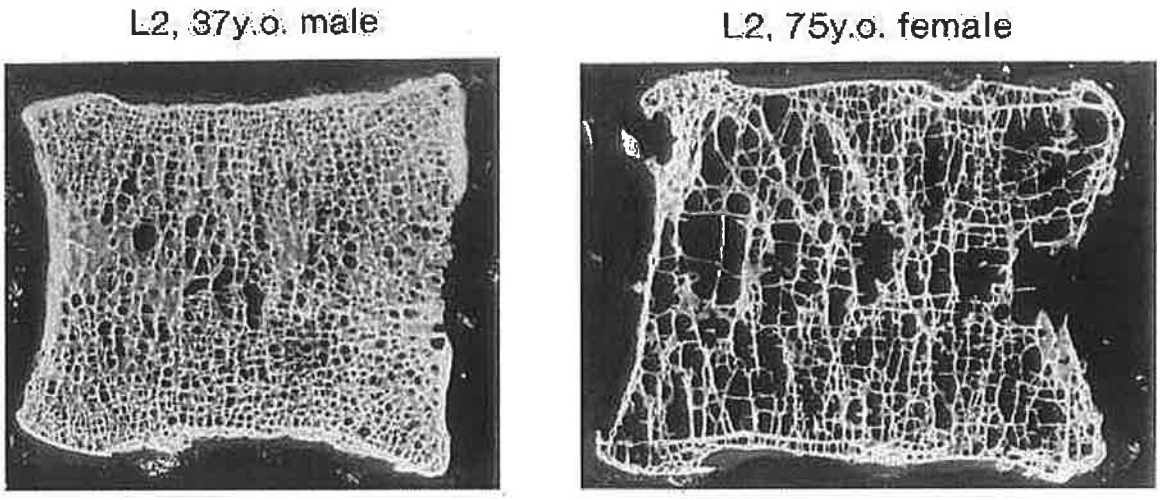

Fig 1.7 Bone loss in osteoporosis; lumbar vertebrae section from a 37 year old healthy man on the left hand side versus a lumbar vertebrae section from a 75 year old woman with osteoporosis (Chiras, 2002).

1.3.7 Bone damage and repair mechanisms

Bone is highly versatile; it has a high level of self regeneration following fractures. Bone fractures can vary on their severity and duration to heal. Healing begins following a fracture with the formation of a blood clot (fracture hematoma) at the region of injury. This region becomes infiltrated with fibroblasts whose function is to secrete collagen. At this stage the clot becomes a callus which contains activated periosteal/endosteal stem cells which differentiate via chondrogenesis and osteogenesis (Soloman, 2005). At the

centre of the external callus hyaline cartilage is formed; at the edges of the external callus osteoblasts lay down osteoid along the collagen struts and mineralise creating a bridge between the fracture. Osteoblasts continue to mineralise the region replacing the cartilage with trabecular bone. The mineralised callus and dead bone fragments become remodelled by osteoclasts returning the bone region to its original state prior to injury (Fig 1.8).

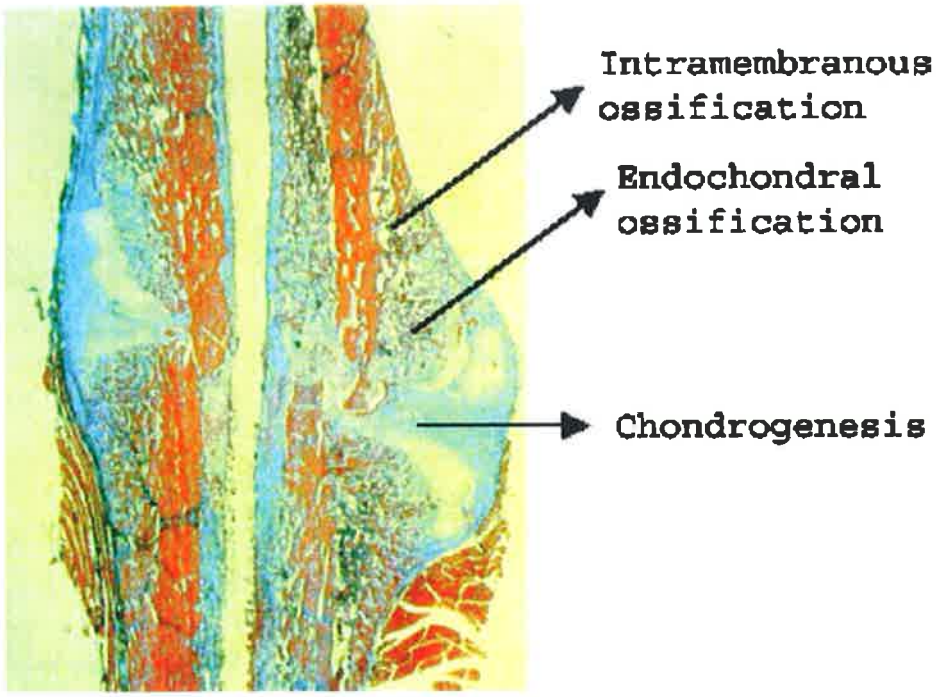

Fig. 1.8 Bone healing and callus formation 9 days post fracture showing mature bone and the cartilaginous callus (Doblare and Garcia, 2003).

1.4 Bone grafts

The main reason that bones may not heal naturally is that the regenerative cells do not receive the stimulatory signalling molecules required from their environment. This may be due the presence of molecular inhibitors or more often due to spatial issues. Bridging gaps is essential to provide cell support, migration, differentiation and maturation. A critical size defect is one which the bone cannot heal as the gap is too large and requires assisted bone healing in the form of a graft. The critical size for a

canine mandible has been reported to be 15mm (Huh *et al.*, 2005). In such cases a bone graft is necessary to aid new bone formation and healing. There are three main types of bone grafts; autograft, allograft and alternative bone graft substitutes.

1.4.1 Autografts

Autografting is the most common and most favoured bone graft. It represents almost 60% of current interventions (Giannoudis *et al.*, 2005). Autografting involves harvesting tissue from three regions in the body; the tibia (cortical grafts), fibula (whole bone transplants) or the iliac crest (superior part of the ileum in the pelvis- cancellous bone) of the patient's body and transplanting this to the injury site. It is the favoured therapy as there are fewer problems regarding tissue rejection and often contains living red bone marrow to aid regeneration. However, disadvantages include: the expense (harvesting an iliac crest is estimated to cost between $1,000 and $9,000 alone), scarce amounts of tissue, inflammation, blood loss, infection, chronic pain as well as requiring two surgical procedures.

1.4.2 Allografts

Allografts on the other hand use tissue from a cadaver and are currently responsible for about 30% of bone grafts. Allografts overcome some of the problems associated with autografts however, there are drawbacks primarily regarding the lack of donors, immunogenicity and a risk of infections like human immunodeficiency virus (HIV) and *Clostridium sordellii* (a gram-positive toxin producing anaerobic bacterium) (Malinin *et al.*, 2003).

1.4.3 Alternative grafts/ bone graft substitutes

Bone graft substitutes aim to provide alternative grafts to combat the limitations of the traditional autograft and allograft methods. They may consist of cells, biomaterials and growth factors, alone or in some varied combination.

Cell-based approaches have originated from the knowledge that stem cells can differentiate into different tissue types like bone and cartilage. They function on the basis of implanting stem cells from bone marrow directly into defect. These methods are limited due to expense, scarcity of tissue which fail to provide support to surrounding tissue within the defect and little success bridging large critical sized defects.

There are a variety of biomaterials used as scaffold supports for a given bone graft substitute. These include ceramics, polymers and metals. Each biomaterial has distinct advantages and disadvantages typically pertaining to osteoconduction[2] and osteoinduction[3], these will be further discussed in section 1.5.

1.5 Biomaterials for bone tissue engineering

A biomaterial was first defined in 1976 by the Consensus Conference of the European Society for Biomaterials as a "material intended to interface with biological systems to evaluate, treat, augment or replace any tissue, organ or function of the body".

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[2] Osteoconductivity refers to the ability of a biomaterial to provide an interconnected structure for cell attachment and migration; Carlisle & Fischgrund (2005) define it as the process that supports the in-growth of cells into a 3-dimensional graft.

[3] Osteoinductivity refers to biomaterials ability to induce cellular differentiation to a mature phenotype

1.5.1 Properties of scaffolds for tissue engineering

According to Lyons *et al.* (2008) scaffolds for tissue engineering applications are designed to perform the following functions: (1) to encourage cell-material interactions *i.e.* cell attachment, differentiation and proliferation, eventually leading to the deposition of extracellular matrix, (2) to permit the transport of nutrients, wastes and biological signalling factors to allow for cell survival, (3) to biodegrade at a controllable rate which approximates the rate of natural tissue regeneration, and (4) to provoke a minimal immune and/or inflammatory response *in vivo*.

There are five paramaters which need to be considered when designing a scaffold for tissue engineering:

1.5.1.1 Biocompatibility

Biocompatibility refers to a scaffolds ability to be functional without eliciting a harmful immune or inflammatory reaction by the host. If the scaffold is non-toxic and degradable, new tissue will eventually replace it; if it is non-toxic and biologically active then the scaffold will integrate with the surrounding tissue. However, if the scaffold is biologically inactive, it may be encapsulated by a fibrous capsule and in the worst case scenario if the scaffold is toxic, rejection of the scaffold and localised death of the surrounding tissue can occur.

1.5.1.2 Biodegradability

The ability of a scaffold to be broken down by the body is an important property when developing a bone graft. During biomaterial design, scaffolds are intended to be completely replaced by the regenerated extracellular matrix thus becoming completely integrated with the surrounding tissue. Ideally, scaffolds should degrade with a controllable degradation rate, (approximating the rate of natural tissue regeneration), as well as with controllable degradation products. Degraded products should be non-

toxic and easily extracted from the body via metabolism or excretion.

1.5.1.3 Scaffold Architecture

Scaffolds with a higher porosity allow for optimal interaction of the scaffold with cells. A scaffold with an open and interconnected pore network, and a high degree of porosity (>90 %) is ideal for the scaffold to interact and integrate with the host tissue (Freyman *et al.*, 2001a).

1.5.1.4 Manufacturing technology

Scaffold fabrication should be commercially viable and cost effective easily stored and transported. They should cater for the end user i.e. the clinician, who generally prefers "off the shelf" products that may be used routinely. The most common methods of manufacturing scaffolds and are summarised below (Freyman *et al.*, 2001a):

a) Fibre bonding involves immersing non-bonded fibres in a non-solvent solution. The solvent is evaporated leaving some interconnections at their junctions. Once heated to above melting point the bonds are formed. The non-solvent solution can be eluted to give a porous scaffold.

b) Foaming uses CO_2 dissolved in a polymer under high pressure (800 psi @ 25°C). Scaffolds produced by this method are quite porous (93%) and have pore sizes of ~ 100 μm.

c) Salt leaching produces a product similar to foaming. A powder polymer like PLGA (poly DL lactic co-glycolic acid) is mixed with NaCl and a solvent such as chloroform. The solvent is evaporated and heated similar to fibre bonding. Once cooled the material is immersed in water to leach out the salt. Although Laurencin *et al.* (2006) successfully used this method to fabricate a poly DL lactic co-glycolic acid hydroxyapatite (PLGA/HA) composite matrix generally the structure is not very reliable and produces a porosity of between 20 and 93% and a limited pore size range of 30 to 120 μm.

d) Three dimensional printing is similar to salt leaching; polymer powder like PLA (polyglycolic acid) is mixed with NaCl, a thin layer of the mixture is then spread onto a powder bed. Using a printhead nozzle to deposit chloroform binding occurs producing the polymer. The step can be repeated to obtain the required size. Once dried the matrix is immersed in water to leach out the salt. Porosity and pore size of 95% and 100μm respectively are obtained with this technique.

e) Lyophilization involves freezing a slurry precipitate under a vacuum. The change in pressure causes ice to sublime producing a highly porous solid. The rate of cooling can control the size and volume fraction of the pores. CG scaffolds produced by this technique gave porosities of 90 to 99% and a pore size range of 100-350μm. In our laboratory we use this method when fabricating CG scaffolds.

1.5.1.5 Mechanical properties

The mechanical properties of the scaffold should be designed to meet the specific requirements of the tissue to be regenerated at the defect site and should be easy to handle during implantation. Immediately after implantation, the scaffold should provide a minimal level of biomechanical function that should progressively improve until normal tissue function has been restored, at which point the construct should have fully integrated with the surrounding host tissue (Partap *et al.*, 2010). One method of improving the mechanical properties of collagen based materials is to introduce cross linking between fibrils.

1.5.2 Biomaterials for bone tissue engineering

There are several different biomaterials presently in use and may be categorised as:

1.5.2.1 Synthetic polymers

Synthetic polymers may be fabricated into fibrous meshes, porous sponges, foams or

hydrogels. They are extremely versatile and can be easily moulded into the shape of the tissue or organ (Stocum, 1998). The biodegradable polycaprolactone family of poly (α-hydroxy acids) polymers like PGA and PLGA (Fig. 1.9) are easily broken down and removed by the body following implantation (Freyman *et al.*, 2001a). Non-biodegradable polymers like polymethyl methacrylate (PMMA), polytetrafluoroethylene (PFTE) and PHEMA (poly(2-hydroxyethyl methacrylate)) can be used as bone cements and have received FDA approval for applications in joint replacement. Disadvantages for bone TE however, include poor cell osteoinductivity and structural strength. Synthetic polymers tend to be acidic in nature which yields them with a high degradation rate, as they are not natural materials they can also elicit an immune response.

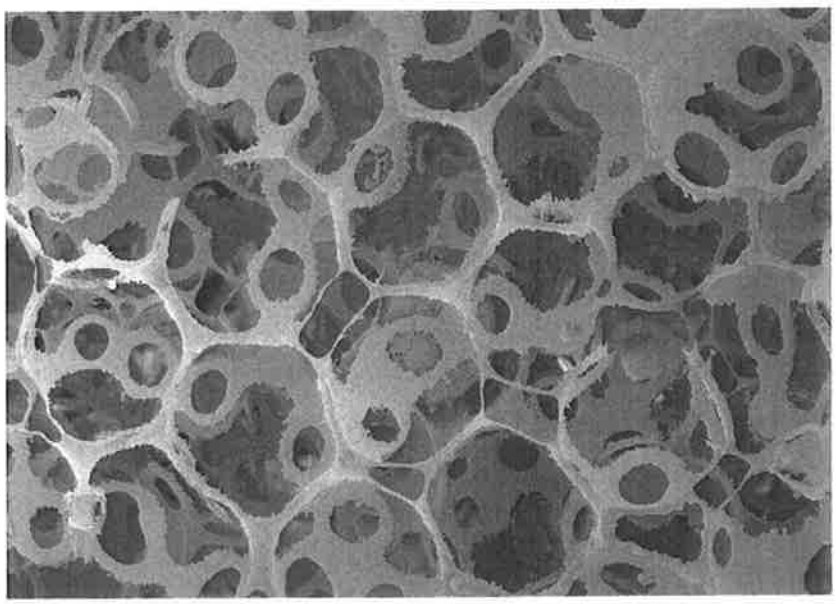

Fig. 1.9 Polymeric scaffold PLGA foams prepared with paraffin spheres with a size range of 420–500 μm (x50 mag) (Liu and Ma, 2004).

1.5.2.2 Ceramics

Ceramic scaffolds are mechanically quite stiff, they tend to comprise of calcium phosphate/sulphates based compounds of which HA and tri calcium phosphate (TCP) are most common (Fig. 1.10). TCP is used as a degradable scaffold, whilst HA, which is non-resorbable and has the added advantage of being osteoinductive, is typically used

for coating biomedical implants to induce bone regeneration, allowing the implant to integrate with the surrounding tissue. For this reason, HA has shown much popularity for use as a scaffold for tissue engineering.

Calcium phosphate cement (CPC) and calcium sulphate (plaster of Paris) are fast setting ceramics with good osteoconductivity but are brittle in nature. They may be reabsorbed after 30-60 days and are resistant to compressive forces but not shear or tension (Laurencin *et al.*, 2006). Since the resorption rate is faster than calcium phosphate, Barrack (2005) considers that calcium sulphate may therefore be also used as a carrier for growth factor or drugs. Bioactive glasses are hard, non-porous materials made of calcium, phosphorous and silicondioxide. They have greater mechanical strength than calcium phosphate ceramics and can resist drilling and shaping; however, they are prone to fractures and may be difficult to fix to the body (Giannoudis *et al.*, 2005). The addition of polymethylmethacrylate to bioactive glass forms bioactive cement which improves mechanical properties.

Used alone ceramics appear to have good osteoconductivity but are too brittle to use as an optimal substitute alone. Commercial examples include OsteoGraf, Norian SRS, ProOsteon, Osteoset (Laurencin *et al.*, 1996). Their applications however are limited due to their brittle nature, may shear easily, be prone to fractures and/or may be difficult to fix to the body (Giannoudis *et al.*, 2005).

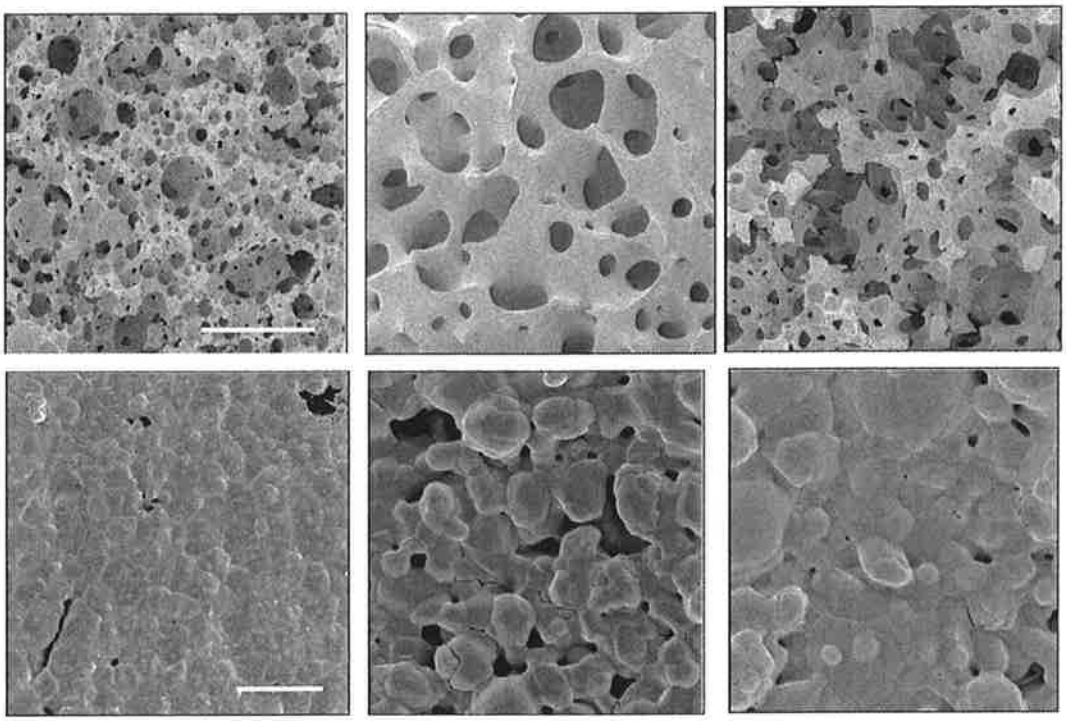

Fig. 1.10 Scanning electron micrographs of some typical ceramic bone graft substitutes. (top) Low power of scaffold macrostructures, 1mm scale bar and (bottom) high power of microstructures and surface topographies, 10 μm scale bar (Hing, 2004).

1.5.2.3 Native polymers

Native polymers include collagen, chitosan, gelatin, silk fibrin, glycosaminoglycans and elastin (Dawson and Oreffo, 2008). They typically exhibit excellent biocompatibility as they are sourced from the extra cellular matrix (ECM) of many tissues and therefore contain many surface ligands and peptides appropriate for cellular attachment and cell-scaffold interactions (Harley *et al.*, 2008). Collagen has been used for skin regeneration and gelatin (denatured collagen) has been examined for cartilage regeneration (Wang *et al.*, 2009). The major limitation of using native polymers in tissue regeneration is they have are low in mechanical strength.

1.6 Collagen glycosaminoglycan scaffolds

CG has been successfully used to regenerate skin for burn victims (Freyman *et al.*, 2001a; Yannas, 2001). Originally developed at the Massachusetts Institute of Technology, this scaffold is fabricated using a blend of bovine collagen and shark chondroitin-6 sulphate which is freeze dried. This produces a highly porous compliant material suitable for organ regeneration (O' Brien *et al.*, 2004) (Fig. 1.11).

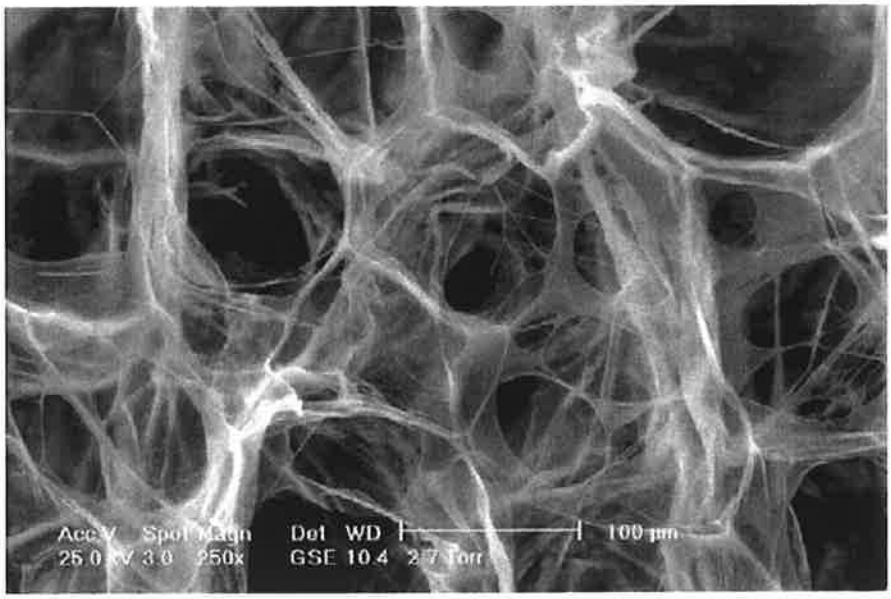

Fig. 1.11 Scanning electron microscopy of the highly porous CG scaffold (O'Brien et al., 2004).

This CG scaffold successfully received FDA approval in 1996 for use as a dermal replacement in burns victims (Fig. 1.12). The dermal treatment was an acellular implant which became infiltrated with surrounding epidermal cells. These cells form a mature epidermis and basement membrane via sequential regeneration (Harley *et al.*, 2008). This produces a fully functional graft that fully replaces the damaged defect.

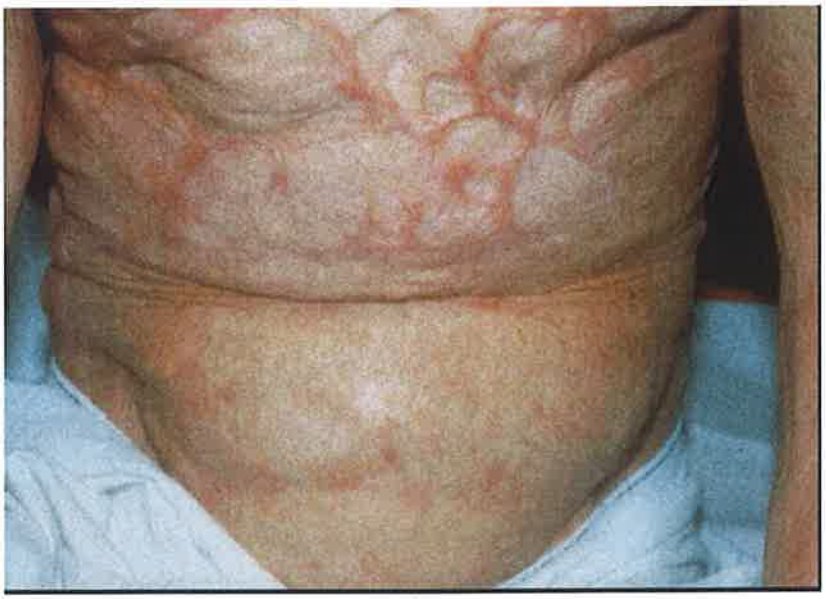

Fig 1.12 Lower abdomen of burn patient treated with CG scaffold, the thorax remained untreated (Freyman *et al.*, 2001a)

CG scaffolds have also made successful advances in the area of peripheral nerve regeneration. NeuraGen® Nerve Guide (Integra products) is a tubular scaffold that allows nerve growth by guided tissue repair. It facilitates the nerve during axon growth across a nerve gap*. The CG based scaffold is fabricated to give hetrogenuous pores. These pores are axially orientated between the distal and proximal ends, which act as a directional guide for peripheral nerve growth. NeuraGen® Nerve Guide has been described to be at the level of the current gold standard (an auto grafted nerve) (Chamberlain *et al.*, 1998a).

1.6.1 CG scaffold fabrication

CG scaffolds are fabricated using a lyophilisation technique (O' Brien *et al.*, 2005). Briefly, CG slurry is prepared by blending bovine collagen type-1, in a chondroitin-6

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* Peripheral nerve regeneration will not proceed naturally if the gap is greater than a few millimetres (Harley *et al.*, 2008)

sulfate acetic acid solution. After blending, the slurry is degassed prior to lyophilisation. The slurry is freeze dried at a cooling rate of 0.9°C/min and a final freezing temperature of -40°C, 50mTorr for 24 hours. As the lyophilisation process commences the slurry begins to freeze, forming ice crystals which surround the CG solution. These ice crystals sublimate leaving a highly porous sponge. By altering the final freezing temperature of the process it is possible to fabricate a range of various sized pores as previously reported (O' Brien *et al.*, 2004) (Fig 1.13).

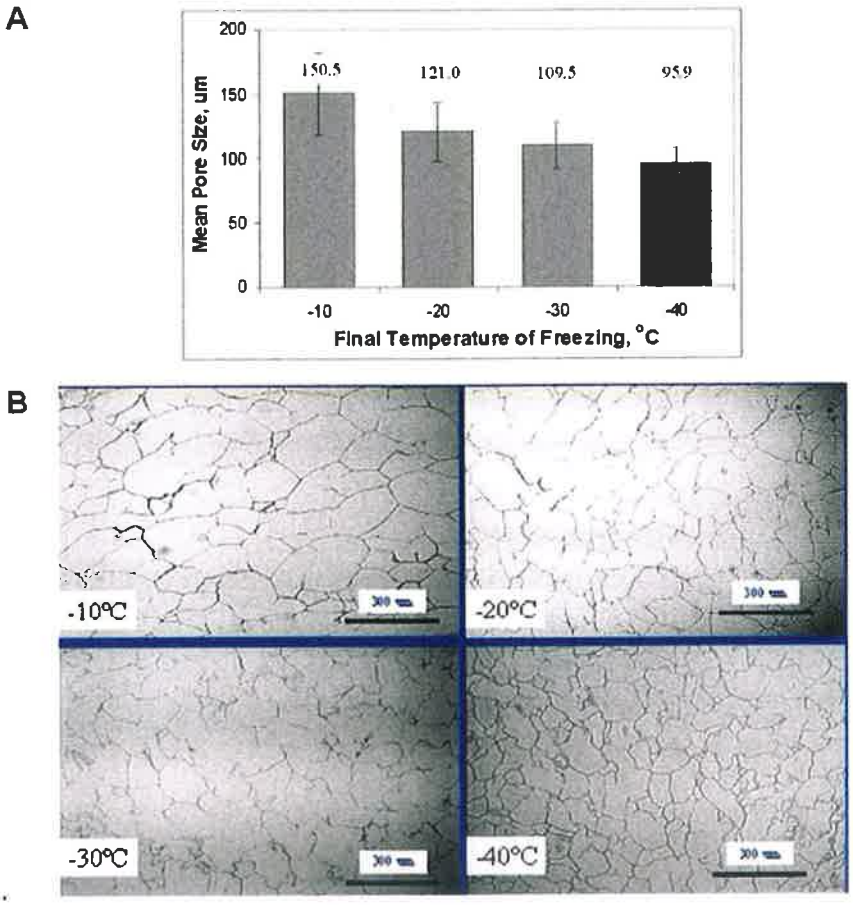

Figure 1.13 Effect of freezing temperature on the mean pore size of a CG scaffold. (a) Illustrates the mean pore sizes ± CV (b) shows the microstructure of the different pore sizes as imaged using 5 μm thick histological samples. Scale bar = 300μm (O' Brien *et al.*, 2005).

1.6.2 CG scaffold porosity

The CG scaffold contains a porosity of 99%. High porosity has been shown to effect cell attachment & growth (O' Brien *et al.*, 2005; O' Brien *et al.*, 2007; Byrne *et al.*, 2008;

Murphy *et al.*, 2010). Small pore sizes (<30μm) limit diffusion rates of nutrients and waste removal and may result in the development of a necrotic core region in the biomaterial. Large pores can limit the surface area required for cellular attachment.

The CG scaffold is highly permeable with a mean value of 10-10m^4/Ns (Harley *et al.*, 2007). Permeability is important as it allows for cell migration and diffusion to occur throughout the construct. High permeability makes it easier for vascularisation to proceed into the scaffold. Vascularisation is vital to allow the graft to become fully integrated with the host bone tissue.

1.6.3 CG scaffold contraction

An important property of CG scaffolds is their ability to contract during *in vitro* culture (Menard *et al.*, 2000; Schulz Torres *et al.*, 2000; Freyman *et al.*, 2001b; Spilker *et al.*, 2001). Schultz Torres (2000) examined CG seeded with fibroblasts and the percentage of contraction up to 21 days of *in vitro* culture. Results showed CG scaffolds identical to those used for skin regeneration contracted by a total of 60% after 21 days.

It is still unclear why typically non-contractile cells contract; however, it is thought that fibroblasts can contract becoming myofibroblasts (fibroblasts which assume a contractile phenotype and express smooth muscle actin) during wound healing and defect closure. Contraction is often clinically viewed as a potential limitation of a scaffold's properties, linked with structural instability within a defect and has been shown to be potentially problematic when designing a repair graft of a particular size (Lee *et al.*, 2001). The action of biomaterial contraction can limit cellular nutrient and waste diffusion due to a reduction in pore size/porosity, potentially limiting cell number and viability.

On the other hand, *in vitro*, contraction has been linked with the production and maintenance of ECM and may benefit tissue formation (Menard *et al.*, 2000). As a result, these contractible CG scaffolds are mechanoactive and have been shown to be beneficial

for chondrogenesis and nerve wound healing but have not been assessed for long term osteogenesis (Lee *et al.*, 2001; Spilker *et al.*, 2001; Zaleskas *et al.* 2004). Contraction of CG scaffolds can be controlled using crosslinking methods; the effect of which on osteogenesis is examined in Chapter 4.

1.6.4 Crosslinking

Crosslinking is a process used to enhance the mechanical properties of biomaterials particularly collagen based ones (Weadock *et al.* 1983; Lee *et al.* 2001). Crosslinking strengthens substrates by introducing extra molecular bonds; in collagen for example this can increase stiffness by restricting collagen fibres from sliding over each other during stress. It can reduce substrate contraction which can limit cellular nutrient and waste diffusion due to a reduction in pore size/porosity. Engler *et al.* (2004) has shown that crosslinking can influence cellular differentiation, adhesion and proliferation within collagen gels. Different crosslinking methods exist and may be classified as being either physical (e.g. ultra violet light) or chemical treatments (e.g. transglutaminase); however, the following methods will be further discussed as they are applied specifically to this thesis:

Dehydrothermal (DHT) crosslinking

DHT is a physical cross-linking process which removes water molecules and creates cross-links by esterification and amide formation (Weadock *et al.*, 1983). The process involves heating freeze dried collagen scaffolds to +90°C under a vacuum. Any bound water molecules are removed and cross linking of the substrate material occurs through condensation reactions (either esterification or amide formation) (Yannas and Olson, 1972). DHT does not use cytotoxic reagents and results in a sterile product. Mechanically DHT can increase the compressive stiffness from 0.2 to 0.5kPA (Harley *et al.*, 2007). One limitation of DHT is its ability to denature collagen. DHT crosslinking density increases with temperature but not with treatment duration; however,

denaturation increases with exposure period (20-60%) (Haugh *et al.*, 2008). This allows a range of collagen based biomaterials to be fabricated varying in levels of denaturation and mechanical stiffness.

1-ethyl-3-(3-dimethyl aminopropyl)-urea (EDAC) crosslinking

EDAC is a carbodiimide that crosslinks adjacent collagen molecules. It acts as a catalyst in the presence of N-hydroxysuccinimide (NHS) and forms bonds between collagen fibres and glycosaminoglycans in CG scaffolds. EDAC reacts with lysine, arginine, aspartic acid, glutamic acid, serine and threonine of which there are 745 reactive residues (Weadock *et al.*, 1983) (Fig. 1.14). Upon reaction it is not incorporated itself into the collagen scaffold but is converted to 1-ethyl-3-(3-dimethyl aminopropyl)-urea. Increased substrate stiffness can also slow down the overall degradation of a material thus improving the structural integrity of the scaffold.

EDAC has been shown to crosslink between the glycosaminoglycan, chondroitin-6 sulphate, and collagen not just collagen to collagen (Pieper *et al.*, 1999) and is therefore an ideal candidate for crosslinking the CG scaffold. Previous studies have shown that EDAC can increase the compressive modulus of a CG scaffold to 1.497kPa a 7 fold increase (Harley *et al.*, 2007).

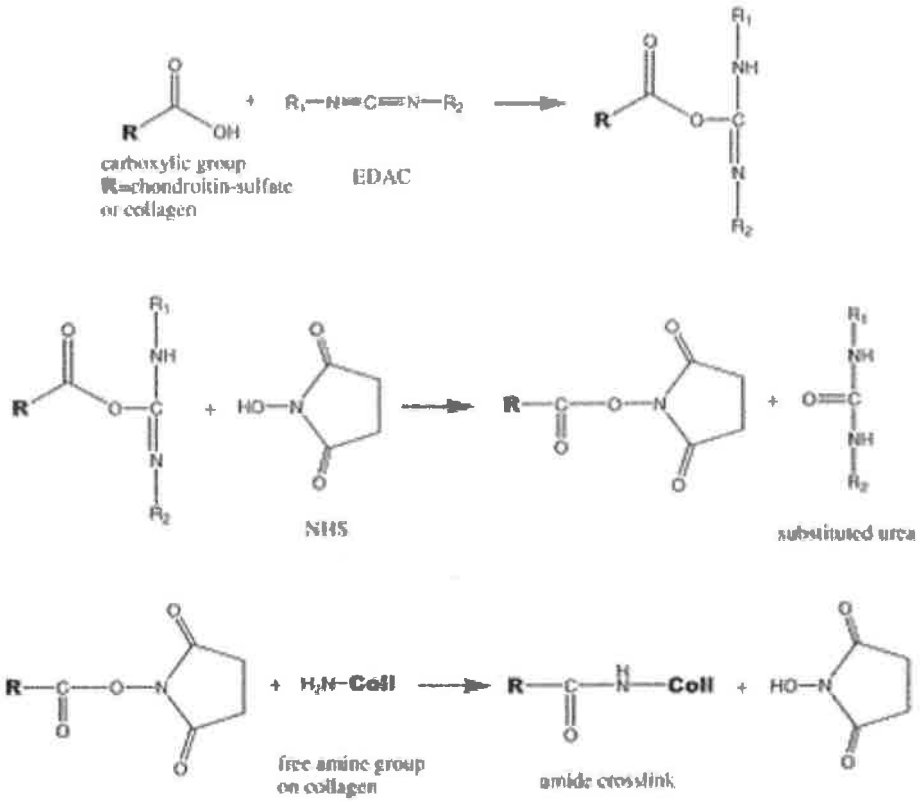

Fig 1.14 Proposed mechanisms for EDAC crosslinking collagen using the catalyst NHS. The reaction results in the production of a substituted urea (Lee *et al.*, 2001).

Glutaraldehyde (GLUT) crosslinking

GLUT is chemical crosslinker which has traditionally been used for reducing the antigenicity of bioprosthesis since the late 1960's and has been shown to be an effective cross-linker of collagen (Olde Damink *et al.*, 1996; Lynn *et al.*, 2004). Unlike EDAC it crosslinks collagen molecules separated by a distance (Zeeman *et al.* 1999); typically, by bridging amine groups between adjacent polypeptide chains of 53 reactive lysine residues (Weadock *et al.* 1983). GLUT may leave aldehyde linkages during the crosslinking process which have caused concern regarding cytotoxicity particularly during the degrading phase of a biomaterial/implant. How clinically relevant this level of cytotoxicity is open to debate as yet no adverse effects have been shown (Kikuchi *et al.,* 2004). GLUT based scaffolds have been shown to provide greater fibroblast infiltration and vascularisation (Wu *et al.*, 2004); however, residual GLUT may also

restrict cell growth and viability (Lee *et al.*, 2001) (Fig. 1.15). A consideration of coating an implant with GLUT is they have been shown that GLUT treated scaffolds have a tendency to cause calcification *in vivo* Jorge-Herrero *et al.* (1994); however, to date no long term cell based studies have been carried out to support these claims.

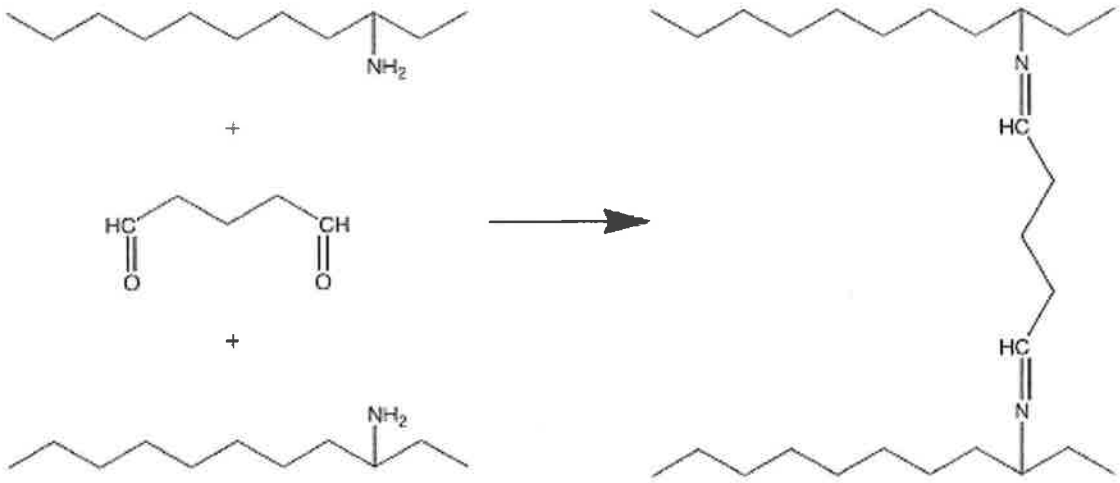

Fig. 1.15 Proposed mechanisms for GLUT crosslinking two collagen fibres (Lee *et al.*, 2001)

1.7 Biophysical stimulation

Other than chemical stimulation cells can be stimulated using biophysical cues. This is because cells can sense and respond to their external environment by applying traction stresses to matrix and then sensing mechanical responses at the cell-matrix interface (Discher, 2010). Examples of biophysical stimuli include; cyclical loading, substrate hydrophobicity, granularity, substrate stiffness, and pericellular fluid flow mechanics.

1.7.1 Cyclical loading, granularity and hydrophobicity

Cyclical loading is critical for bone maintenance and formation *in vivo* (Wan *et al.*, 2006). Changes in muscle mass which occur following mechanical stresses causes

muscle and bone to respond by thickening and strengthening (Srouji and Livne, 2005). Studies carried out examining the effects of continuous mechanical stretching on osteogenesis found that after 8, 16 and 24 hours there was an increase in the expression levels of growth factors (but not cytokines) attributed to the direct effect from physical strain (Cillo *et al.*, 2000). Granularity is important as studies have shown that smooth surfaces for example tend to increase osteoconduction whereas rough surfaces increase osteoinduction. Linez-Bataillion *et al.* (2002) studied MC3T3s grown on various polished surfaces and noticed that rough surfaces resulted in cells spending most of their time producing adhesion proteins and less time proliferating. Whereas smooth surfaces caused cells to spread out more increasing proliferation. Lim *et al.* (2008) showed that hydrophobic surfaces increased morphology cell spreading in hFOB cells grown on quartz (Fig. 1.16).

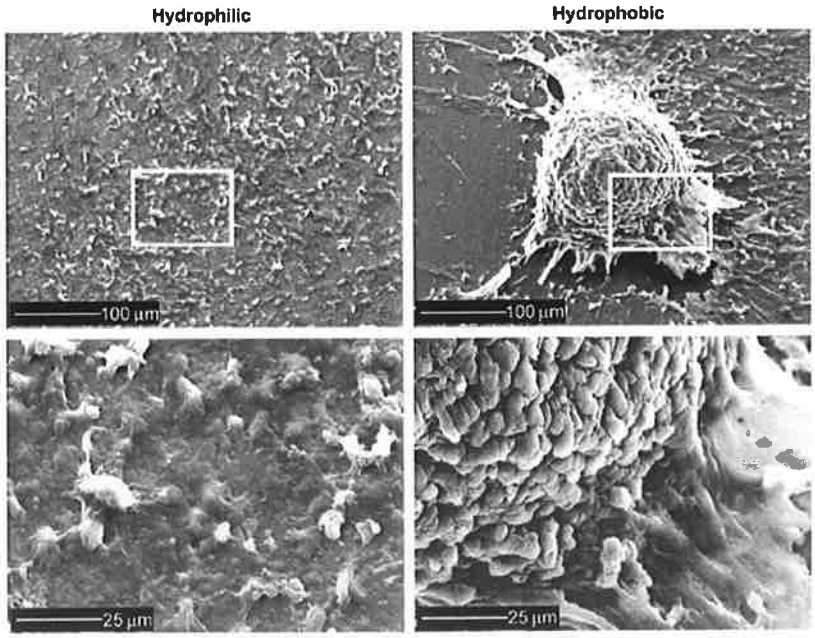

Fig 1.16 Scanning electron micrograph of hFOB cells cultured for 21 days on plasma treated quartz. Lower images show increased magnification from white rectangular spots in the upper images (Lim *et al.*, 2008).

1.7.2 Substrate stiffness

Substrate stiffness has been shown to affect cellular adhesion, proliferation, infiltration and phagocytotic cellular processes (Leipzig and Shoichet, 2009). Discher *et al.* (2005) demonstrated that substrate stiffness resulted in myoblast differentiation on a soft gel unlike cells grown on glass which failed to express myosin filaments (Fig. 1.17). In a recent MSC substrate differentiation study soft substrates were shown to differentiate down a neurogenic lineage whereas stiffer bone-like substrates (>34kPa) committed to osteogenic lineages (Pek *et al.,* 2010; Reilly and Engler 2010). Guo *et al.* (2006) also showed that cells respond to substrate stiffness; however, in this study it was demonstrate that if the physical signals from the substrate are stronger than those exerted by the cells then cells migrate away from each other resulting in greater surface spreading and cell proliferation. However, where substrates are softer than the cell-cell mechanical interactions they act more like *in vivo* tissue and aggregate into multicellular tissue-like structures which can lead to greater levels of differentiation.

1.7.3 Fluid flow

Fluid flow dynamics can effect cell growth and development. In bone for example osteocytes are typically exposed to bouts of pulsatile and oscillatory flow of pericellular fluid through the canaliculi as a result of movement. Several *in vitro* studies have been carried out to examine the effects of fluid flow on cellular proliferation and differentiation.

Several stress-induced mechanoregulatory pathways have been identified including extracellular signal-regulated kinases (ERK) pathway (PD98059 and U0126), the nitric oxide synthase pathway (N(omega)-nitro-L-arginine methyl ester), the cyclo-oxygenase pathway (indomethacin), and the Gi/o pathway (Kapur *et al.*, 2003).

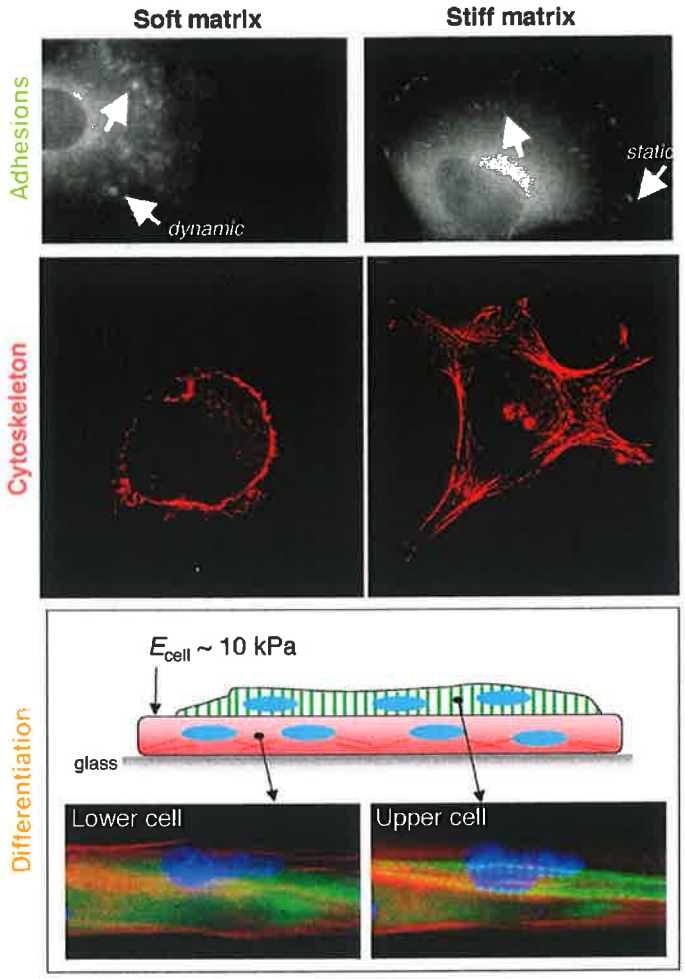

Fig. 1.17 Substrate stiffness influences adhesion structures and dynamics, cytoskeleton assembly and cell spreading, and differentiation processes such as striation of myotubes. (Top) The arrows point to dynamic adhesions on soft gels and static, focal adhesions on stiff gels. (Middle) The actin cytoskeleton. (Bottom) A cell-on-cell layering in which the lower layer is attached first to glass so that the upper layer, which fuses from myoblasts that are added later, perceives a soft, cellular substrate (Discher *et al.*, 2005).

Bioreactors can provide biomechanical stimulation using fluid flow dynamics and have been used to aid *in vitro* tissue formation of several organs including prostate, gut, heart and liver formation (Margolis *et al.*, 1999; Kim *et al.*, 2007; Yates *et al.*, 2007; Syedain and Tranquillo, 2009).

Several types of bioreactors exist including spinner flasks (Frith *et al.*, 2009), rotating wall vessels (Wang *et al.*, 2009), hydrostatic (Elder and Athanasiou, 2009), dynamic compression (Chowdhury *et al.*, 2008) and flow perfusion bioreactors (Jaasma

et al., 2008). They all aim to improve on traditional static culturing methods by providing improved mass transfer to enable efficient nutrient delivery and waste removal under controlled settings.

Static culture tends to rely on chemical factors for cell stimulation; bioreactors can provide mechanical coupled with chemical stimulation which can be beneficial for improving cell activity and maturation of cells in both 2D and 3D cultures (Owan *et al.*, 1997; Wiesmann *et al.*, 2004a; Zhu *et al.*, 2009). Mechanical stimulation has been shown to alter gene expression of the bone formation markers ALP, Op and Oc (Wiesmann *et al.*, 2004a). As a result bioreactors may be used to produce more functional constructs for tissue engineering applications.

1.7.4 Flow perfusion bioreactor

A flow perfusion bioreactor acts by forcing media through the interior of a cell seeded scaffold and has previously been shown to improve cell seeding and distribution within 3-dimensional biomaterials (Glowacki *et al.*, 1998; Goldstein *et al.*, 2001; Janssen *et al.*, 2006). They generally comprise of a perforated scaffold chamber to hold the construct linked with tubing to a media reservoir on one side and a pump on the other (Fig. 1.18).

The set-up is sterile as they are within a closed system by which gas exchange can pass across low permeable tubing. As flow perfusion bioreactors provide the best fluid flow throughout a construct compared to other bioreactors, they are an ideal choice to mechanically stimulate cells such as osteoblasts. Specifically for bone tissue engineering, the flow perfusion bioreactor has been shown to be beneficial for enhancing osteoblast growth, differentiation and mineralisation (Bancroft *et al.*, 2002; Goldstein *et al.*, 2001). Different versions of flow perfusion reactors exist for bone tissue engineering which are either specifically developed for stiffer scaffolds such as titanium mesh (Bancroft *et al.*,

2002) or for compliant scaffolds such as the CG scaffold (Jaasma *et al.*, 2008).

Recently we have looked at the use of a flow perfusion bioreactor and how fluid flow can influence osteoblastic activities for cells seeded within a CG scaffold (Jaasma *et al.*, 2008; Partap *et al.*, 2010). In this thesis Chapter 5 will further examine the use of the flow perfusion bioreactor.

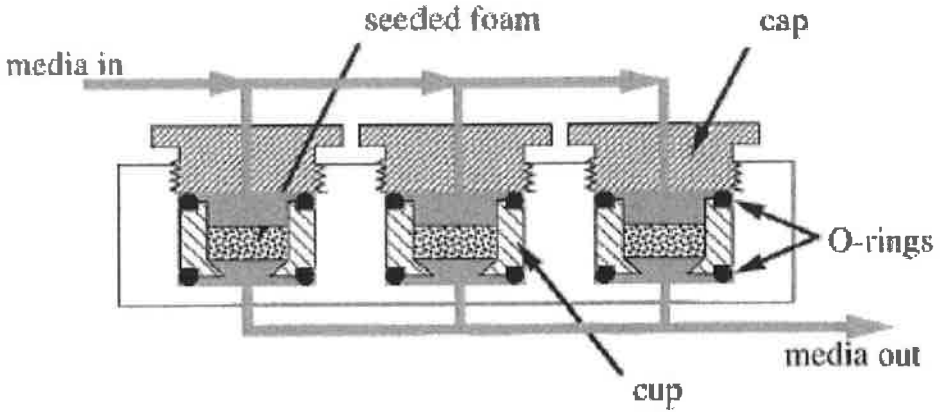

Fig. 1.18 Schismatic of a perfusion bioreactor (Goldstein *et al.*, 2001).

1.8 Biochemical stimulation: bioactive proteins and growth factors for bone tissue engineering

Bioactive proteins and growth factors have been used to induce growth and proliferation between cells *in vitro*. They act by binding to cell receptors resulting in a cascade of events which can induce the cellular processes of transcription, translation and protein synthesis. In doing so these chemical and biochemical stimulants may influence the cell state by altering stages of DNA synthesis and differentiation.

There are three requirements of *in* vitro culture studies that must be in place before osteogenesis and mineralisation can occur: the presence of collagen, a phosphate source and the absence of inhibitors (Allori *et al.*, 2008). In the first instance collagen is a basic requirement for mineralisation and is upregulated in osteoblasts by the addition of ascorbic acid (vitamin C). Exogenous phosphate is an *in vitro* requirement provided by the addition of beta glycerolphosphate. These two compounds are the basic formulation

for inducing osteogenesis; however, several compounds have been used alone or in combination to induce osteogenesis; below are some of the more common factors (Fig 1.19):

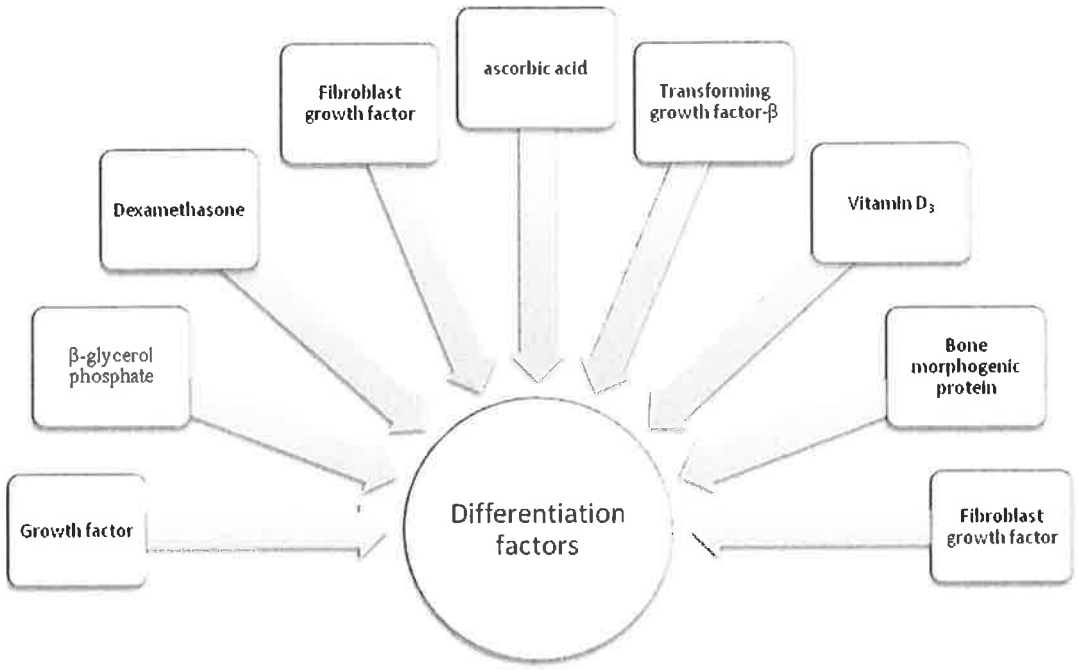

Fig. 1.19 Common differentiation factors involved with bone formation

1.8.1 Ascorbic acid & β-glycerophosphate

Ascorbic acid induces osteoblasts to produce collagen an essential prerequisite for mineralisation. Franceschi and Young (1990) reported that when using human MG-63 cells that ascorbic acid is an essential factor for differentiation of osteoblastic cells. In rat foetal parietal bones ascorbic acid deficiency prevented mineralisation (Ganta *et al.*, 1997). Ascorbic acid has been shown to be dose dependent (Takamizawa *et al.*, 2004) the standard concentration; however, is 50μg/ml. It acts as a cofactor for proline hydroxylase and lysine hydroxylase (both compounds which hydroxylate collagen).

β-glycerophosphate is an *in vitro* requirement for osteogenesis; it acts as a phosphate donor to aid mineralization (Laurencin *et al.*, 1996). Typically a 10mM solution of β-glycerophosphate is used during *in vitro* osteogenic culture.

1.8.2 Dexamethasone

Generally corticosteroids increase collagenase synthesis and inhibit collagen type 1 and Oc production. However, the glucocorticoid Dexamethasone (Dex) can induce osteoblast maturation and matrix production (Ramoshebi *et al.*, 2002). Dex has also been shown to induce osteoporosis when given *in vivo* at supraphysiological concentrations (Watrous and Andrews, 1989) and may therefore act as a growth inducer as well as an inhibitor depending on concentration, duration of exposure and cell state.

1.8.3 Fibroblast growth factor

Bone matrix contains heparin-binding fibroblastic growth factor (FGF) which is important for inducing mitosis. Previous studies had shown that by administering FGF systemically caused an increase in bone formation of animals; however, in vitro studies have demonstrated that FGF inhibits mineralization. It was hypothesised that this inconsistency must depend on the differentiation stage of osteoblasts and the length of exposure to FGF. It was concluded that FGF enhances osteogenesis by selectively expanding osteogenic cells as well as down-regulating the expression of inhibitors such as noggin (an antagonist of BMP-2).

1.8.4 Growth hormone

GH can stimulate bone formation; it is produced in the pituitary gland and circulates the body producing anabolic effects on cells. It has been shown to induce IGF production in osteoblasts which can induce synthesis of collagen and non collagenous proteins (Watrous and Andrews, 1989).

1.8.5 Cytokines

Cytokines are small bioactive protein communicators also called interleukins (IL). There are well over 200 documented cytokines with varying cell effects. Cytokines are generally autocrine and paracrine therefore they can cause an effect locally when produced. These molecules bind to specific cytokine receptors forming a ligand-receptor complex. This complex can in turn cause a cascade of effects inside the cell usually resulting in the up and/or down regulation of certain genes.

The most common cytokines important for bone growth and proliferation are IL-1, IL-6, IL-7, and IL-11. IL-1 is known for the activation, proliferation and differentiation of most cell types. Temporary IL-1 exposure can result in bone formation; however, prolonged effects appear to show inhibitory effects (Mundy, 1995). Interlukin-1 (IL-1) can stimulate bone formation by increasing DNA synthesis and osteoblast proliferation. It is described as and endogenous collagenase which blocks the activity of ascorbic acid which increases collagen matrix production (Hayami *et al.*, 2006). IL-1 may be best suited initially for increasing the rate of proliferation of the graft; however, it appears to not be suitable for inducing differentiation. IL-6 is described as an osteo-resorptive factor produced by osteoblasts. It activates glycoprotein-130-IL-6 subunit receptor important for osteoclast growth and differentiation but normally has little or no effect on osteoblasts (Kudo *et al.*, 2003). The exception however, is that there could even be some evidence that IL-6 under conditions of high bone turnover may play some role in osteoblasts generation (Franchimont *et al.*, 2005).

1.8.6 Bone morphogenetic proteins

BMPs are osteoinductive factors derived from the TGF-β superfamily. There are approximately 17 different BMPS, found in the ECM of bone of which the most important bone formation potential are BMP-2, -3, -4, -7 and -8 (Franchimont *et al.*,

2005). Research has shown that BMP-2 significantly increases expression levels of TGF-β1 and BMP-7 increases expression levels of collagen type 1 mRNA by almost 2.5 fold when compared to a control (Li *et al.*, 2004).

Similar to TGF-β, BMPs act synergistically with vitmin D; Jorgensen et al. (2004) demonstrated that the addition of BMP-2 in combination with 1,25-dihydroxyvitamin D enhance a more differentiated osteoblasts phenotype. However, in contrast, it seems that combining BMPs with growth factors like TGF-β does not have a synergistic effect, instead factors may actually antagonise the activity of the other.

1.8.7 1,25 dihydroxyvitamin D_3

The physiologically active polar form of vitamin D is 1,25-$(OH)_2D_3$. Vitamin D stimulates osteoblasts in a similar manner as parathyroid hormone (PTH) (Watrous and Andrews, 1989). It binds to the osteoblast intercellular receptors 3.2S and 3.7S and can act on a systemic level to improve bone formation (Mundy, 1995). Vitamin D binds to intracellular vitamin D receptors (VDRs) on osteoblasts. It causes a down-regulation in early bone formation markers ALP and Col type I but increases Oc production and is therefore regarded as a key factor for calcification. This is evident in people with vitamin D deficiencies result in rickets (in children) and osteomalcia (in adults) due to the prevention of mineralization. *In vitro* studies have shown that vitamin D in combination with other growth factors can improve bone development (Ramoshebi *et al.*, 2002).

1.8.8 Transforming growth factor-β (TGF-β)

TGF-β is produced by osteoblasts where it is stored in the bone matrix. It is both structurally and biologically similar to BMPs with variations only in the c-terminal amino acid sequence; providing properties such as loss of contact inhibition, bone formation and resorption. There are three isomers of TGF in humans: TGF-β_1, β_2 and β_3

and once in its active form they can bind to the cell bound integrin receptors TGF-β I, II, and III.

As well as inducing embryogenesis, normal cellular physiology, inflammation and tissue repair; TGF-β also enhances proliferation of MSCs and osteoblasts (Solheim et al 1998). Osteoblasts have the highest number of these TGF-β receptors than other cells in the body.

Vitamin D_3, testosterone and PTH have also been reported to increase TGF-β expression (Linkhart *et al.*, 1996). Yang et al. (2000) demonstrated that 4ng/ml increased osteoblast proliferation after 12 hours. Mice injected with 1μg TGF-β daily for three days can lead to a 40% increase in the width of the calvarial bone within one month. This is also the case for *in vitro* studies; however, prolonged exposure inhibits differentiation and mineralization (Mundy, 1995). Therefore temporary exposure of the growth factor may be most advantageous. TGF-β has also been shown to produce bone when in combination with other growth factors such as FGF which enhances the expression of TGF-β in osteoblasts (Simmons *et al.*, 2004).

1.9 Cells in bone tissue engineering

1.9.1 Primary cells

Primary cells are cells taken directly from living tissue; they are finite in lifespan and heterogenous in nature. Primary cells used in bone tissue engineering may be differentiated (e.g. osteoblasts) or undifferentiated (e.g. MSCs). MSCs require harvesting (from aspirates of bone marrow) and cell sorting to obtain a purified culture; this may be done by either magnetic or fluorescent activated cell sorting. MSCs are easier to extract than harvesting osteoblasts making them popular for use in TE. Allogenic cells may be banked; however, they can illicit an immune response if detected by the bodies immune system (Stocum, 1998).

1.9.2 Cell lines

Cells may also be transfected with viral DNA such as adenoviral vectors coding for a particular phenotypic outcome. Cells lines have been used as they are easily purified and it is possible to infect a range of cell types. Immunological reactions can clear/destroy cells expressing viral proteins by recognising them as not 'self' limiting *in vivo* applications. However, for *in vitro* studies such as assessing the potential of a biomaterial they provide an excellent alternative over primary cells.

1.9.3 hFOB 1.19

In order to assess osteogenesis of a CG scaffold, a homogenous cell phenotype with controlled growth rate is beneficial which is a limitation of using primary human bone cell cultures. Expanding a primary cell population in vitro can result in them losing their ability to differentiate (Ter Brugge and Jansen, 2002). An alternative is to use osteosarcoma cell lines due to their homogeneity; however, they may be limited phenotypically by responding differently to growth hormones and factors (Subramaniam *et al.*, 2002). These clonal human osteoblast cell line hFOB offers another alternative to reflect human bone biology. hFOB is a conditionally immortalised cell line developed and described by Harris *et al.* (1995). They are oncogene transformed** pre-osteoblasts from a spontaneous miscarriage which were transfected with a temperature sensitive plasmid (pucsvtsAS3) containing neomycin resistance (PSV2-Neo). This allows hFOB to be controlled between states of cellular growth; at 34.5°C the SV40 TAg gene is optimally expressed providing a proliferative phenotype whereas at 39°C it is not expressed, preventing proliferation but initiating differentiation. Clones were made and

** Oncogenic transformation is the process where by normal wild type cells can be altered to become immortalised. This may be with the aid of a viral carrier like the SV-40 plasmid.

the strain hFOB 1.19 was selected as it expressed the highest levels of ALP. The cells have adherent growth properties and the ability to differentiate into osteoblasts expressing the normal osteoblasts phenotype. hFOB have been shown to express high levels of ALP, collagen type 1, Op, On, BSP and Oc. In 2D cultures, once confluent, they can form mineralized nodules and because of this they are a model cell line for studying human osteogenesis on our support scaffold (Harris *et al.*, 1995; Donahue *et al.*, 2000).

This cell line will be used during this thesis as a model for studying human osteoblasts within our CG scaffold.

1.9.4 MC3T3

Another cell line which will be used in this work is the MC3T3 cell line. MC3T3 is a *Mus musculus* (mouse) calvaria clonal cell line with phenotypic characteristics of preosteoblast cells. They were cultivated in the early 1960's from mouse embryo fibroblasts and spontaneously mutated using cell density assays. MC3T3s have been shown to express ALP, produce Col type-I and can form mineralised nodules once in the presence of ascorbic acid and a phosphate source. They have been shown to attach, grow and support osteogenesis on biomaterials such as the CG scaffold making them a suitable cell line for studying osteogenesis (Tierney *et al.*, 2009).

1.10 Thesis hypothesis

The objective of this thesis was to examine the ability of a novel biomaterial; the CG scaffold; to support the growth, development and maturation of osteoblasts, to assess the effects of substrate stiffness on osteogenesis on cells in the scaffold and to examine the effects of a bioreactor to improve cell distribution and osteogenesis of cells within the CG scaffold.

The specific objectives were:

- The study set out by assessing cell growth, attachment, proliferation and viability of hFOB 1.19 on the CG scaffold. Optimisation parameters were carried out prior to an examination into the ability of a CG scaffold to support human osteoblast up to 35 days (Chapter 2)
- An examination of the ability of a CG scaffold to support human osteoblast growth and differentiation and how recombinant human transforming growth factor-beta (TGF-β_1) may enhance long term (49 days culture) *in vitro* bone formation was carried out in long term culture. The study also examined the benefit of introducing an initial high treatment of TGF-β_1 (10ng/ml) followed by a low continuous treatment (0.2ng/ml) to enhance human osteogenesis on the scaffold (Chapter 3).
- Once the osteogenic potential of the CG scaffold was known; an assessment into how anchorage dependent cells respond to mechanical and physical properties of the biomaterial was carried out. This study compared the osteogenic potential of CG scaffolds with varying stiffness up to 6 weeks in culture. The mechanical stiffness of CG scaffolds was varied by crosslinking via physical dehydrothermal (DHT) and chemical, 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDAC) and glutaraldehyde (GLUT) methods (Chapter 4).
- Finally, the use of a bioreactor was considered, as a way of producing a more

functional construct for tissue engineering applications. This study examined the use of a flow perfusion bioreactor to improve human osteoblast cell distribution and osteogenesis within a CG scaffold for bone tissue engineering (Chapter 5).

CHAPTER 2

Analysis of the ability of a novel collagen glycosaminoglycan scaffold to support human osteoblast growth

2.1 Introduction

The collagen glycosaminoglycan (CG) scaffold is a highly porous sponge, typically composed of type 1 collagen, an abundant connective protein in bone, and the glycosaminoglycan, chondroitin-6 sulfate, a proteoglycan commonly found in bone matrix. Both components have been shown to be important factors for cell attachment, proliferation and differentiation (Pieper et al., 1999). Thus, the constituents of this scaffold make it more analogous to a physiological substrate than scaffolds composed of synthetic polymers, bioceramics or metals. These scaffolds are typically fabricated by lyophilising collagen-based slurries and crosslinking them using dehydrothermal (DHT) treatments to form a highly porous sterile sponge. Recent studies have optimised the fabrication process in order to produce a more homogenous scaffold intended for bone tissue engineering (O' Brien *et al.*, 2004).

When developing a biomaterial for bone regeneration ideally it should possess osteoconductive properties to support cell growth and viability. The CG scaffold has recently successfully shown its ability to support attachment and proliferation of various animal cell types including fibroblasts, chondrocytes, neurons and osteoblasts (Freyman *et al.*, 2001a; Farrell *et al.*, 2006; Mcmahon *et al.*, 2008). As the CG scaffold is intended for human applications, the aim of this chapter is to examine the CG scaffold's

ability to support growth and development of human pre-osteoblasts.; this will become the main focus of the chapter.

2.1.1 Attachment and cell distribution onto CG scaffolds

One of the first steps in developing a tissue engineered construct for bone is to optimise cell seeding conditions on the scaffold. This is important as it provides the graft with the best start *in vitro,* a prerequisite for tissue integration (Wilke *et al.*, 1998). Optimal cell attachment can reduce the culture duration thus reducing the overall time taken in developing a tissue engineered construct from bench to bedside. Currently, there is no standardised cell seeding procedure when seeding CG scaffolds and previous studies have used several variations in CG scaffold size, cellular densities[4] and the duration of cell seeding; all important factors of cell attachment (Table 2.1). This may be due to differences in cell type and therefore optimisation of seeding parameters should be carried out accordingly. Optimising cell seeding is one of the objectives in this study.

Author (Year)	CG scaffold size, cell density and duration of seeding
Lee *et al.* (2001)	9-mm diameter disks, 3.5 mm thick CG scaffolds seeded with $2x10^6$ chondrocytes (canine, articular cartilage)
O'Brien *et al.* (2005)	10x10mm, 3.5 mm thick CG dry seeded with 1ml of $3x10^6$ MC3T3-E1 osteoblasts, per side incubating for 10 min
Farrell *et al.* (2006)	8x8 mm, 3.5 mm thick CG scaffold dry seeded with $3x10^5$ rat bone marrow stromal cells, incubating for 12 minutes

Table 2.1 Previous studies using CG scaffolds with varying scaffold sizes, cell densities and seeding durations.

Cell adhesion occurs via regulated cell membrane bound integrins[5] binding to a substrate. This process takes time therefore the duration of cell adhesion should be considered. Cell seeding density is another important consideration as it has been

[4] Cell density may be defined as the number of cells seeded per unit area of scaffold (Bueno *et al.* 2007)

[5] Integrins are cell surface adhesion points (motifs) which help the cell bind to a substrate; examples of osteoblastic integrins include $\alpha1\beta1$ $\alpha2\beta2$ and $\alpha3\beta1$.

shown to affect the ability to form tissue (Vunjak-Novakovic and Radisic, 2004). Cell density can affect the overall uniformity of the construct. Seeding a scaffold with too few cells may result in a heterogeneous cell distribution which can lead to regions of the scaffold remaining acellular. On the other hand, too many cells may prevent cell attachment by blocking binding sites or by causing cell-cell rather than cell-scaffold binding i.e. clumping of cells.

The method of cell seeding also needs to be considered. There are several different seeding methods which are generally termed as being either static or dynamic. Static seeding generally refers to a cell suspension seeded onto a scaffold in a drop-wise manner using a pipette. Dynamic seeding can be performed in a spinner flask, shaker, rotator or using a flow perfusion system. Although dynamic seeding may better reflect *in vivo* conditions, for example flow perfusion systems can mimic the physiological conditions of blood vessels; the most common method of cell seeding remains to be static seeding. A recent study in our research group compared several seeding techniques on CG scaffolds and found no significant improvement was observed between the static seeding methods (Murphy *et al.,* 2007) (as a result this thesis will consider only static seeding). Properties of the CG scaffold have also been shown to effect cell adhesion; these may include the pore size (O' Brien *et al.*, 2005; Murphy *et al.*, 2010), its composition (Tierney *et al.*, 2008) and substrate stiffness (Lee *et al.*, 2001).

In this thesis, the CG scaffold as described by O' Brien *et al.* (2004) is assessed for its potential to support growth and development of human cells. Thus, the human foetal osteoblast cell line hFOB 1.19 developed by Harris *et al.* (1995) was chosen as a model to study osteoblast functionality on the scaffold. hFOB 1.19 cells were derived from biopsies obtained from a spontaneous miscarriage. Primary cultures were isolated from the foetal tissue and immortalised by transfecting with the temperature sensitive mutant (tsA58) of the SV40 large T antigen. hFOB cells are ideal for studying human osteoblast growth and development as they have been shown to express high levels of

the bone formation marker alkaline phosphatase and form mineralised nodules (Kartsogiannis and Ng, 2004). Similarly, they are a model cell line for studying osteoblast growth and development as they are a highly reproducible non-transformed immortalised cell-line (Subramaniam, 2002).

Other than osteoblast markers, the hFOB cell line have been shown to express Oct3/4 an embryonic stem cell marker, indicating their ability to mimic the differentiation of progenitor cells and have been shown to trans-differentiate to adipocytes and chondroblastic lineages if stimulated accordingly (Yen *et al.*, 2007). Therefore, these cells appear to be highly responsive to environmental stimulation. As a result the hFOB cell is often used to represent human pre-osteoblast attachment to various biomaterials (Liu *et al.*, 2007; Setzer *et al.*, 2009; Stangl *et al.*, 2001). Many of these studies tend to be short term 2D analyses; with a few recent studies that have examined their growth response in 3D biomaterials (Cuddihy and Kotov, 2008; Dhurjati *et al.*, 2006; Ponader *et al.*, 2008). To date, none however, have examined their growth and development on a CG scaffold.

2.1.2 Cellular viability in a tissue engineered construct

As the tissue engineered construct develops *in vitro* it is important to be able to monitor its viability during growth and differentiation. Several methods of assessing cellular proliferation and viability exist (Table 2.2). These tend to be designed for 2D end point assays but may be adapted for the 3-dimensional (3D) environment in certain cases. The assays are either proliferation[6] assays or viability[7] assays; however, within the literature these terms tend to be used interchangeably. Most of these proliferation/viability assays result in the destruction of either the cell or the substrate

[6] Proliferation assays tend to operate on the basis of a biochemical marker that is present in proliferating cells and absent in non-proliferating cells.

[7] Viability assays work by either excluding a dye from a viable cell or by using a dye to stain living cells.

they exist on e.g. gel/scaffold. This limits experimental analysis to a series of end points and therefore is an important consideration when developing a TE construct.

Name	***Mode of action***
H-thymidine	A radioactive substance that binds to recently synthesised DNA. May require immunohistochemistry for quantification. It is a destructive end point assay.
5-bromodeoxyuridine (BrdU)	Similar to H-thymidine incorporation BrdU binds to DNA to identify recently proliferative cells. It also requires Immunohistochemistry antibody quantification.
Hoecht 33258	Proliferation may be assayed by the amount of fluorescence emitted by this dye when bound to double stranded DNA. Cell number may be calculated by comparing to a standard curve.
Tetrazolium salt	This assay works on the basis that the inside of proliferating cells is more reduced than non- proliferating cells which can be measured with tetrazolium salts like MTT. These salts are often cytotoxic and may not be soluble in culture medium. These assays are end point.
alamar^{tm} blue	Similar to tetrazolium salt assay alamar blue also calculated cell proliferation on the reducing environment of cells. The reagent is media soluble and non-toxic therefore allowing for real time assessment.
Neutral Red staining	Stained cells are cultured in microtitre plates. Cells may be adherent or in suspension. Absorbance is read at 550nm. Viable cells absorb the dye and concentrate it in the lysosomes.
Trypan blue exclusion	This non-toxic real time assay uses a dye that can enter cell membranes that have become damaged or ruptured. Live cells exclude the dye. Live dead cell numbers may be calculated using a haemocytometer.
Propidium Iodide	Similar to the trypan blue exclusion assay living cells also exclude propidium iodine staining only dead cells. Cells incubated in the reagent for 10mins. Fluorescence is carried out at 485 excitation and 580nm emission.
CFDA staining	This fluorescent dye can selectively stain viable cells. The dye passes the cell membrane where it is de-acetylated and then concentrated. Using an Attofluor imaging device fluorescence can be read at 488nm and 495 (excitation and emission respectively).
Crystal Violet Inclusion	Crystal violet stains viable cells adhered to a culture vessel (non viable cells are non adherent and therefore removed). It is an end point detection assay.
51Cr Release	Viable cells incorporate and store the radio labelled cells. As the cells die the chromium is released into the surrounding culture medium. This assay can be expensive and hazardous.
Europium Titriplex V	Cells can take up europium titriplex in the presence of dextran sulphate. That retains the reagent when treated with $CaCl_2$ and glucose. Fluorescence is read and compared to a standard curve.
Lactic Dehydrogenase (LDH)	This enzymatic assay can provide information on structural damage to the cell. Cells undergo freeze thaw action to release LDH which can be enzymatically measured. LDH levels are normalised to a total cell death reference value.

Table 2.2 Summary of cell proliferation and viability assays

The traditional method of analysing cell viability in 3D CG scaffolds as described by O' Brien *et al.* (2005) has been to enzymatically digest the scaffold using collagenase or dispase[8]. Once the cells are harvested from the scaffold, a cell count is performed using trypan blue. Trypan blue is excluded from viable cells and therefore one can distinguish between viable and non-viable cells. As alluded to above one limitation with this method is it requires the destruction of the tissue engineering construct.

An alternative is to use a non-destructive real-time assay. One such assay is the alamar blue assay (also known as the resazurin fluorescent dye assay) which quantitatively measures the proliferation and viability of cells (human, animal, bacterial and fungal) (Mo *et al.*, 2008; O' Brien *et al.*, 2000). The assay is user-friendly, particularly due to the fact that the dye is water soluble so no washing/fixation and extraction steps are required. It works via a REDOX reaction which fluoresces and changes colour following chemical reduction of growth medium as a result of cell metabolism. In order to use alamar blue to assess cell viability, a standard curve is required. Using this curve one can assess the optimal detection time point to determine viability levels and cell number. Obtaining a standard curve for cells on scaffold and analysing the ability of alamar blue to measure real-time viability and cell numbers is one of the objectives of the study.

[8] Dispase is a neutral protease which cleaves collagens I and IV, and fibronectin.

2.1.3 Research question

The aims of these preliminary studies are to define the optimal cell growth and proliferation conditions in order to assess long term osteogenesis on the scaffold. Specifically, it is intended to (i) optimise cell seeding conditions on the scaffold, (ii) to optimise a real time viability assay using alamar blue for cells on CG scaffolds; (iii) to investigate the ability of the CG scaffold to support long term human cell attachment, migration, distribution and viability *in vitro* under standard growth conditions up to 35 days and (iv) to examine the ability of a CG scaffold to support hFOB cells in maintaining an osteogenic phenotype up to 35 days.

2.2 Materials and methods

2.2.1 Standard methods and basic tissue culture conditions

The following standard cell culture techniques were used prior to subsequent long term analysis of cells on CG scaffold:

2.2.1.1 Cell thawing

Cryogenic vials containing hFOB 1.19 (ATCC, MA) pre-osteoblastic cells were swabbed with 70% ethanol (EtOH) and cells were thawed by rolling the vial gently between the hands until liquid was visible on the sides. The cells were then decanted into a 30ml centrifuge tube (Sarstedt, Germany) containing 10ml of prewarmed medium and centrifuged @ 280g for 5 minutes. The supernatant was removed and the pellet re-suspended in 10ml of prewarmed medium before being gently vortexed. The cells were pipetted into a labelled T25 flask (Sarstedt, Germany) and 5ml media was added.

2.2.1.2 Preparation of complete growth media

hFOB 1.19 (ATCC, MA) pre-osteoblastic cells were cultured under standard conditions (5% CO_2, at 37°C). Cells were routinely grown to 80% confluency in T175 culture flasks (Sarstedt, Germany) containing culture media; a 1:1 ratio of Hams F12 and DMEM (without phenol red) (Gibco, UK), 0.3mg/ml G418 antibiotic (Gibco, UK), 10% heat inactivated FBS (Sigma-Aldrich, Germany) and 1% (10mg/ml) penicillin/streptomycin (Sigma-Aldrich, Germany).

2.2.1.3 Cell feeding

Non-confluent cells were fed every 2/3 days by decanting spent medium, washing with 5ml of prewarmed phosphate buffered saline (PBS) (Gibco, UK) and replenishing with pre-warmed medium (5, 7, 12ml for T25, 75 and 175 respectively).

2.2.1.4 Cell sub culturing

Cells were checked for confluency (80-90%) using a Nikon E800m light microscope. Spent medium was decanted into waste containers. Cells were rinsed with pre-warmed PBS (Gibco, UK) to remove any trypsin inhibitors. 3ml of prewarmed trypsin (Sigma-Aldrich, Germany) was added and culture flasks were incubated for 5 min at 37°C. 7ml of pre-warmed medium was added to neutralise the effects of the trypsin. Cells were spun down using a centrifuge (280G for 5 minutes) and the cell pellet was resuspended in prewarmed medium. Typically cells were split in ratios no greater than 1:4. Flasks were labelled including name, date, cell-type and *passage* number. All experiments were carried out between 5 to 26 *passages.*

2.2.1.5 Cell freezing

In order to maintain cell stocks at a low *passage* number; spare cells were frozen down. For this a freeze medium was prepared using the following ratios of reagents (according to ATCC protocol): medium 72%, foetal bovine serum 20%, dimethyl sulfoxide (DMSO) 8%. Cells were sub cultured as per section 2.2.1.4 and resuspended at 1-3x10^6 cells/ml of freeze medium into each cryogenic vial. Cryogenic vials were initially frozen by placing at -20°C for 30mins, then at -80°C overnight before being transported to liquid nitrogen for long term storage.

2.2.2 Analysis of cell attachment to CG scaffold

2.2.2.1 Scaffold fabrication and cross-linking

CG scaffolds were fabricated using a lyophilisation technique as described previously by O'Brien *et al.* (2005). Collagen slurry was prepared from defrosted (wet) bovine collagen type-1 (Integra Life Sciences, Plainsboro, NJ) by blending 3.4g (dry weight) collagen with 600ml 0.05M acetic acid (Sigma-Aldrich, Germany) at 15,000 rpm

for 90 minutes (4°C) using an Ultra Turrax T18 overhead blender. 0.32g of shark derived glycosaminoglycan 6-chondroitin sulfate (Sigma-Aldrich, Germany) was dissolved in 120ml 0.05M acetic acid (HOAc) at pH3.2 and stirred for 10 minutes. This solution was added dropwise for 25 minutes using a peristaltic pump. The slurry was blended for an additional 90 minutes before degassing the mixture in a vacuum oven for 60 minutes. The slurry was poured into a stainless steel tray and freeze dried at -40°C for approximately 24 hours, 50 mTorr. During this time ice crystals were formed via sublimation which resulted in the production of a porous structure. Scaffolds were then cross linked by dihydrothermal (DHT) cross-linking at 105°C for 24 hours (VacuCell 22). Sterile sheets of the finished product (thickness = 3.5mm; mean pore diameter = 96μm; porosity =99.5%) were aseptically cut to size (10x10mm or as otherwise stated).

2.2.2.2 Cell seeding onto CG scaffold

hFOB cells from confluent tissue culture flasks were tripsinised as per section 2.2.1.4 and resuspended in medium to form a cell suspension of $1x10^6$ cells/ml. 150μl of this cell suspension was seeded dropwise onto one side of the CG scaffold. Scaffolds were incubated at 34°C for 10 minutes prior to being turned over and reseeding with a further 150μl of $1x10^6$ cells/ml cell suspension and incubated for 2 minutes. 2ml of standard medium was added to each well and incubated at 34°C for 48 hours.

2.2.2.3 Dispase digest of CG scaffolds

Following cell culture, constructs (n=3) were rinsed in prewarmed PBS before being placed in a sterile 15ml falcon tube. 3ml of 2U/ml prewarmed and pre-filtered dispase (Gibco, UK) was added to digest the scaffold and release the cells into suspension. Tubes were gently vortexed every 5 minutes for 30min at 37°C. A cell count was obtained as per section 2.2.1.4.

2.2.2.4 Cell counting (trypan blue exclusion method)

Cells were trypsinised and resuspended in medium as above (2.2.1.4). A 1:1 ration of cell suspension (100µl) and trypan blue (100µl) were added to a 0.5ml eppendorf tube. The sample was mixed thoroughly by gentle pipetteing. 10µl of which was pipetted into the haemocytometer.

Cells which did not take up the dye in the haemocytometer squares A, B, C, D were counted at low power (10x) using the light microscope and the sum total inserted to the equation:

Number of cells = ($\sum$ ABCD/ #squares counted) x dilution rate x 10,000 x volume (ml)

The outcome of which gave an average of the total number of viable cells.

2.2.2.5 Histological processing of CG scaffold

Following the incubation period scaffolds were removed from the medium and rinsed in pre-warmed sterile PBS (Gibco, UK). Samples were then fixed in 3ml of 4% paraformaldehyde (Sigma-Aldrich, Germany) for 30 minutes followed by rinsing in PBS. Post fixation, samples were then placed into tissue processing cassettes prior to beginning a 16 hour automatic series of dehydration, equilibration and infiltration steps using the LEICA ASP300 Processor. These steps were

1. Dehydration:
    - 60 min cycle in formalin (x2)
    - 60 min cycle in 70% ethanol
    - 60 min cycle in 90% ethanol
    - 60 min cycle in absolute ethanol (x3)
2. Equilibration
    - 60 min cycle in xylene (x3)
3. Infiltration
    - 60 min cycle in histowax at 60 °C

Cassettes containing samples were then placed in the warming drawer of the LEICA EG1 140 H&C wax embedding machine (Leica, Germany). Approximately 1ml of 60°C wax was poured into the provided metal moulds. Each scaffold sample was then transferred using forceps into the centre of the mould containing wax. The mould was then placed onto a cold plate to fix the scaffold into place. The back of the cassette was placed onto the mould and filled up with more hot wax. Moulds were allowed to cool before being stored at 4°C prior to sample slicing.

Wax embedded scaffolds were mounted onto the vice grips of a LEICA RTV 1225 microtome for sectioning. Initially a 25μm slice was removed to trim the block. Subsequent sections were sliced at 10μm. Sections were then placed into a water bath (40°C) before mounting onto pre-subbed poly-lysine slides (VWR, Germany). Slides were labelled with both the sample and slide number. They were allowed to dry on a heated slide rack before being placed into a tissue drying oven for 12 hours at 58°C in order to remove the paraffin wax. Coronal sections of scaffold between a third and half way from the scaffold surface were used for further analysis (unless otherwise stated).

2.2.2.6 Haematoxylin & eosin staining (H&E) for cell distribution within a CG scaffold

Sections were removed from the tissue drying oven and placed in xylene to remove any remaining wax (Sigma-Aldrich, Germany) for 10 minutes. Excess xylene was removed by dipping slides into a series of alcohol washes for 5 minutes each; these were 100%, 95%, 80%, and 70% EtOH respectively. Slides were re-hydrated by placing into distilled water water for 5 minutes. At this point slides were ready for staining and were placed into a rack containing hematoxylin (to stain the nuclei of cells) for 10 minutes. Slides were then dipped into distilled water to remove excess dye then into tap water for 5 minutes to allow 'bluing' to occur. Excess dye was further removed by dipping three times into a solution of 0.25% hydrochloric acid (Sigma-Aldrich, Germany) in 70% EtOH. Once again slides were dipped into distilled water for 5 minutes. At this point

slides were placed into 1% eosin (Sigma-Aldrich, Germany) (to stain protein/ cellular cytoplasm) dye for 3 minutes then 10 dips into 100% EtOH, 5 dips into acetone (Sigma-Aldrich, Germany) and finally 10 dips into xylene. Slides were allowed to dry in the fume hood. Once dry two drops of the histological mountant DPX was placed on the centre of the slide and gently mounted using a forceps with appropriate sized coverslips (VWR, Germany).

2.2.2.7 Image analysis

Slides were viewed using the Nikon E800M (Nikon, Japan) optical microscope at various magnifications (5x, 10x, 20x and 40x). Images were transferred to a PC using LUCIA measurement software where images could be filled and saved.

2.2.3 Study 1: Investigation of hFOB cell seeding and attachment within a CG scaffold

In order to optimise cell conditions on the CG scaffold a series of preliminary studies were carried out prior to long term culturing of cells on CG scaffolds:

2.2.3.1 Analysis of seeding volume used on CG scaffold

50, 100, 150, 200 & 250µl volumes of pre-warmed media were pipetted on both wet (prehydrated in PBS) and dry scaffolds (10x10x3mm). The optimal seeding volume was calculated by measuring the amount of saturation of the scaffold which occured.

2.2.3.2 Optimisation of hFOB cell seeding density on CG scaffolds

hFOB cell suspensions were seeded dropwise at densities of 0.5, 1, 1.5, 2, & 2.5 $x10^6$ in 200µl volumes per side of scaffold (n=6). Cell seeded CG constructs were incubated at 34°C for 10 minutes and reseeded on the opposite side with a further 200µl of 0.5, 1, 1.5, 2, & 2.5 $x10^6$ cells; incubating for a further 2 minutes. This resulted in a final cell seeding density range of 1, 2, 3, 4 & 5 $x10^6$ cells per scaffold. Cell seeded CG constructs were incubated with 2ml media at 34°C for 48 hours. Constructs were digested and a cell

count obtained as per section 2.2.2.3 & 2.2.2.4.

2.2.3.3 Optimisation of cell seeding duration of hFOB on CG scaffolds

Optimal cell attachment was assessed by seeding 200µl of pre warmed media containing $2x10^6$ hFOB cells drop-wise onto each side of the CG scaffold (n=6). Cell seeded CG constructs were incubated at 34°C for 10 minutes prior to being turned over and reseeding with a further 200µl of $2x10^6$ cells and incubated for 0, 5, 20, 35 and 50 minutes. Cell seeded CG constructs were incubated with 2ml media at 34°C for 48 hours. This gave a total seeding duration of 10, 15, 30, 45 & 60 mins respectively.

2.2.4 Study 2: Optimisation of a non invasive real time viability assay (alamar blue) for hFOB 1.19 cells on a CG scaffold

2.2.4.1 Optimisation of alamar blue standard curve for hFOB cells on CG scaffold

A total of 1, 2, 3, 4 and 5 million hFOB cells were seeded in a dropwise manner onto CG scaffolds and incubated at 34°C. Firstly, 150µl of a cell suspension containing 0.5, 1, 1.5, 2 and 2.5 million hFOB cells were seeded for 10 minutes then a further 150µl of a cell suspension containing another 0.5, 1, 1.5, 2 and 2.5 million hFOB cells were seeded onto the opposite side of the scaffold and incubated for 20 minutes (n=6). 3ml of media containing 10% alamar blue (Biosource, Germany) was added to each well. Plates were placed on a Biorad 3D orbital shaker set at 90° rotation incubating at 34°C for 24 hours to enhance rates of diffusion through the construct. 100µl aliquots of each well were then taken in triplicate at various time points up to 3 hours. In order to maintain a constant volume, 300µl of medium containing alamar blue was added following sampling. Samples were read on a spectrometer at 540nm and 620nm and results recorded. Average absorbance values were calculated and the percentage reduction was determined as follows:

Firstly, the correction factor (R_0) was calculated: $R_0 = AO_{LW}/AO_{HW}$

Where, AO_{LW} = absorbance of oxidised form at 540nm;

AO_{HW}= absorbance of oxidised form at 620nm

The percentage reduction[9] was then calculated as: % Reduction = [A540-(A620 x R_0)] x 100%

2.2.5 Study 3: Long term analysis of hFOB growth and distribution on a CG scaffold

2.2.5.1 hFOB cell seeding and culturing on CG scaffold

2x10^6 hFOB cells were seeded in a dropwise manner onto CG scaffolds and incubated at 34^{o}C for 10 minutes. A further 2x10^6 hFOB cells were seeded onto the opposite side of the scaffold and incubated for 20 minutes (n=6). 5mls of standard media was added and scaffolds were cultured for 4, 7, 14, 21, 28 & 35 days at 34^{o}C. Following cell culture samples were fixed, tissue processed and analysed as per sections 2.2.2.6.

2.2.5.2 hFOB cell viability on scaffold: alamar blue

Metabolic activity was assessed using the alamar blue assay. Media surrounding scaffolds was removed and replenished with that containing 10% alamar blue dye (Biosource, Germany) at days 4, 7, 14, 21, 28 and 35 (n=6). Constructs were incubated on an orbital shaker for 4 hours. 100µl of media was read using a spectrophotometer at 570nm and 610nm. The percentage of reduced dye was calculated in accordance with section 2.2.4.1.

2.2.5.3 hFOB cell viability on scaffold: dispase digest

Cell seeded constructs were digested and cell number counted (as per section 2.2. 2.3) at 4, 7, 14, 21, 28 and 35 days (n=3).

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[9] An increase in percentage reduction of alamar blue correlates to an increase in metabolic activity.

2.2.5.4 Gene expression of hFOB cells on CG scaffold

RNA isolation: Cell seeded constructs were rinsed with PBS before RNA isolation in 1ml RLT lysis buffer (Qiagen, UK) using a cell scraper (n=3). RNA was extracted using the RNeasy mini kit (Qiagen, UK) according to the manufacturer's instructions. The RNA concentration per scaffold was quantified using a spectrophotometer (abs 260nm).

Gene expression: Following RNA extraction Real time reverse transcription PCR was carried out by firstly removing trace genomic gDNA and then reverse transcribing 400ng total RNA with an RT kit (QuantiTect RT Kit, Qiagen, UK) according to the manufacturer's instructions. Realtime PCR was then carried out using the 7500 Real-Time polymerase chain reaction (PCR) System (Applied Biosystems). The QuantiTect SYBR Green PCR Kit (Qiagen, UK) was used, according to the manufacturer's instructions (Qiagen, UK), with QuantiTect Primers (Qiagen, UK). Expression levels for oct 3/4 (a marker embryonic stem cells), collagen type II (a marker of chondrocytes), E11 (a marker of osteocytes), LPL (a marker for adipocytes), alkaline phosphatase (a marker of early stage bone formation) and osteocalcin (a marker of late stage bone formation) were assessed using the relative quantification ΔΔCt method. β-actin acted as a house keeping control.

2.3 Results

2.3.1 hFOB cell attachment to CG scaffold

H&E staining showed a pink collagenous highly porous scaffold containing some purple stained nuclei along the edge of the scaffold after 48 hours incubation (Fig. 2.1). Cells attached to all sides of the scaffold.

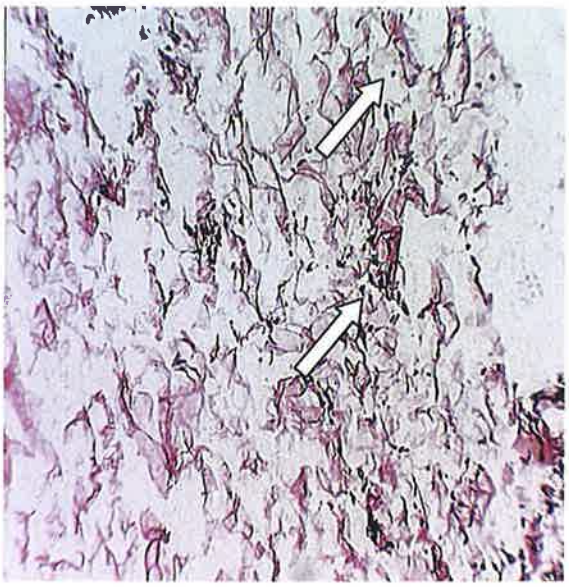

Fig. 2.1 H&E staining of CG scaffold post 48 hours seeding with hFOB1.19 cells (n=3; mag 10x). Sections were taken approximately 40%/650μm deep from the surface of the scaffold. Nuclei identified with white arrows.

2.3.2 Study 1: Optimisation of hFOB cell seeding and attachment within a novel CG scaffold

2.3.2.1 Optimisation of seeding volume used on CG scaffold

Optimal volume for seeding was found to be 200μl per side of dry scaffold (10x10x3mm). Below this volume the scaffolds were not fully saturated resulting in dry regions on the periphery. Above this volume scaffolds became over saturated resulting in a loss of sample.

2.3.2.2 Optimisation of cell seeding density

The cell density curve found that following a 48 hour culture period the highest cell count from dispase digested scaffolds was recorded to be 391,250 (Fig. 2.2). There is a

steady rise in cell number up to this point after which a decline is observed; however, no significant difference existed between the groups. The seeding efficiency using $4x10^6$ cells/CG scaffold was a ~10% attachment efficiency.

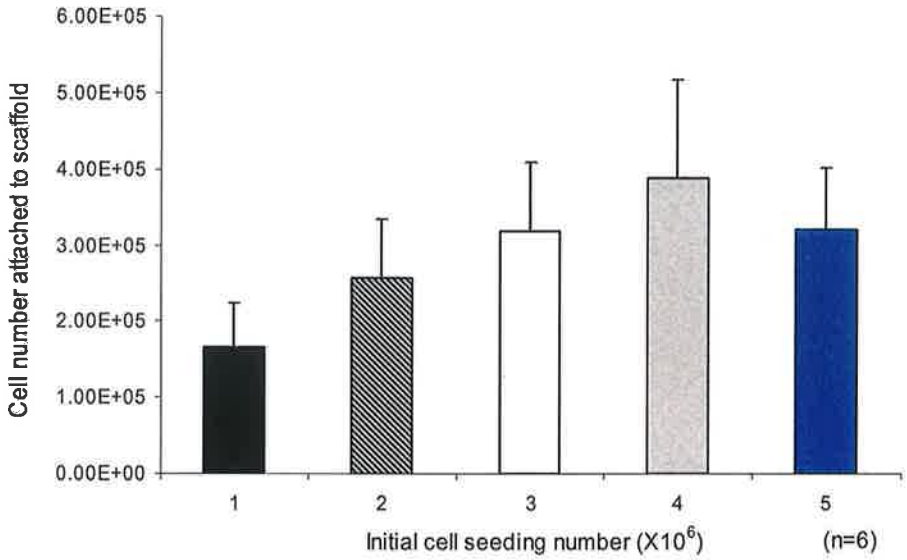

Fig. 2.2 Optimal seeding density curve for cells seeded on CG scaffold post 48 hour incubation. Seeding scaffolds with 4 million cells gave the highest attachment.

2.3.2.3 Optimisation of cell seeding duration

Figure 2.3 demonstrates the optimal seeding attachment duration to be at 30 minutes. Using this seeding duration an increase from 10 to 37% hFOB cell attachment was observed. There was however, no statistical significant difference between the groups.

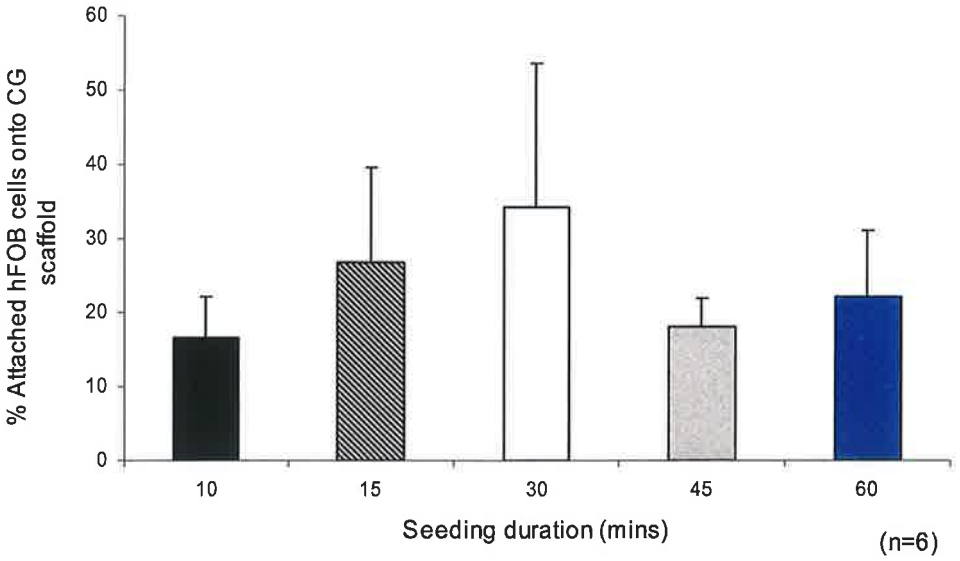

Fig. 2.3 Optimal seeding attachment duration curve for hFOB cells on CG scaffold; post 48 hour incubation. Incubating for 30 minutes was the optimal seeding duration.

2.3.2 Optimisation of a non invasive viability assay (alamar blue) for hFOB cells on scaffold

Figure 2.4 represents the colour change of the cell seeded constructs and surrounding media over time. This colour change is based on the metabolic breakdown of the alamar blue dye. The percentage reduction of dye in the spent media; was plotted against cell number to produce a standard curve of metabolic viability (Fig. 2.5).

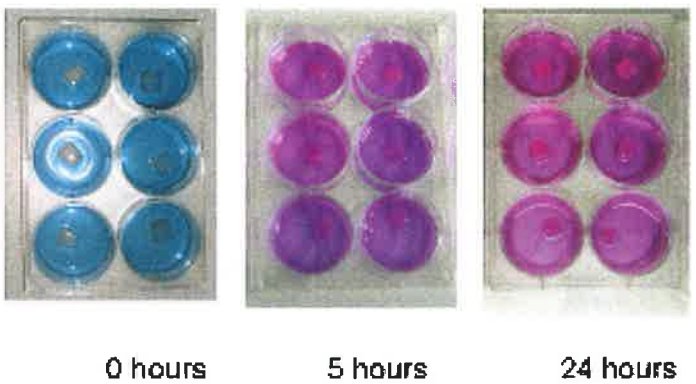

Fig. 2.4 Photographs of alamar blue viability assay of hFOB cells on CG scaffold up to 24 hours culture.

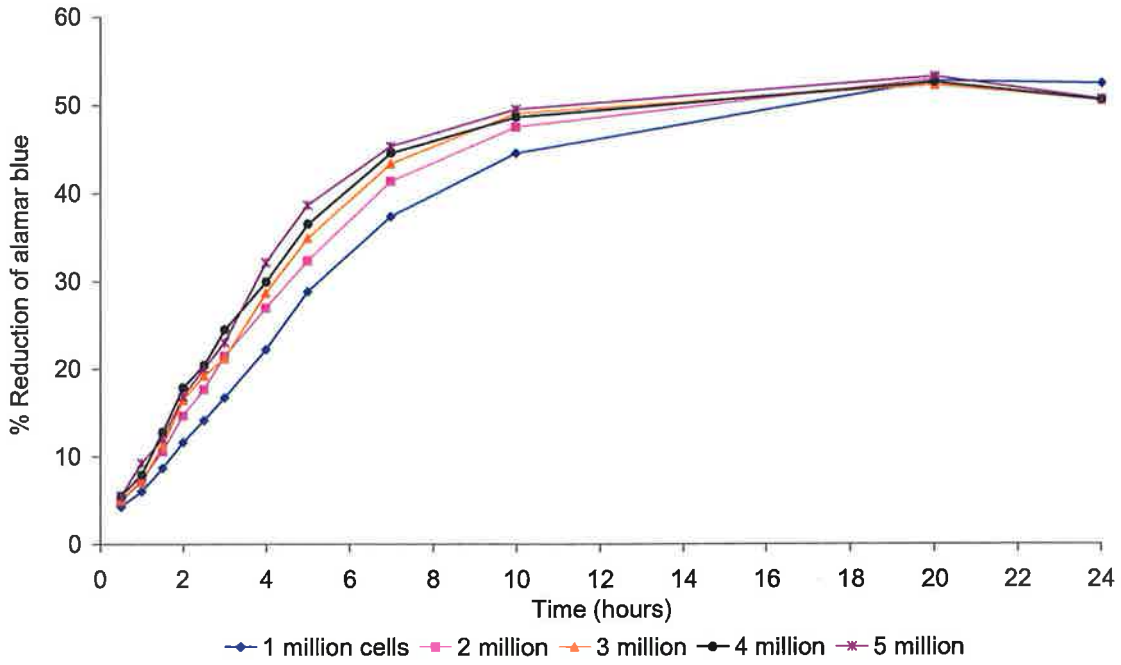

Fig. 2.5 hFOB metabolic viability curve for cells on CG scaffold using alamar blue (n=6).

Figure 5 demonstrated that the assay could discriminate best between differences in cell number between 3 and 8 hours of incubation with dye. When we compare this to with the highest linear fit (Fig. 2.6), both 4 and 5 hours gave good separation and a higher linear fit (R^2 0.9866 & 0.99 respectively).

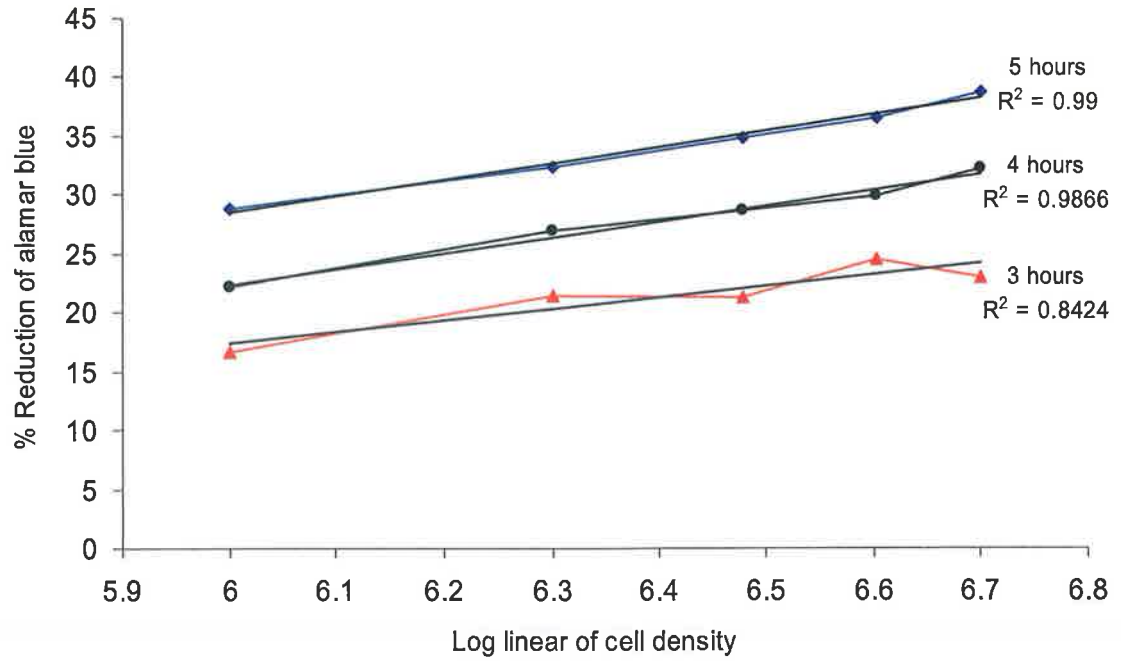

Fig. 2.6 Log linear standard curve of hFOB metabolic viability on CG scaffold using alamar blue (n=6).

Given that a shorter assay duration is preferential therefore, 4 hours was chosen to be the optimal detection time for hFOB cells on the CG scaffold. The equation of the line at 4 hours was determined as $y = 13.423x - 58.132$. Using this equation it is possible to obtain a measure of the number of cells attached from the percentage of reduced alamar blue dye.

2.3.3 Analysis of CG scaffold to support hFOB cell attachment and growth

2.3.3.1 Long term cell viability on the scaffold

An increased percentage reduction of dye indicates increased cell viability cells were shown to be viable on the CG scaffold up to 35 days of culture; with levels of reduced dye increasing between 15 and 22% up to 7 days ($p<0.001$) (Fig 2.7). After this point a decline was observed which stabilised thereafter; the difference between day 7 and days 21 to 35 was statistically significant ($p<0.004$).

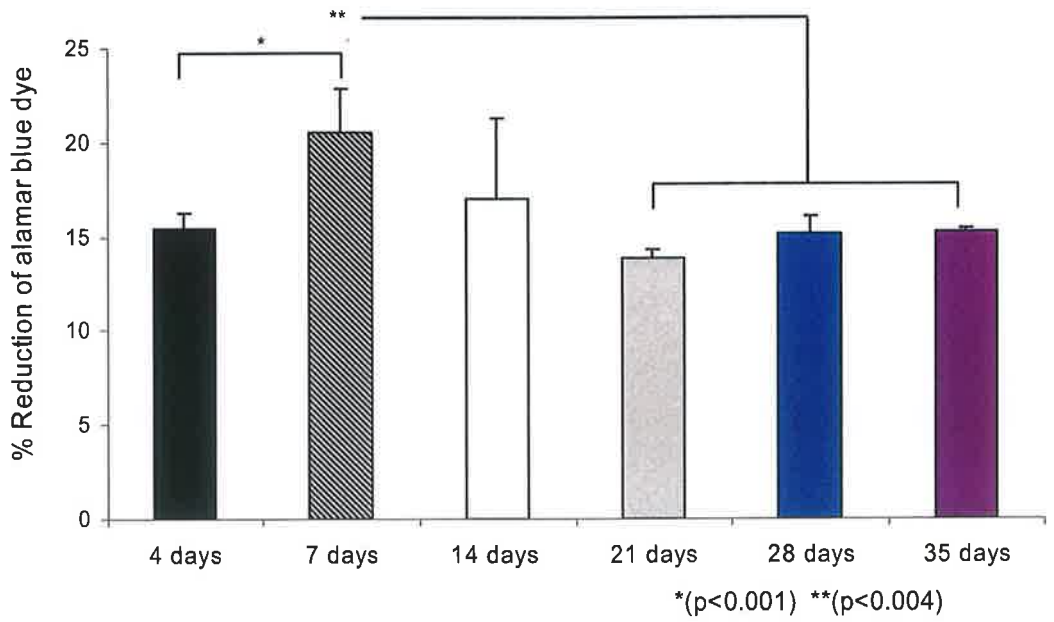

Fig 2.7 Metabolic cell activity determined by alamar blue assay. Cells remained viable on the CG scaffolds with metabolic viability levels of hFOB cells on CG scaffold using alamar blue fluctuated between 15 and 20% reduced dye.

Fig. 2.8 shows a comparison of alamar blue cell number (as obtained from the reduction of dye) and cell number using the traditional dispase digest method. Cell number as monitored by the alamar blue assay increased up to 7 days to a maximum of $8x10^5$ cells and reduced to stabilise at $3x10^5$ cells. Cell number using the dispase digest method, gave a more gradual rise in number from $\sim 3x10^5$, to give a maximum peak after 28 days of $1.4x10^6$ where a reduction of cell number to $8x10^5$ was observed at 35 days. Statistically significant differences were observed at both the early and late time points between each assay ($p<0.01$; $p<0.001$ and $p<0.001$; $p<0.01$ respectively)

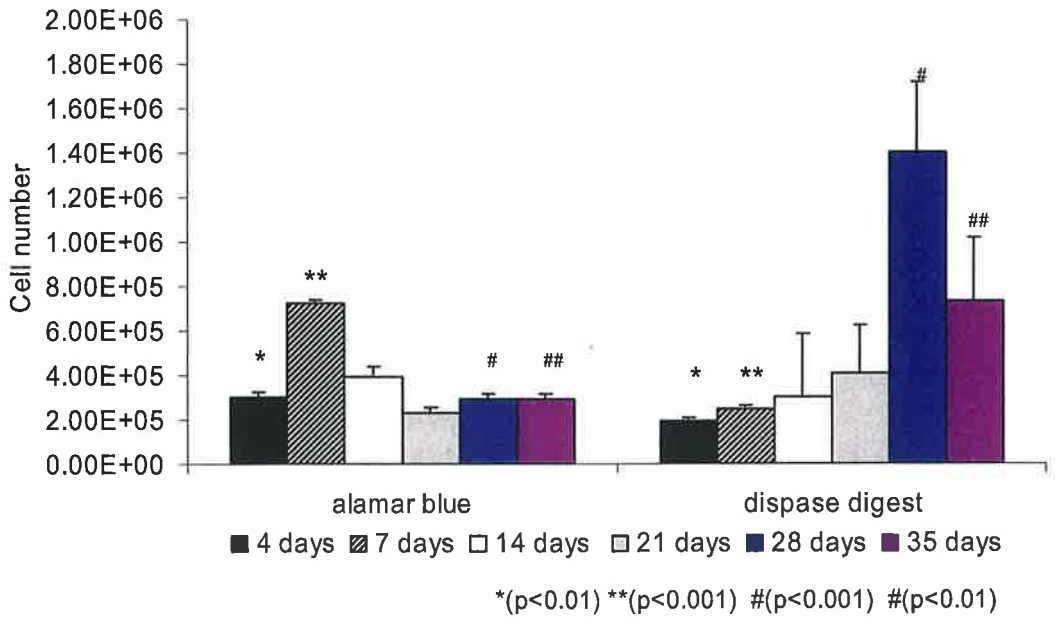

Fig. 2.8 hFOB cell viability on CG scaffold up to 35 days in standard culture using both alamar blue and dispase digest viability assays (n=6 and n=3 respectively). A clear difference in cell number between assays was evident from 21 days of culture.

2.3.3.2 Histological analysis of hFOB growth and distribution on a CG scaffold

hFOB cells were found to attach and infiltrate the scaffold with time (Fig. 2.9 a-h). Between 4 and 7 days cells resided on the scaffold edge increasing in number (Fig. 2.9 a-

b). Cells had migrated into the scaffold centre after 14 days (Fig. 2.9 d). Scaffold pores contained numerous cells and matrix deposition was evident at 21 days along the surface of the tissue engineered construct. Changes in structural integrity were also observed with construct size and pore structure reduced with time particularly between 28 and 35 days (Fig. 2.9 f-h). Cell seeded CG constructs became confluent with a homogeneous cell distribution by 35 days; (Fig. 2.9 g-h); ECM deposition increased with time which coincided with a reduction in porosity notably at 35 days even in the absence of osteogenic growth factors .

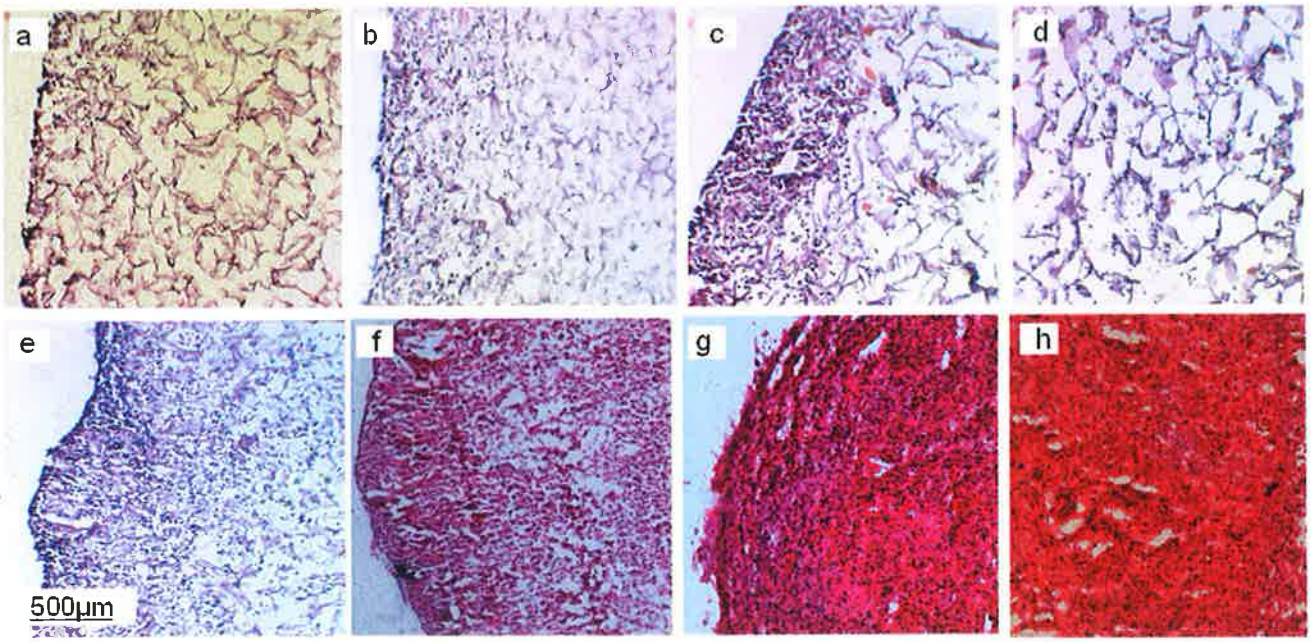

Fig. 2.9 (a-h) H&E staining of the cell seeded CG scaffold showed a clear infiltration, augmentation and matrix deposition by hFOB cells within the scaffold up to 35 days (10x; n=3). Where (a-c) represented day 4,7 and 14, (d) day 14 centre; (e-g) represented day 21, 28 and day 35 and (h) day 35 centre respectively. Sections were taken approximately 40%/650µm deep from the surface of the scaffold.

2.3.4.3 Gene expression analysis of hFOB cell phenotype following culture on CG scaffold

Gene expression results showed that hFOB maintain pre-osteoblast phenotype during standard cultivation after 28 days of culture. In order to examine possible trans-differentiation non-osteogenic markers were tested and included; E11 an osteocyte marker, Oct3/4 an embryonic stem cell marker, collagen type II a marker of

chondrogenesis, and lipo-protein lipase a marker of adipogenesis. No expression levels were detected for these markers. Only expression levels of bone formation markers were detected; including alkaline phosphatase and osteocalcin (Fig. 2.10).

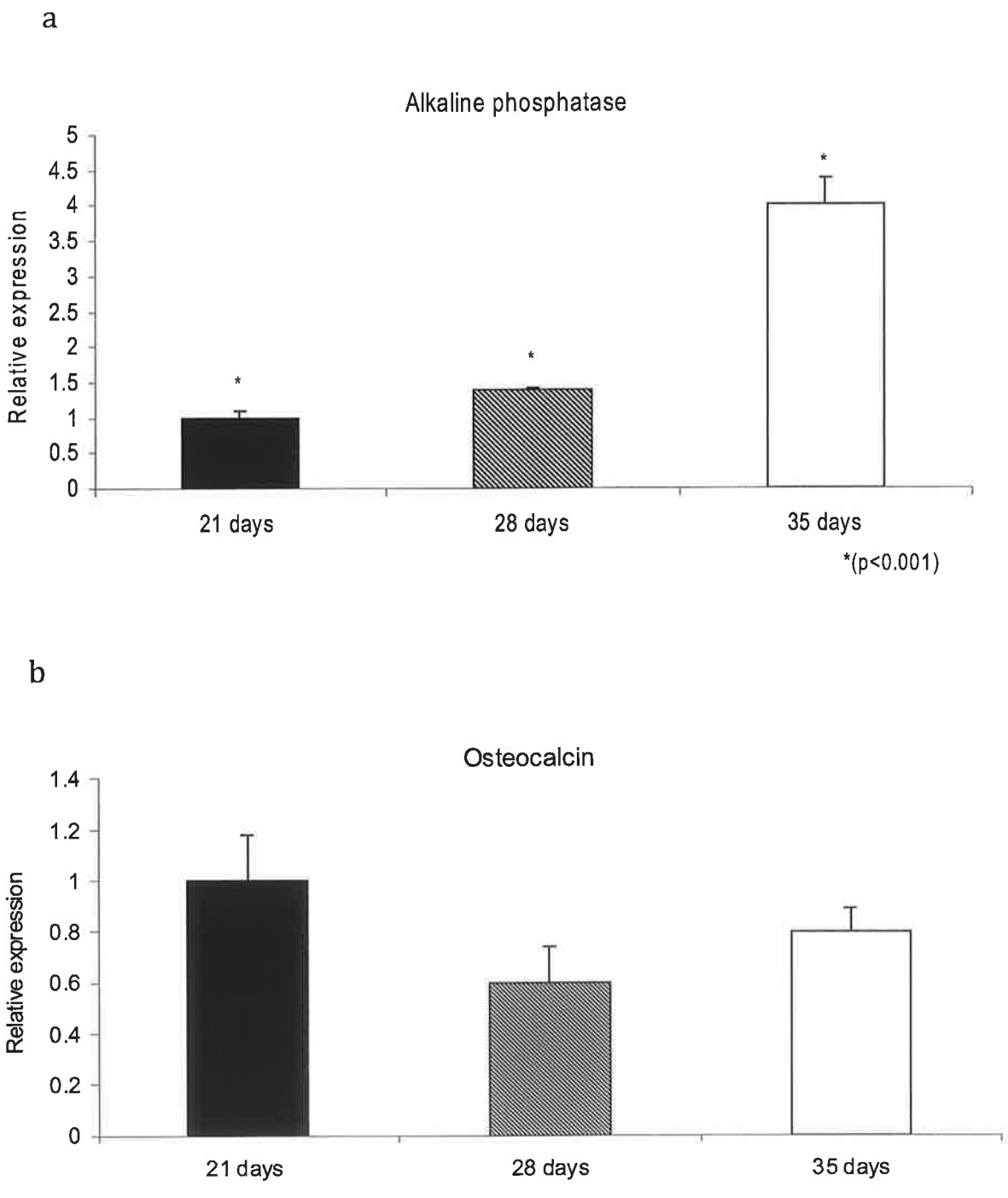

Fig. 2.10 (a) Alkaline phosphatase expression of hFOB cells on CG scaffold; an increase is observed in expression over time; (b) Expression levels of osteocalcin were detected; however, no change was observed between groups. (n=3) * $p<0.01$.

The early bone formation marker alkaline phosphatase increased in expression level with time. Osteocalcin the late stage marker for osteogenesis amplified however, no statistically significant difference was observed over time; suggesting that osteogenic factors are required in order to stimulate cells to synthesize matrix.

2.4 Discussion

The collagen glycosaminoglycan (CG) scaffold was originally developed for the dermal regeneration of burn victims. More recently, the scaffold has been modified for bone regeneration (O' Brien *et al.*, 2004). Recent studies in our laboratory have examined the ability of the CG scaffold to support the growth and development of various animal cells specifically for bone tissue engineering (Farrell *et al.*, 2006; Murphy *et al.*, 2010; Tierney *et al.*, 2008). As the CG scaffold is intended for clinical use the aim of this study was to assess its ability to support human cell growth and development *in vitro.*

Prior to beginning to investigate the CG scaffolds ability to support growth of human cells a series of preliminary studies were carried out. These were performed in order to optimise the *in vitro* culture conditions for human hFOB 1.19 cells on the CG scaffold. The parameters investigated included cell seeding density, cell attachment duration, suspension volume and cell attachment efficiency. These preliminary studies are important as such initial optimisation aims to provide the graft with a reasonably high cell density *in vitro,* which has been shown to correlate with tissue development and formation (Wilke *et al.*, 1998). Optimisation of the culture conditions should be carried out for specific tissue engineered constructs, as both cell and scaffold types may vary.

Cell seeding density is an important consideration for developing functional tissue engineered constructs as it effects cell proliferation, matrix production and therefore cell state (Zhu *et al.*, 2009). The optimal cell seeding density was determined to be $4x10^6$ cells/scaffold (where the scaffold is 10x10x3mm). This seeding density is higher than those reported in previous studies (which have used similar sized CG scaffolds) (Farrell *et al.*, 2006). Results from this study show a cell type specific seeding density, a similar finding to previous studies (Table 2.1). The value obtained in this study coincides with other studies, who have suggested that at least 4 million cells per square centimetre of scaffold should be a minimum seeding density for tissue engineered constructs (of

similar scaffold size) as it provides for spatial uniformity during seeding.

Studies have reported higher cell attachment efficiencies of 40%-50% using fewer than $4x10^6$ cells (O' Brien *et al.*, 2005). The cell attachment efficiency is more important when considering the use of primary cells on the scaffold rather than for a cell line like hFOB. This is because only finite numbers of cells such as primary osteoblasts or MSCs can be harvested from patients, whereas cell lines like hFOB's are immortalised so there are no restrictions on numbers. However, it is worth considering that in this study although the cell attachment efficiency was initially only 10% with $4x10^6$ cells, it was possible to improve this value by increasing the duration of initial seeding from 12 min to 30 minutes. This resulted in an increase of the cell attachment efficiency from 10 to 37%. This result coincided with that by Pinho (2003) who found 30 minutes to be the optimal time required for hFOB cells to adhere to standard tissue culture plates.

As well as optimising initial cell seeding and attachment conditions it was of interest to assess cell viability on the scaffold. As the traditional dispase digest assay requires the scaffold to be destroyed in order to carry out the assessment, an investigation into using the metabolite alamar blue was carried out to measure hFOB cell viability on CG scaffold in real-time. A standard curve for hFOB cells on CG scaffold was developed from which the optimal detection time point was determined to be a 4 hour incubation period on an orbital shaker. Previous studies using alamar blue in 2D cultures reported shorter incubation periods (O' Brien *et al.*, 2000). This is likely to be due to differences in diffusion rates between 2D and 3D assays; where diffusion from a 2D monolayer would be quicker than dye diffusing throughout a 3D scaffold.

The long term study of hFOB 1.19 cells on CG scaffolds revealed that the CG scaffold could support hFOB cell attachment, migration and viability up to 35 days *in vitro*. Cells migrated gradually from the construct periphery towards the centre within 14 days. Cell proliferation continued until the construct became fully confluent with a homogenous distribution of cells by 35 days of culture. Previous studies examining animal osteoblasts

on CG constructs have not shown such levels of confluency throughout the scaffold (Farrell *et al.*, 2006; Tierney *et al.*, 2008).

Histological analysis also revealed structural changes within the CG construct over time, which included reduced porosities, an increase in matrix deposition and a reduction in the overall size of the construct. The changes in scaffold shape may explain the variation in cell viability as shown by the alamar blue and the dispase digest assays. A reduction in pore size caused reduced rates of diffusion of alamar blue dye during the viability analysis. As a result cell number as measured by the alamar blue assay peaked at 7 days with a reduction thereafter whereas cell number as identified by the dispase digest increased steadily up to 28 days. The reduction in cell number (from dispase digest assay) between 28 and 35 days may be associated with increased matrix deposition as it was observed that it was more difficult to digest the scaffolds using dispase at 35 days to release cells form the CG construct.

The optimised real-time alamar blue assay and the traditional dispase digest assays were unable to accurately identify cell number at the longer incubations as a result of high levels of construct contraction and matrix deposition which reduced diffusion rates within the CG construct. Similarly, when using a metabolic assay such as alamar blue to identify cell number inaccuracies may occur where cells undergo stress can result in an increased metabolic activity. Therefore, the alamar blue assay is not suitable to detect accurate cell numbers on the scaffold; however, it can be used to indicate overall cell metabolic activity in real-time. As a result of these limitations another viability assay should be considered particularly for long term culture analysis. An alternative assay is the Hoechst DNA 33258 assay. As Hoechst 33258 is a fluorescent dye that binds to double stranded DNA, it is therefore not affected by construct contraction, matrix deposition or rates of diffusion making it a preferred method under such long term culture conditions. However, the Hoechst 33258 assay is not a real time assay and does require the destruction of both cell and CG construct.

As well as examining cell viability, distribution and structural changes an analysis of the hFOB phenotype on the CG scaffold was carried out. Previous studies have shown the hFOB's ability to trans-differentiate towards adipogenic and chondrogenic lineages when stimulated accordingly (Yen *et al.*, 2007). Therefore, it was important to identify the inductive nature of the CG scaffold for hFOB cells.

Under un-stimulated culture conditions (i.e. in standard growth medium) hFOB's have been shown to express the embryonic stem cell marker Oct3/4; once stimulated however this marker should cease to be expressed (Yen *et al.*, 2007). Similarly, alkaline phosphatase an early bone formation marker has been shown to become expressed with osteogenic stimulation using growth factors (Harris *et al.*, 1995). Results showed that hFOB expressed alkaline phosphatase and osteocalcin and ceased to express Oct3/4 when seeded onto the CG scaffold. This finding is interesting as it indicates the osteoinductive nature of CG scaffolds which have typically been reported to be osteoconductive but not osteoinductive. Under standard 2D conditions hFOB cell do not express these markers but require some osteogenic stimulus be it chemical or physical; therefore this will be the focus in Chapter 3 (chemical stimulation); Chapter 4 (substrate properties) and Chapter 5 (physical- fluid flow stimulation). In this current study the osteogenic stimulation is most likely a result of the presence of collagen type I within the scaffold. The implication that the scaffold was osteoinductive was further supported by the fact that other markers previously associated with hFOB trans-differentiation were not expressed; including chondrogenic (collagen type II), osteocytic (E11) and adipogenic (LPL) markers. Therefore these results indicate the CG scaffold provided both osteoconductive and osteoinductive qualities of human cells under standard growth conditions.

2.5 Conclusions

The results of this study demonstrate that the CG scaffold can successfully support the attachment, infiltration, migration and viability of human hFOB pre-osteoblast cells up to 35 days, under standard growth conditions *in vitro*.

Following initial optimisation of hFOB cells on CG scaffold, a viability assay - alamar blue was adapted for use with the hFOB cell line on CG scaffolds. Ultimately, it was shown to be a more useful as a tool to identify general cell viability rather than to accurately calculate cell numbers.

Long term culture of hFOB cells on CG scaffold demonstrated that cells migrated to the centre of the scaffold by 14 days, resulting in a homogenous, confluent construct by 35 days which displayed both osteoconductive and osteoinductive qualities.

CHAPTER 3

Analysis of the ability of a novel collagen GAG scaffold to support osteogenesis by human cells

3.1 Introduction

The CG scaffold has been successfully shown to support attachment and proliferation of various animal cell types including fibroblasts, chondrocytes and neurons (Freyman *et al.*, 2001a; Mc Mahon *et al.*, 2008). Similarly, studies have also shown that CG scaffolds may support *in vitro* osteogenesis of rat MSCs and pre-osteoblasts (Byrne *et al.*, 2008; Jaasma *et al.* 2008). As the CG scaffold is intended for use as a bone graft substitute for patients it is important to gain knowledge regarding its effect on cell behaviour and bone formation by human cells. To date; however, no studies have examined the ability of this CG scaffold to support long term human osteogenesis; this is the focus of the current chapter.

In the previous chapter an analysis was carried out to determine the ability of the CG scaffold to support human pre-osteoblast attachment, proliferation and viability in a long term *in vitro* culture. This chapter builds on these findings to examine if the CG scaffold can support osteogenesis using human cells in a long term *in vitro* culture.

3.1.1 Osteogenesis

Osteogenesis[10] is the process by which a cell differentiates into an osteoblast i.e. a bone forming cell (Fig 3.1).

Fig 3.1 Schematic of MSC differentiation and osteoblast maturation via osteogenesis; modified from Krishnan *et al.* (2006).

In vivo MSCs become directed towards the osteogenic lineage by a combination of chemical and biomechanical cues. The stem cell differentiates towards a pre-osteoblast lineage before maturing into an osteoblast capable of secreting osteoid and mineralising it into bone. The process of osteogenesis may be characterised by identifying temporally regulated bone formation markers such as alkaline phosphatase, collagen type 1, osteopontin, bone sialoprotein, osteonectin, biglycan and osteocalcin. Alkaline phosphatase and collagen type 1 may be described as early bone formation markers and can appear *in vitro* within approximately 2 weeks of culturing under osteogenic conditions (Donahue *et al.*, 2000). Mid stage markers include osteopontin, bone sialo protein and osteonectin, these are often involved with the onset of crystal nucleation. Late stage markers include osteocalcin and the small leucine rich proteoglycan, biglycan, and are expressed in bones and teeth and are important in regulating mineralization (Setzer *et al.*, 2009).

3.1.2 In vitro osteogenesis

Typically, under both 2D and 3D conditions once cells have reached a level of

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[10] For more information on osteogenesis see section 1.8.1

confluency and are stimulated accordingly they can begin to differentiate towards an osteoblastic lineage; at this stage cells cease to proliferate and begin to differentiate. *In vitro* osteogenesis occurs typically after 10 days in 2D and is identified by the same early mid and late stage bone formation markers as to those *in vivo* (Donahue *et al.*, 2000). Mineralisation may be detected using the calcium binding dye alizarin red or the phosphate binding chemical silver nitrate[11] (von Kossa assay). In 3D culture studies the temporal sequence of osteogenesis *in vitro* may vary to that of 2D, with longer culture durations being more common; as a result analysis in 3D usually occurs after 28 days incubation. For example, Donzelli *et al.* (2007) showed that following rat MSC differentiation on tissue culture plates stained for mineral by 14 days whereas Farrell *et al.* (2006) showed mineralisation of rat MSCs on the CG scaffold after 4 weeks.

3.1.3 hFOB 1.19 as a model cell line to study in vitro osteogenesis

The typical bone tissue engineering triad proposes to use the patient's own primary cells (or a primary cell source that will not immunologically compromise the graft) combined with a scaffold to induce healing within a bone defect. Ideally, primary osteoblasts or MSCs would be used in developing the graft; however, for the purposes of assessing osteogenesis it is easier to use a cell line. In order to assess osteogenesis of human cells *in vitro*, a homogenous cell phenotype with controlled growth rate is beneficial. The hFOB cell line was chosen to mimic human osteoblast growth and differentiation on the CG scaffold. These cells are of interest when examining studies related to osteogenesis as they contain a temperature sensitive plasmid (pUCSVtsA48) which allows the user to control levels of proliferation and differentiation; at 34°C hFOB cells exhibit optimal proliferation and at 39°C optimal differentiation (Harris *et al.*, 1995). Once stimulated under osteogenic conditions hFOB have been shown to express

[11] Mineralised matrix in bone is predominantly calcium-phosphate based.

high levels of alkaline phosphatase, collagen type 1, osteopontin, osteonectin, bone sialo-protein and osteocalcin. This sequential expression of osteogenic markers and their ability to form mineralized nodules make them ideal for studying osteogenesis (Donahue *et al.*, 2000).

3.1.4 Osteogenic growth media

Pre-osteoblasts and undifferentiated MSCs require osteogenic stimulation to undergo osteogenesis. These cells can be cultured in a standard growth medium which allows for cell division but does not induce differentiation. Several standard growth media formulations exist and are usually supplemented with 10% foetal calf serum and antibiotics such as penicillin or streptomycin. Without osteogenic factors some cells such as cell lines can be maintained in an immature pre-osteoblastic phenotypic state indefinitely; primary cells on the other hand tend to become more fibroblastic-like and become less responsive to cell stimulation with time.

In vitro osteogenic stimulation is typically provided by the addition of osteogenic growth factors such as ascorbic acid and a phosphate donor to the cell's standard growth medium. Ascorbate (vitamin C) has been shown to aid collagen synthesis (Coelho *et al.*, 2000; Laurencin *et al.*, 1996). A phosphate donor such as β-glycerol phosphate is an *in vitro* requirement for cell maturation and may be required for nucleation (Fratzl-Zelman *et al.*, 1998). Other factors that may be used include vitamin D_3 and K_3, dexamethasone, growth hormone and bone morphogenetic proteins (Jorgensen *et al.*, 2004).

Another factor of interest in stimulating osteogenesis on the CG scaffold is transforming growth factor-β_1 (TGF-β_1). TGF-β_1 is a key regulator of bone, produced by osteoblasts and stored in bone during its formation (Eichner *et al.*, 2002). Most cells in the body respond to TGF where it generally causes inhibitory and immunosuppressive effects to cells; however osteoblasts and chondrocytes become stimulated to up-regulate

cell division and matrix deposition. TGF has been linked with inducing embryogenesis, normal cellular physiology, inflammation and tissue repair. Several studies have shown its importance in bone formation by increasing osteoblast (Yang *et al.*, 2000) and MSC populations (Solheim, 1998). As well as increased cell proliferation TGF has been shown to increase levels of the early bone formation marker alkaline phosphatase of dental pulp cells (Sloan and Smith, 1999; Sloan *et al.*, 1999) and increasing the production of ECM (Shiba *et al.*, 1998). Nie *et al.* (2006) showed that TGF-β_1 formed increased levels of mineralized nodules which were noticeable from 14 days of culture.

TGF-β has been shown to have both a stimulatory or inhibitory effect on cells depending on the concentration used. As a result, different concentrations have been reported as being optimal within the literature for osteogenesis *in vitro*. Centrella *et al.* (1987) found that TGF-β displayed a biphasic effect in subconfluent foetal rat calvarial cell cultures; those cells which were exposed to 1.5 ng/ml of TGF-β significantly increased DNA synthesis and collagen synthesis while treatment with 0.15 ng and 15 ng/ml decreased both parameters. Kenneth (1989) found using mouse pre-osteoblasts that low concentrations (<1ng/ml) provided greatest osteogenic stimulation over high treatments (1-5ng/ml). Zang *et al.* (2003) demonstrated that both primary human osteoblasts and SAOS-2 (osteosarcoma cell line) cells cultured on tivanium (Ti-6Al-4V) implants when exposed to either a low continuous dose e.g. 0.2ng/ml of TGF-β_1 (if given continuously for at least 2 weeks) or by one treatment containing a single high concentration to induce osteogenesis e.g. 10ng/ml provided an optimal osteogenic response. As a result optimal culture conditions for TGF-β_1 need to be established.

3.1.5 Research question

In the previous chapter we showed the CG scaffold was capable of supporting long term osteoblast viability and migration of human pre-osteoblasts under standard growth conditions. In lieu of developing a bone graft substitute, the aim of this study is to investigate the potential of the CG scaffold as a suitable biomaterial to support osteogenesis of human cells with applications for bone tissue engineering. As such this chapter may be described as having two parts; firstly, we aim to examine the ability of the hFOB cell line to undergo osteogenesis in 2D using two osteogenic media formulations versus a standard control media. The osteogenic media formulation used by Harris *et. al* (1995) will be compared with and without the addition of TGF. This is intended to identify the optimal osteogenic growth conditions for the hFOB cell line. Secondly, it is intended to apply this knowledge to determine if the CG scaffold can support osteogenesis of human cells *in vitro* up to 49 days of culture. Analysis will include cell viability, histology, mineralisation, and effects on contraction and mechanical stiffness. Within this study it is envisaged to examine the effect of exposing cells on a CG scaffold to a continuous low dosage of 0.2ng/ml TGF-β_1 versus an exposure using an initial high dosage of 10ng/ml for 7 days and then reducing it to the continuous lower concentration.

3.2 Materials and methods

3.2.1 Optimisation of osteogenic growth media formulations for the hFOB 1.19 cell line (2-dimensional analysis)

3.2.1.1 hFOB seeding and long term osteogenic cell culture (2-dimensions)

$1x10^5$ hFOB cells were seeded per well on a 12 well plate (Nunc, UK). 2ml of prewarmed standard media was added and incubated at 34°C until confluent. Cells were separated into three groups; (1) cells in standard non-osteogenic growth media (as described in section 2.2.1.1) served as a control and will be referred to as the standard group; (2) cells in osteogenic media according to Harris *et al.* (1995); as a result this group will be referred to as the Harris group. This media comprised of standard growth media supplemented with 50µg/ml ascorbic acid, 10mM β-glycerophosphate, 10nM vitamin D_3, and 50nM vitamin K_3 and (3) a third group was formulated using the Harris media plus an additional 0.2ng/ml TGF-β_1[12] (R&D systems, Ireland); known as the Harris +TGF group; once confluent, cultures were incubated at 39°C (for optimal differentiation) up to 21 days. Two thirds of spent media was replenished every 3-4 days.

3.2.1.2 Analysis of osteogenesis in 2-dimensional cultures: alkaline phosphatase assay

The early bone formation membrane bound protein alkaline phosphatase was identified using the substrate p-nitrophenyl phosphate disodium salt (pNPP) (Sigma-Aldrich, UK). The substrate reacts with alkaline phosphatase to produce a water soluble yellow product which absorbs light at 405nm. Following 21 days of culture, the spent media was removed and cells were rinsed with prewarmed PBS. 2ml of a 20% pNPP solution was prepared in prewarmed PBS and added to each well. Plates were incubated

[12] 0.2ml TGF was chosen as it was shown to be beneficial for stimulating human osteoblasts *in vitro* (Zhang *et. al* 2003)

for 30 minutes and samples read at 405nm on a spectrophotometer.

3.2.1.3 Analysis of osteogenesis in 2-dimensional cultures: alizarin red staining and quantification for mineralisation

Following 21 days of culture, the spent media was removed and cells were rinsed with prewarmed PBS. Cells were fixed using iced absolute ethanol for 30 minutes at -20°C. 2ml of a 2% filtered alizarin red (Sigma-Aldrich, UK) solution, pH 4.2 was added to each well for 1 minute. Wells were rinsed several times with distilled H_20. Images were taken using a digital camera. Quantification was carried out by adding 2ml of a prewarmed 10% cetyl-pyridine chloride (Sigma-Aldrich, UK) aqueous solution to each well. Plates were incubated for 10 minutes. Samples were taken and read at 570nm on a spectrophotometer.

3.2.2 Analysis of the ability of a CG scaffold to support human osteogenesis

3.2.2.1 Scaffold seeding and cell culture

CG scaffolds were fabricated as per section 2.2.2.1. A total of $4x10^6$ cells were seeded onto the scaffolds as described in section 2.2.2.2. Culturing took place at 34°C for 14 days to allow for proliferation and migration to the centre of the scaffold and at 39°C thereafter to allow for optimal differentiation. After 14 days cell seeded constructs were replenished with osteogenic media to induce cellular differentiation (this modification was a result from Chapter 2 and was intended to produce a graft with homogenous cell distribution throughout). This comprised of standard media (growth media for proliferation and migration) supplemented with 100µg/ml ascorbic acid, 10mM β-glycerolphosphate, recombinant human TGF-β_1 (R&D Systems, Ireland), 10nM Vitamin D_3 (Sigma-Aldrich, UK) and 50nM Vitamin K_3 (Sigma-Aldrich, UK). To this formulation one of two different TGF-β_1 concentrations were added: a 'low TGF' group which

contained 0.2ng/ml TGF-β_1 throughout exposure period or a 'high TGF' group which contained an initial 10ng/ml TGF-β_1 for 7 days reduced to 0.2ng/ml for the remainder of the experiment. 2/3 of the media was replenished every 3 days and cultures were maintained for 35 and 49 days in total.

3.2.2.2 Cell viability of hFOB cells on CG scaffold:

Metabolic cell viability on the scaffold was determined by replacing media surrounding the cell seeded constructs and replenished with that containing 10% alamar blue dye (Bioscience, Ireland). Scaffolds up to 49 days of culture were incubated on an orbital shaker for 4 hours (n=6). 100µl of media was read using a spectrophotometer at 570nm and 610nm. The percentage of reduced dye was calculated in accordance with manufacturer's recommendations.

In order to determine cell number a Hoechst 33258 DNA assay was carried out. Cell-seeded constructs were digested and homogenised using lysis buffer from the RNA quantification kit (Section 2.2.5.4) and 30µl of the digested scaffolds were mixed with 600µl of a working dye solution made up of Tris, Na_2EDTA, NaCl, distilled water and Hoechst dye solution (Sigma-Aldrich, Germany). 210µl of this mixture was pipetted into the wells of a 96 well plate, providing triplicates of all readings. The fluorescence of the samples was measured at 460nm after excitation at 355nm in the Wallac Victor2™ 1420 multilabel counter (Perkin Elmer Life Sciences, Finland). Fluorescence readings were compared to a standard curve (Appendix A) to give cell number.

3.2.2.3 Analysis of cell distribution and mineralisation within a CG scaffold

Hematoxylin & eosin staining (H&E) was used to analyse cell attachment, infiltration and distribution on the scaffold (n=3) following tissue processing as per sections 2.2.2.5 to 2.2.2.7. Alizarin S red staining for mineralisation on the scaffold (n=3) was carried out following paraffin wax embedding and microtome sectioning (10µm thick); sections were then deparaffinised and stained with 2% alizarin red (Sigma-Aldrich, UK) filtered

solution for 2 minutes. Sections were rinsed several times with dH_20, dehydrated in xylene and mounted with DPX.

Von Kossa staining for mineralisation on the cell seeded CG construct (n=3) was carried out following 35 and 49 days of culture. Scaffolds were tissue processed, paraffin wax embedded and sectioned (10μm thick) as per section 2.2.2.6; sections were then deparaffinised and stained with 2% silver nitrate (Sigma-Aldrich, UK) aqueous solution for 1 hour under bright light. The reaction was stopped by adding the developing solution 1% sodium thiosulphate (Sigma-Aldrich, UK) for 1 minute. Sections were counterstained with 0.5% nuclear fast red (Sigma-Aldrich, UK). Images were taken using a digital camera.

3.2.2.4 Gene expression analysis of hFOB cells on CG scaffold

Cell seeded constructs were flash frozen in liquid nitrogen at each time point and stored at -80°C (n=3). RNA isolation from the constructs was carried out by homogenisation in RLT lysis buffer (Qiagen, Ireland) using a rotor-stator homogeniser (Omni International, Germany). Cell lysates were centrifuged using QI shredder columns (Qiagen, Ireland) and RNA extracted using the RNeasy Mini Kit (Qiagen, Ireland) according to the manufacturers' instructions. RNA concentration per scaffold was quantified using a spectrophotometer (abs 260nm).

Following RNA extraction real-time reverse-transcription polymerase chain reaction was carried out for gene expression analysis as per section 2.2.5.4. The following markers were measured: collagen type I, alkaline phosphatase, osteopontin, osteonectin, biglycan, and osteocalcin. Expression levels were assessed using the relative quantification ΔΔCt method. β-actin[13] acted as a house keeping control.

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[13] β-actin was chosen as the house keeping gene as per Subramaniam *et al.* (2002) which examined the genetic characterisation of the hFOB cell line.

3.2.2.5 Analysis of cell mediated CG scaffold contraction

CG scaffold contraction was analysed aseptically using a sterile vernier calliper to determine cell mediated scaffold contraction (n=3). An average was taken of three sides of each scaffold at 35 days and 49 days post incubation and compared to the original diameter.

3.2.2.6 Analysis of CG scaffold biomechanical stiffness

Wet compression mechanical testing was carried out in order to assess mechanical properties of the constructs following long term osteogenic culture. Cell seeded CG constructs and unseeded CG scaffold controls (n=3) were measured following 49 days of culture; using a Z050 mechanical testing machine (Z050, Zwick/Reoll) fitted with a 5-N load cell (n=6). Constructs from each culture dish were immersed in a PBS bath and tested to 10% strain/minute unconfined wet compression testing. An unseeded CG scaffold was used as a control. The modulus was calculated as the slope of a linear fit to the stress-strain curve over 2%-5% strain.

3.2.2.7 Statistical analysis

Statistical analysis was determined using Sigma statistical software package SigmaStat 3.0. The statistical differences between 2 groups were calculated using the Students t test and multiple groups were calculated using Kruskal-Wallis One Way Analysis of Variance on Ranks (ANOVA). Statistical significance was declared at $p<0.05$.

3.3 Results

3.3.1 Optimisation of osteogenic media formulation for hFOB 1.19 cells: A 2-dimensional differentiation study

3.3.1.1 Alkaline phosphatase analysis for hFOB osteogenesis

Expression levels of the early bone formation marker alkaline phosphatase increased 5 fold in all groups between 7 and 21 days ($p<0.01$); with standard and Harris media groups showed higher levels of ALP expression at 21 days than Harris + TGF ($p<0.01$) (Fig. 3.2). Harris +TGF was statistically significantly lower than standard media or Harris alone at 21 days ($p<0.01$).

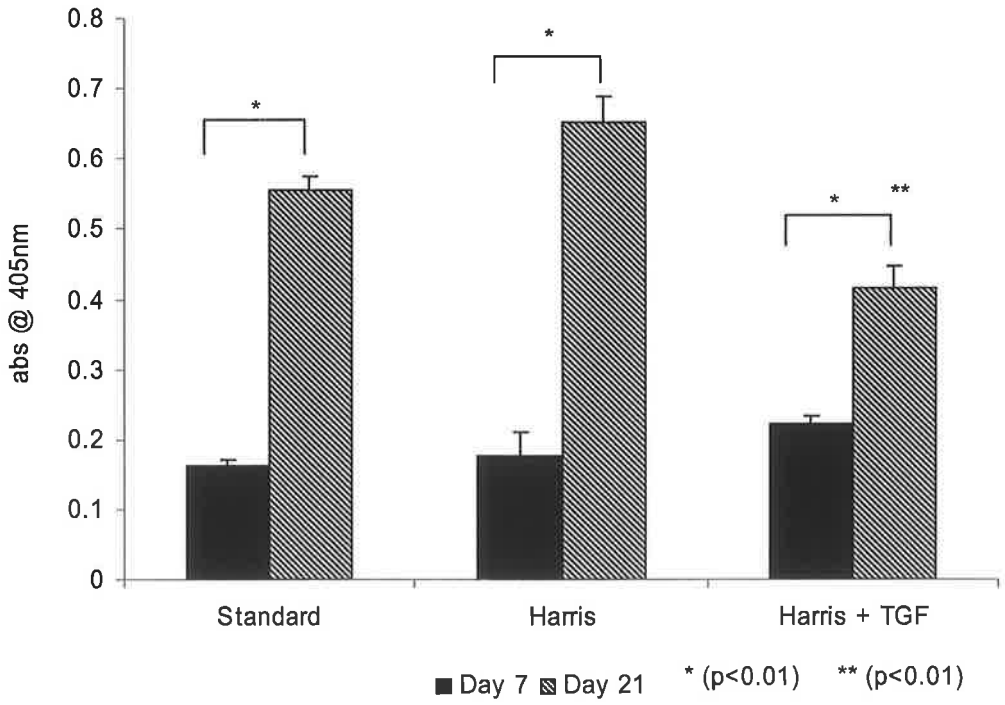


Fig. 3.2 Alkaline phosphatase activity of hFOB cells post 7 & 21 days in Standard growth media, Harris osteogenic and Harris osteogenic +TGF media (n=3). An increase in alkaline phosphatase levels was observed in all groups over time.

3.3.1.2 Alizarin red staining and quantification for mineralisation of hFOB cells

Fig. 3.3 shows visible mineralised nodules formation by mature hFOB cells in the osteogenic Harris and Harris +TGF groups (b & c); but not in the unstimulated standard group (a) following 21 days of culture. Harris + TGF displayed (visibly) greater levels of positive mineral staining (Fig 3.3 f) than standard media alone (Fig. 3.3 d) or with Harris media alone (Fig. 3.3 e).

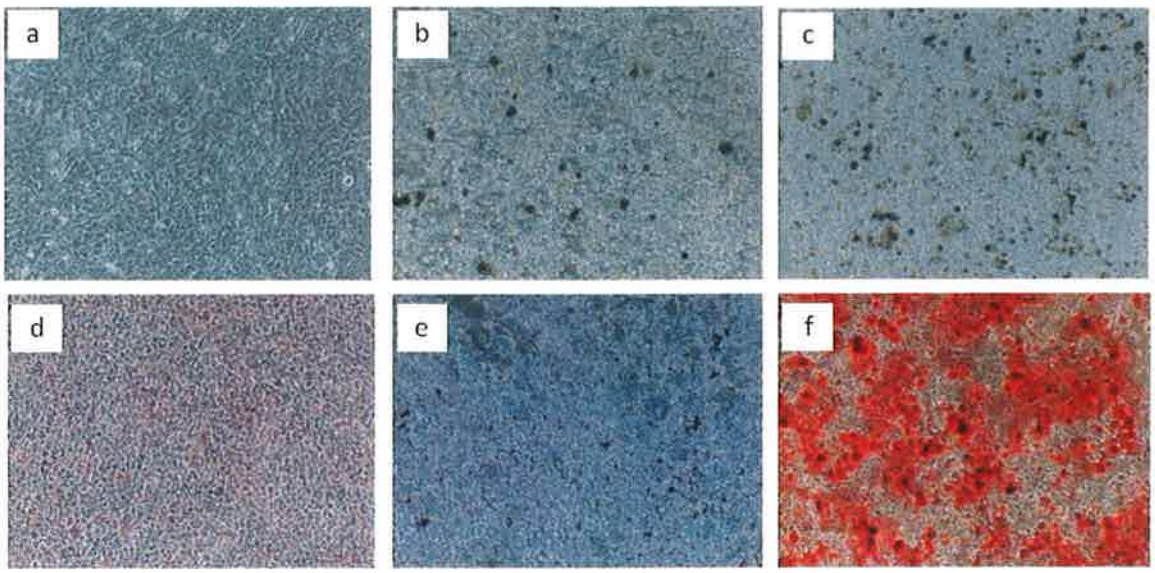


Fig. 3.3 Analysis of nodule formation and mineralisation in hFOB 1.19 following 21 days culture (n=3). (a-c) Unstained wells (d-f) and alizarin red stained wells. Wells were stimulated as follows: (a & d) un-stimulated standard media (b & e) Harris osteogenic media and (c & f) Harris osteogenic media +TGF-β, (n=3; mag 10x).

Figure 3.4 demonstrates an increase in quantified levels of mineral staining at 21 days of culture. Both osteogenic groups provided higher levels of alizarin red mineralisation than standard media alone; quantified as a 4-fold increase between Harris + TGF over standard and a 2 fold increase of Harris over standard ($p<0.01$).

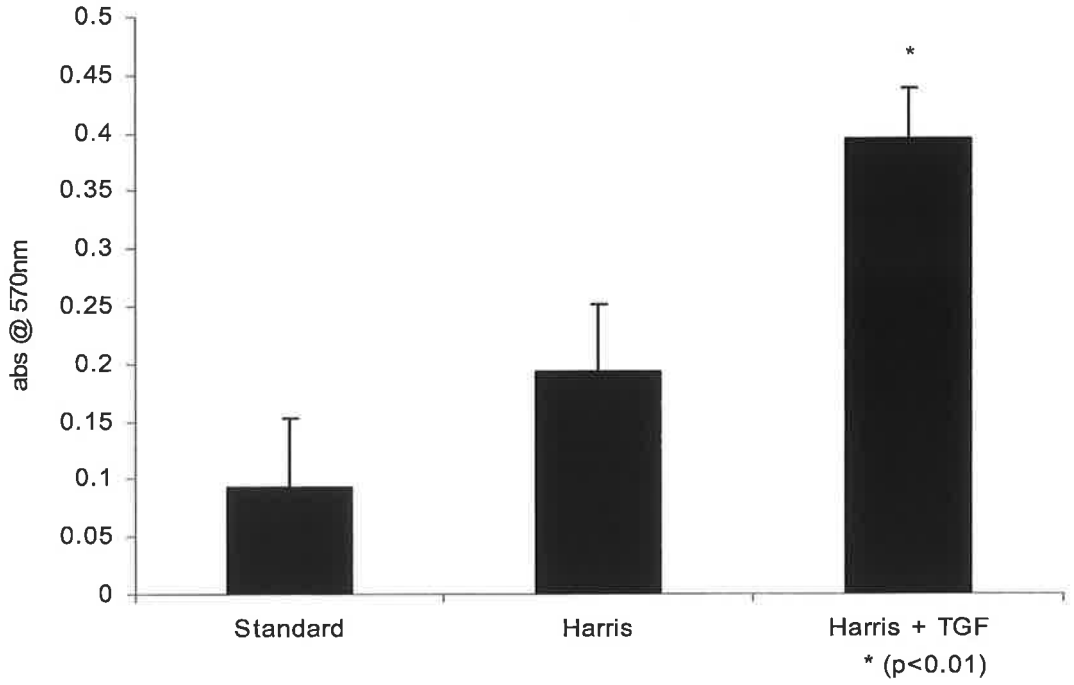


Fig. 3.4 Alizarin red quantification of hFOB 1.19 cells alone following 21 days in Standard growth media, Harris osteogenic and Harris osteogenic +TGF media. Greatest levels were observed in the Harris +TGF group (n=3).

3.3.2 Analysis of CG scaffold to support hFOB long term osteogenesis

3.3.2.1 Viability of hFOB cells on CG scaffold

Alamar blue metabolic cell viability was greater at 35 days than at 49 days for both treatment groups ($p<0.01$) (Fig 3.5). However, no significant difference was observed between TGF groups.

Using the Hoechst DNA standard curve the slope of the line as determined in Appendix A was calculated to be $y = 0.5996x + 4097.7$ ($R^2 = 0.993$). From this the number of hFOB cells on scaffold revealed a decrease in cell number over time with both low and high TGF groups. Cell number reduced between 35 and 49 days in high TGF ($p<0.007$). A 0.46 fold reduction was also observed over time between low and high TGF groups at 49 days of culture ($p<0.01$) (Fig. 3.6).

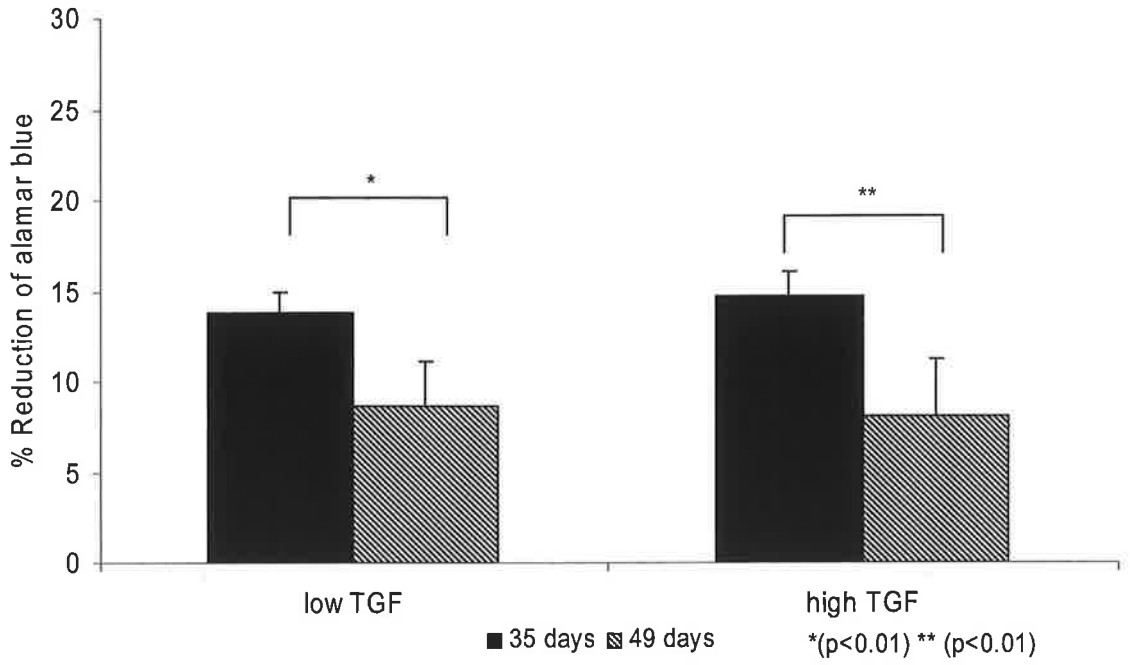


Fig. 3.5 Alamar blue cell viability for hFOB cells on CG scaffold post osteogenic TGF exposure. A significant reduction in metabolic activity was observed between both low and high TGF groups from 35 to 49 days (n=6).

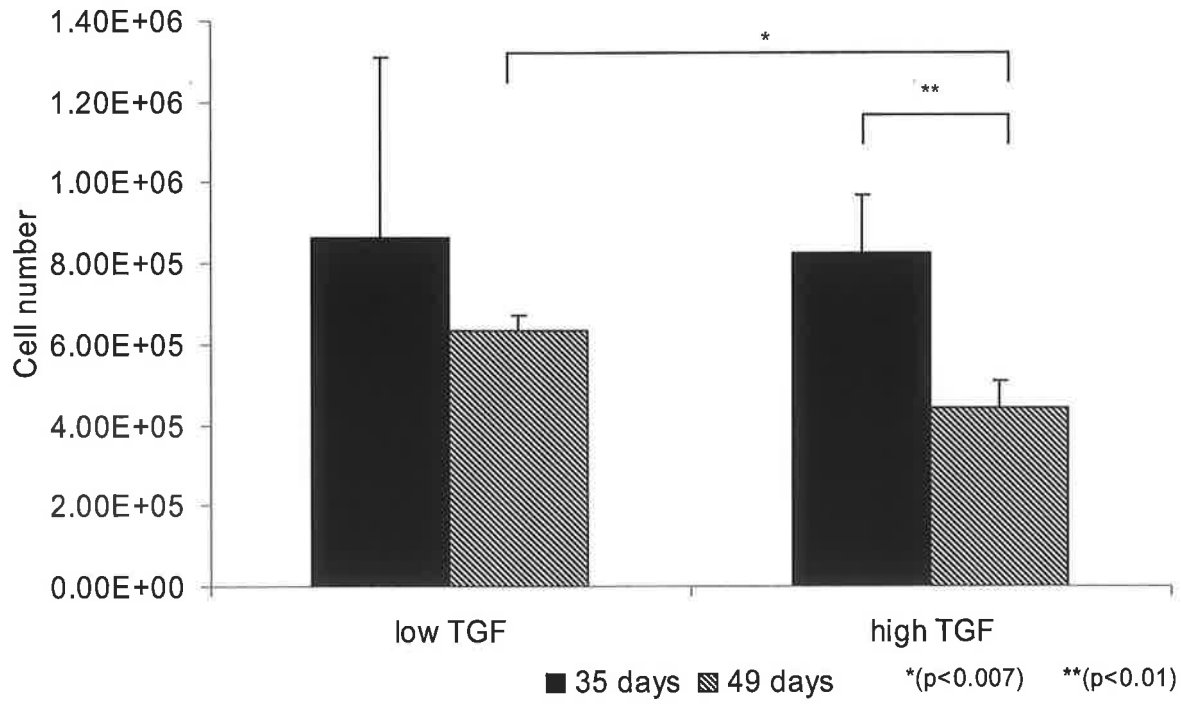


Fig. 3.6 Number of hFOB cells on CG scaffolds following 49 day culture in osteogenic media. A significant reduction in cell number was observed between low and high TGF from 35 to 49 days (n=3).

3.3.2.2 Histological distribution and mineralisation analysis of hFOB cells on CG scaffold

Histological results showed cell attachment, infiltration and uniform distribution throughout the constructs at 35 and 49 days (Fig. 3.7 a-d). An external layer or capsule of cells developed on the surface of the cell seeded construct becoming more prominent with time. There was no histological difference between low or high TGF-β_1 exposure between time points. ECM deposition and a reduction in the original shape and porosity of the constructs were observed at 35 and 49 days. After 35 and 49 days incubation positive mineralisation staining was observed in both TGF-β_1 treatment groups however, high TGF-β_1 treatment resulted in greater mineral staining than low TGF-β_1 as evident by both alizarin red and von Kossa staining (Fig. 3.7 f, j, h, l).

A notable finding of the study was the level of degradation within the construct over time. The homogenous pore structure was obsolete after 35 days of culture due to cell mediated contraction and matrix deposition. By 49 days constructs became hollow at the centre irrespective of treatment groups although it appeared that high TGF groups demonstrated more extensive core degradation than low TGF groups (Fig. 3.8 a-c). Regions in which there are viable cells (Fig. 3.8 a) of the construct surrounded the degraded core; however, mineralization remained evident on the construct (Fig. 3.8 b-c).

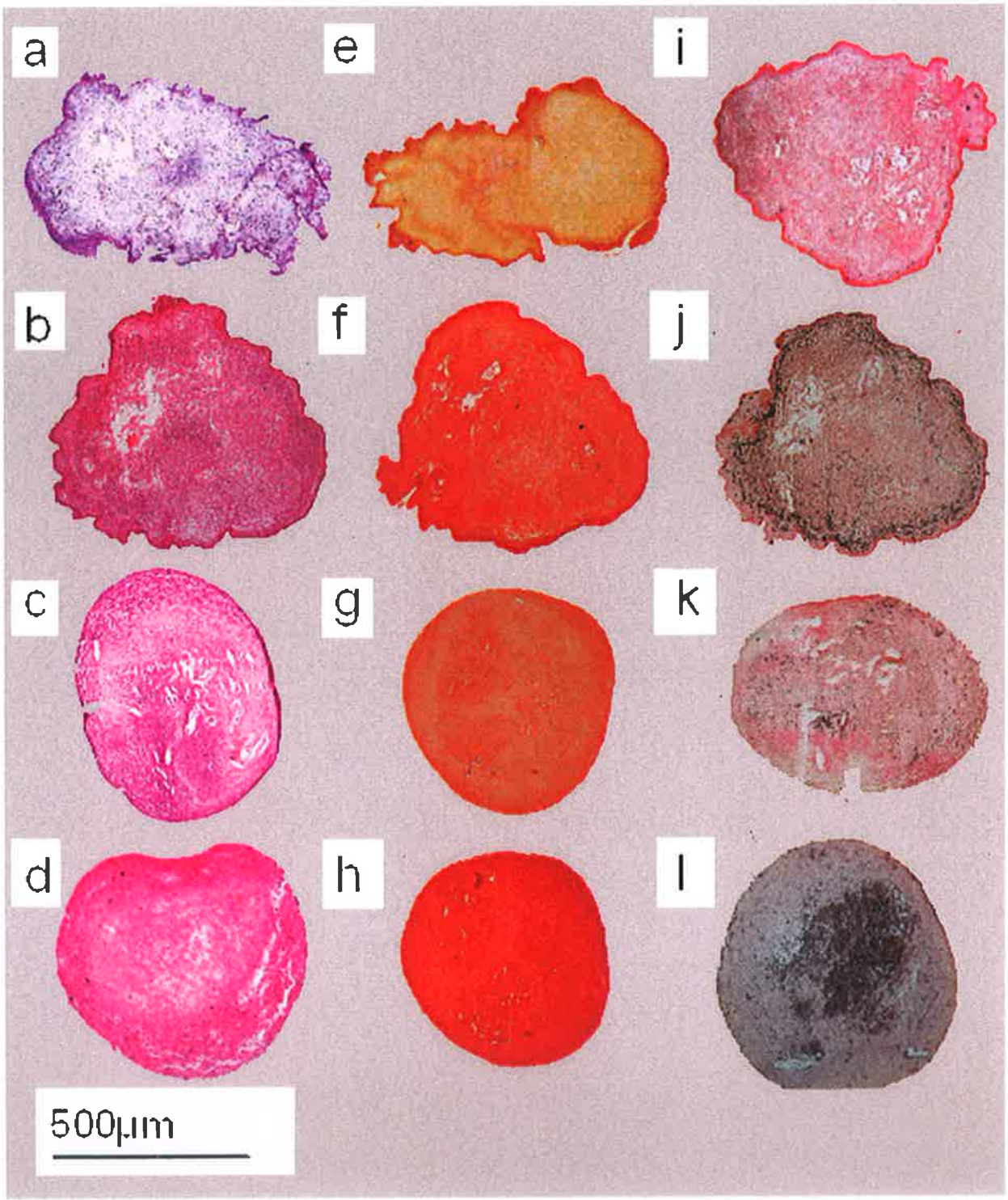


Fig. 3.7 (a-l) Histological and mineral staining of the cell seeded collagen GAG scaffold (magnification 4x). Sections illustrate (a-d) H&E staining (e-h) Alizarin red and (i-l) von Kossa staining for mineralisation. Low TGF-β_1 (a, e, i) and high TGF-β_1 (b, f, j) represent 35 day cultures with 49 days represented as low TGF-β_1 (c, g, k) and high TGF-β_1 (d, h, l). H&E staining (a-d) illustrate highly infiltrated cell seeded constructs at all time points. Alizarin red and von Kossa staining showed greater staining for groups containing high treatment of TGF-β_1; (f, j) at 35 days and (h, l) at 49 days respectively (n=3).

3.3.2.3 hFOB cell mediated scaffold contraction

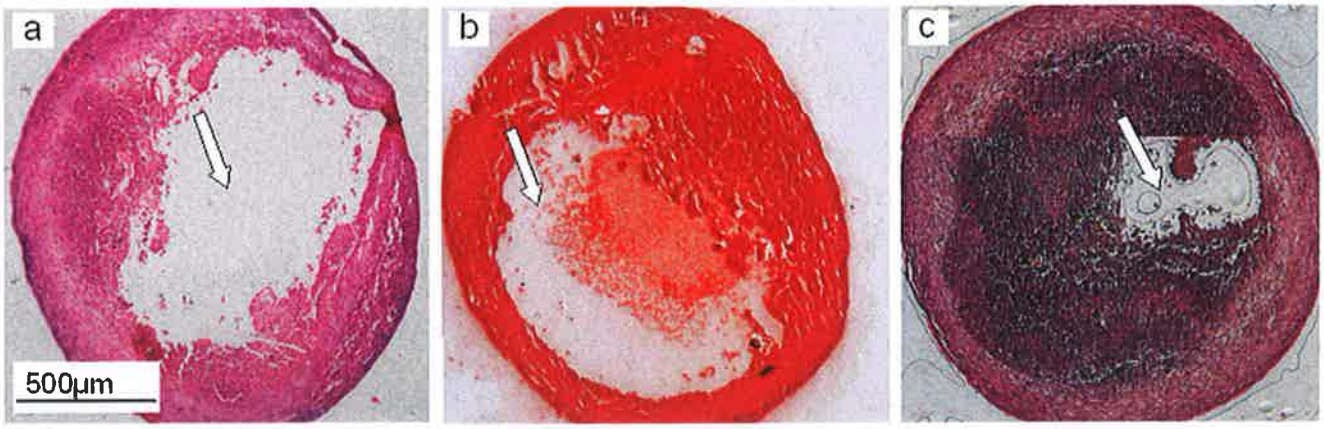


Fig. 3.8 (a-c) Sections of the scaffold centre following a 49 day culture under high TGF-β_1 conditions showing necrotic core region (illustrated by white arrows) surrounded by (a) H&E stained cellular capsule that stained positive for mineralised tissue formation, (b) Alizarin red and (c) Von Kossa staining (n=3; magnification 4x).

Another interesting finding was the level of scaffold contraction over the 49 day culture period (Fig. 3.9). Un-seeded control scaffolds contracted in overall size by 50% of the original size; however, this was more pronounced in cell seeded constructs (by 70% of the original size) at 49 days incubation. Similarly, the scaffolds shape changed over time with the construct becoming more oval at 49 days (~3.5x3mm) from their original rectangular shape (10x10x3mm). Comparable changes in scaffold size and shape were observed in both low and high TGF-β_1 treatment groups.

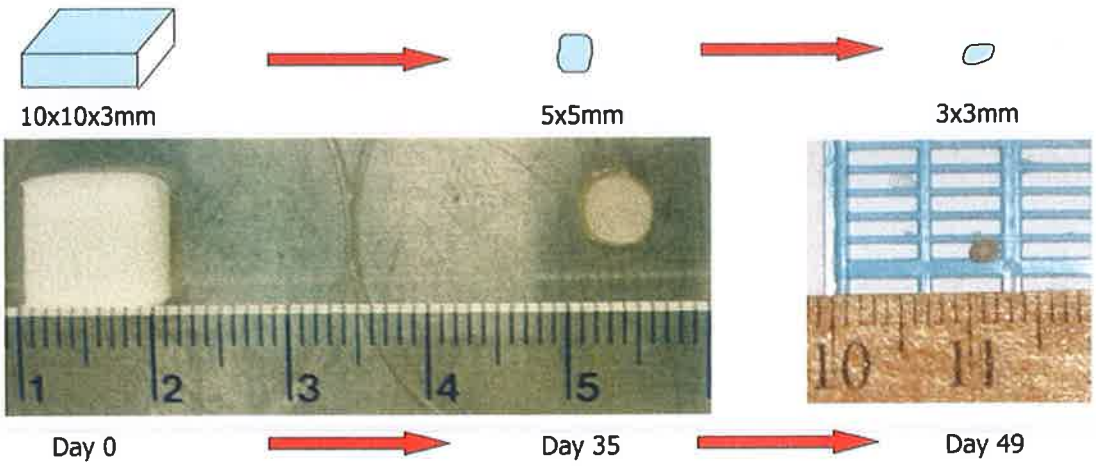


Fig. 3.9 Photographs of scaffold contraction with time; constructs contracted by 70% after 49 days (n=3).

3.3.2.4 Gene expression analysis of hFOB cells on CG scaffold

Collagen type 1 a marker for early stage osteogenesis was expressed in all cultured constructs. Low TGF-β_1 groups at day 35 and day 49 gave greater expression levels of collagen type I than high TGF-β_1 groups; this difference was significant at both 35 and 49 days, reducing by 54% and 60% respectively (p=0.023; p=0.026) (Fig. 3.10 a).

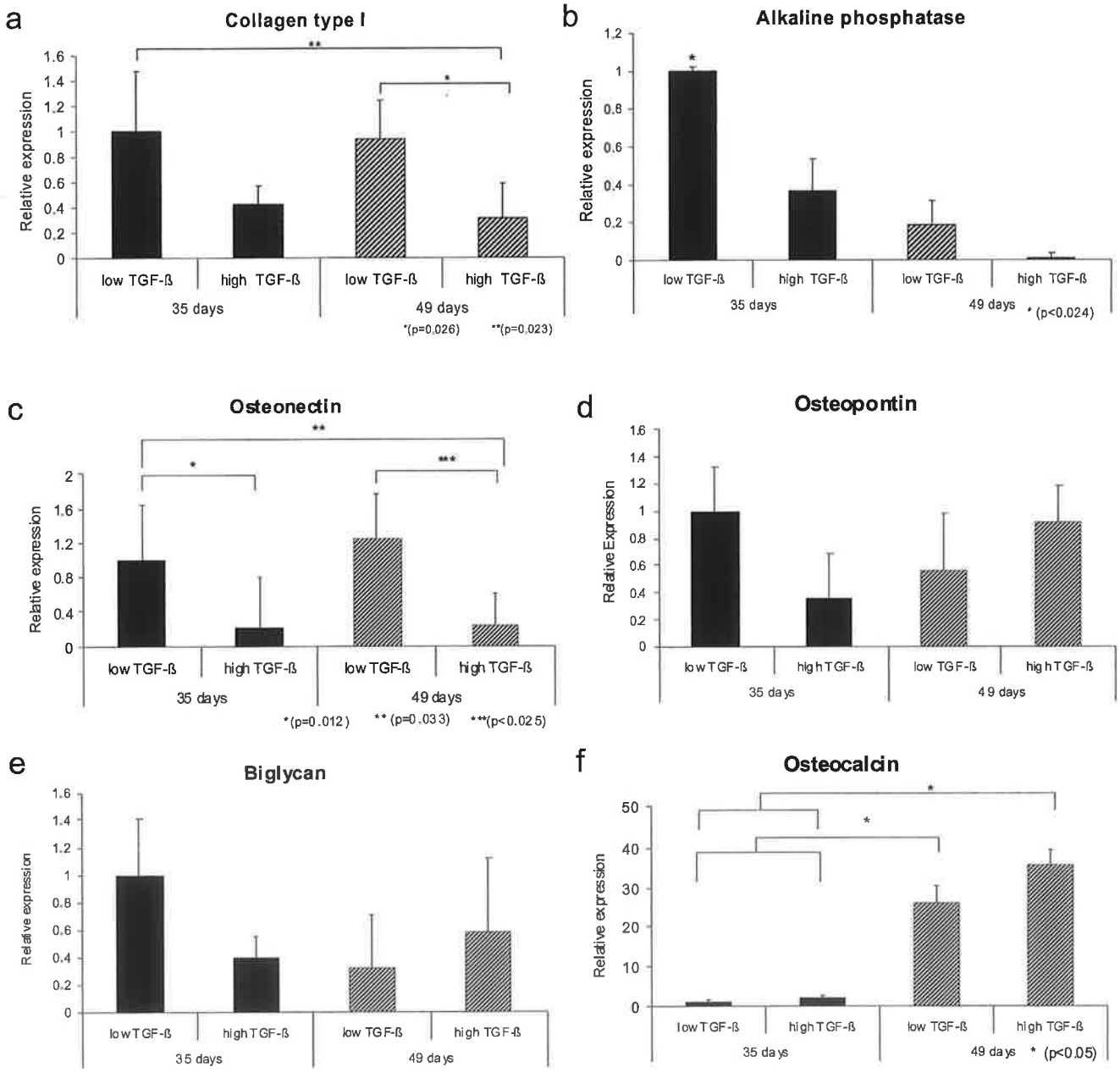


Fig. 3.10 (a-f) Gene expression levels of osteogenic markers for hFOB cells on CG scaffold following 35 and 49 days culture (n=3). High TGF yielded a more mature osteoblastic phenotype with greater expression of the late stage bone formation marker osteocalcin. Expression levels are relative to low TGF 35 days.

Another marker of early osteogenesis, alkaline phosphatase showed similar trends to that of collagen type 1. There was a statistically significant difference in alkaline phosphatase expression between treatment groups; at 35 days an initial high treatment of TGF-β_1 reduced expression levels relative to low treatment of TGF-β_1 by 60% ($p<0.024$). This trend followed at 49 days where both treatments were statistically significantly reduced by 80% (low TGF-β_1) and 99% (high TGF-β_1) when compared to day 35 low TGF-β_1 ($p=0.021$, $p=0.005$) (Fig. 3.10 b).

Osteonectin is an important matrix protein expressed during osteoblast maturation. Expression levels typically show similar trends to that of the early stage markers alkaline phosphatase and collagen type 1. Expression levels of osteonectin decrease by 70% and 84% with high TGF-β_1 over low TGF-β_1 treatment at 35 and 49 days respectively ($p=0.012$; $p=0.025$) (Fig. 3.10 c). Both osteopontin and the TGF-β_1 regulated matrix protein biglycan showed trends of increasing expression levels by 38% and 57% respectively, for high TGF-β_1 treatment over low TGF treatment at 49 days (Fig. 3.10 d-e). Osteocalcin, a marker of late stage osteogenesis and mineralisation, showed a statistically significant increase in expression with time ($p< 0.05$). The higher dose of TGF-β_1 resulted in a 40% increase in the expression of osteocalcin over that of the low dose at 49 days and a 700% increase between day 35 and day 49 with high TGF-β_1 treatment (Fig. 3.10 f).

3.3.2.5 Biomechanical stiffness of CG scaffold constructs

Biomechanical testing of cell seeded CG constructs following 49 days of culture found a 10 fold increase in the stiffness of the tissue engineered constructs for both low ($p= 0.01$) and high ($p< 0.0291$) treatments of TGF-β_1 when compared to unseeded control scaffolds (Fig. 3.11).

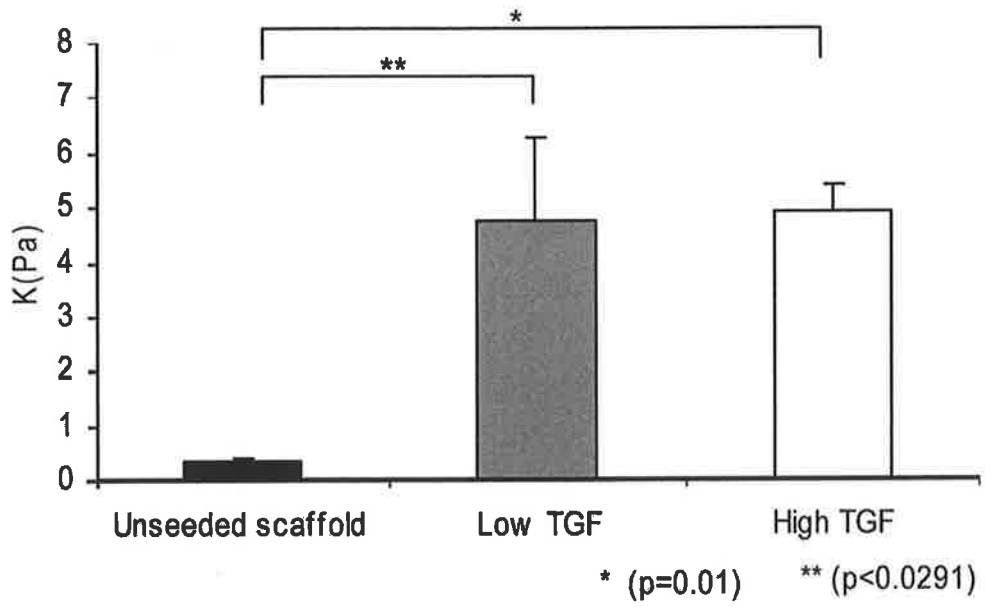


Fig. 3.11 Biomechanical stiffness (in kilo Pascals) of hFOB cells seeded onto CG scaffolds following 49 days culture versus an unseeded CG scaffold control; a 10 fold increase was observed for cell seeded constructs over an unseeded control (n=6).

3.4 Discussion

The aim of this study was to examine the CG scaffolds' ability to support osteogenesis of human cells *in vitro.* Using the human cell line hFOB 1.19, an initial examination to identify the osteogenic potential of these cells in 2D was carried out before applying this to the 3D CG scaffold setting. Results from the 2D hFOB osteogenic analysis showed that hFOB cells can support osteogenesis as previously reported (Harris *et al.*, 1995) This result provided the basis for further analysis of human osteogenesis on the CG scaffold. Although the hFOB cell line has been described as a model cell line for osteogenesis with an ability to form mineralised nodules (Harris *et al.*, 1995); not all studies report this capability (Dhurjati *et al.*, 2006). However, this study demonstrated that hFOB cells could mineralise and produce calcified nodules when stimulated appropriately; similarly, that the addition of TGF-β_1 could enhance the effects of mineralisation following a 21 day culture in the Harris based osteogenic media.

In 2D culture hFOB osteogenesis was confirmed by identifying the early bone formation protein marker alkaline phosphatase and staining for mineralisation using the calcium binding dye alizarin red. Many bone formation markers have been shown to be expressed during osteogenesis in a stepwise sequential manner including alkaline phosphatase (Stein and Lian, 1993). Studies show alkaline phosphatase expression to rise within the first few days of differentiation and usually will peak at about 2 weeks, after which a decline is observed (Donahue *et al.*, 2000). This reduction in expression reflects the maturation of the cell towards a mineralising phenotype, due to the fact that enough phosphate has been released by the action of alkaline phosphatase. In this study a continuous rise in alkaline phosphatase expression in each group was observed up to 21 days of culture without any decline. There was however, less alkaline phosphatase expression in the Harris +TGF group at 21 days when compared with standard or Harris alone; indicating a more mature phenotype for the Harris +TGF group at this stage which was reflected in the mineralisation results .

hFOB cells required osteogenic stimulation. This was evident at 21 days where mineralised nodules were visible only in osteogenic groups; no nodules were identified in standard (un-stimulated) groups (Fig. 3.3). Greater levels of nodules were observed in Harris + TGF-β_1 which also provided greater alizarin red staining over Harris alone. The level of this mineralisation was quantified using the solvent cetyl-pyridinine chloride and as expected cells exposed to Harris + TGF-β_1 provided highest levels of mineralisation; 4-fold greater than standard and 2 fold greater than Harris alone media. The findings from the 2D results were applied to examine the ability of the CG scaffold to support osteogenesis of human cells.

In the 3D osteogenic CG scaffold study it was shown that the CG scaffold can support osteogenesis by human cells. The cell seeded constructs remained viable up to 49 days, and osteogenesis was confirmed by the sequential expression of major bone formation markers coupled with positive staining for mineralisation. The culture period was extended from 35 days to 49 days in total as to accommodate a 14 day pre-culture which allowed the hFOB cells to infiltrate to the centre of the scaffold. This modification occurred from the histological analysis in Chapter 2 and was intended to reduce the development of acellular regions within the construct during differentiation. After 14 days of pre-culture in standard media at 34^{0}C (for optimal proliferation) hFOB cells on the CG scaffold were exposed to osteogenic conditioned media at the raised temperature of 39^{0}C (for optimal differentiation) for a further 21 and 35 days (i.e. total culture period 35 and 49 days).

This osteogenic media contained TGF-β which was shown to effect osteogenesis of the human cells on the scaffold. Where previous studies have reported the use of high concentrations of TGF to inhibit cell differentiation (Kenneth *et al.*, 1989) results of this study showed clearly that providing an initial dose of 10ng/ml was beneficial to simulate osteogenesis a similar finding to that of Zhang *et al.* (2003). High TGF yielded lower levels of the early stage bone formation markers collagen type 1 and alkaline

phosphatase and greater levels of osteocalcin the late stage marker of bone formation with greater staining for mineralisation; indicating that early bone formation markers peaked before 35 days of culture in this group. As the only difference between the two groups (low and high TGF-β_1) was this additional 10ng/ml for the first 7 days of osteogenic stimulation it is possible to report high TGF-β_1 as being beneficial to enhance osteogenesis of hFOB cells on a CG scaffold.

The development of a cell capsule was evident on the surface of the constructs and became particularly noticeable at 49 days. The problem with cells along the periphery of a scaffold occurs when they cause a reduction in diffusion rates of nutrients and wastes (Partap *et al.*, 2009). This becomes a greater issue when cells are stimulated to produce matrix as has been previously reported (Shea *et al.*, 2000). These limited rates of diffusion of nutrients and waste would explain the reduction in cell number over time in both low and high TGF groups and was likely to be a reason for the central core degradation which occurred at 49 days.

Core degradation occurred in both TGF-β_1 groups; however, it was observed to be more pronounced in the higher TGF-β_1 group at 49 days, this would also elucidate to the significant decrease in cell number of high TGF-β_1 over that of low TGF-β_1. This is an important finding when developing a tissue engineered construct and may signify an end point of 35 days for *in vitro* analysis on a CG scaffold given the fact that osteogenesis was identified also at this time point. Knowing the ideal experimental end point for *in vitro* osteogenesis can help with experimental design and should be considered for similar studies. Clinically speaking, both core degradation and cell capsule formation on a biomaterial are best avoided. A bone graft substitute which contains a necrotic central core surrounded by cells would not be beneficial for bone regeneration as it could 1) impede angiogenesis 2) be mechanically unstable and 3) result in fibrosis between the defect edges and construct edges itself. In order to overcome capsule formation and the development of core degradation, custom designed bioreactors may be used to

encourage cellular infiltration at early stages of culture so that scaffolds may become confluent with cells earlier, thus shortening the duration for the onset of osteogenesis. Perfusion flow bioreactors can also be used to enhance nutrient diffusion throughout the scaffold (Jaasma *et al.,* 2008). The ability of a flow perfusion bioreactor to enhance scaffold functionality will be examined in Chapter 5.

The location of the mineral staining reflected cell distribution. Previous studies using rat MSCs on the CG scaffold have also reported this finding; where mineralisation resided along the cellular periphery of the scaffold (Farrell *et al.*, 2006). As the 14 day pre-culture allowed cells to migrate into the centre of the CG construct, histological staining revealed that by 35 days there was a homogenous cell distribution throughout the construct which in turn stained for mineral.

A significant reduction in construct size was observed during the long term incubation; where a reduction in size of 70% was observed at 49 days irrespective of TGF treatment. CG construct contraction may result in a loss of contact between the implanted graft and the surrounding host tissue making integration of the repair tissue difficult (Lee *et al.*, 2001). Contraction therefore could be limited by increasing the initial stiffness of the construct using crosslinking methods which should reduce scaffold contraction during culture; this is the focus of Chapter 4. It should be noted that some construct contraction may however, be beneficial to promote osteogenesis by providing mechanical stimuli to cells during contraction *in vitro* (Freyman *et al.*, 2001b). Coupled with construct contraction, there was an increase in mechanical strength at 49 days of culture. This increase in stiffness was attributed to a combination of matrix formation following the induction of osteogenesis on the scaffold and contraction of the scaffold assisted by the action of the osteoblasts and is likely to be linked with the observed lower cell viability and increased contraction levels in the mature osteogenic mineralised cell seeded CG construct.

3.5 Conclusion

The results from this study demonstrate that the CG scaffold can successfully support osteogenesis of human hFOB cells and form mineralised tissue *in vitro*. Osteogenesis of hFOB was shown to occur in a temporal sequential manner in both 2D and 3D environments.

The effect of TGF-β_1 was examined and found to enhance osteogenesis. In the CG scaffold optimal osteogenic results occurred when using an initially high exposure of 10ng/ml TGF for 7 days and reducing this to 0.2ng/ml thereafter. Cell seeded CG constructs were found to remain viable with fully infiltrated homogenous cell distribution up to 49 days. Cell seeded constructs were found to undergo high levels of cell mediated contraction and increased in compressive modulus with time. However even in the presence of this core degradation mineralisation was shown to be uninhibited with highest levels late stage osteogenic gene expression and mineralisation detected in the highly confluent cellular outer region of the scaffold. In conclusion these findings successfully show the ability of the CG scaffold to support osteogenesis of human cells.

CHAPTER 4

Substrate stiffness and contractile behaviour modulate the functional maturation of osteoblasts on a collagen GAG scaffold

4.1 Introduction

In the previous chapters growth and osteogenesis of human cells were shown to be supported on a CG scaffold. During these long term incubations, changes in the scaffold structure and size were observed with constructs contracting in culture up to 70%. Given that physical properties of a scaffold like pore structure, construct contraction and mechanical strength can have important roles in cellular growth and function (Lo *et al.*, 2000; O' Brien *et al.*, 2005); this chapter is concerned with investigating the effect of substrate stiffness and cell mediated contractile behaviour on osteogenesis within the CG scaffold.

4.1.1 Substrate stiffness

A substrate's stiffness is a physical property known to affect cellular adhesion, proliferation, infiltration and phagocytic cellular processes (Leipzig and Shoichet, 2009). Cells bind to substrates (scaffold/biomaterial or ECM) at specific recognised adhesive ligands using transmembrane receptors called integrins (Fig. 4.1). Force sensing and transduction is assessed by these cellular contacts and cells can respond via mechanoregulatory processes which are being currently examined by the scientific community. *Hapotaxis* is a substrate adhesion model which refers to the tendency of cells to migrate along gradients of substrate adhesion ligand density. Similarly, *durotaxis*

refers to the tendency of cells to migrate along gradients of substrate stiffness (Engler *et al.*, 2004a). Not all cells respond to substrate stiffness in the same way; however, MSCs, fibroblasts and epithelial cells show increased cell spreading, adhesion and proliferation on stiffer scaffolds.

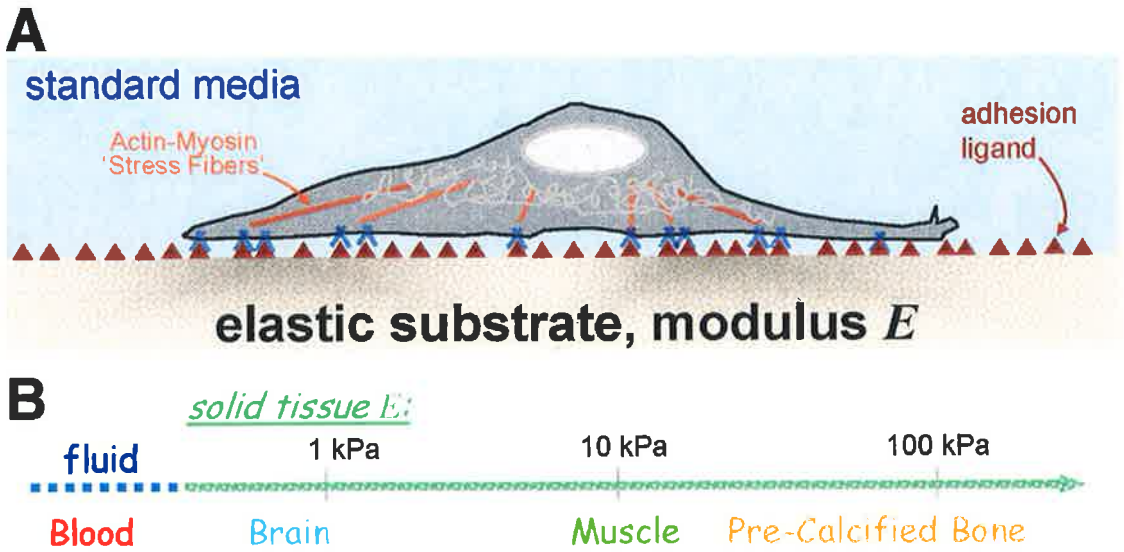


Fig 4.1 Cells on elastic substrates that model tissue elasticity. A) Sketch of a model in vitro environment of a cell on a substrate of elasticity E, coated with ligands that are specifically recognized by cell adhesion receptors. Force sensing and transduction is mediated by these contacts. Biochemical stimuli are also provided by factors in the surrounding media. B) Elasticity of various solid tissues, and blood as a "fluid tissue" (Rehfeldt *et al.*, 2007).

In 2D substrate studies it has been shown that a relationship exists between stiffness and cell differentiation; where soft substrates were shown to compel a neurogenic lineage whereas stiffer bone-like substrates (>34kPa) commit to osteogenic lineages (Pek *et al.* 2010; Reilly and Engler 2010). Other studies have shown that more compliant substrates appear to promote cellular differentiation over cell proliferation and motility e.g. MSC neural differentiation has been shown to be best at a low substrate stiffness of less than 7 kPa; stiffness levels higher than this resulted in an increased level of proliferation (Leipzig and Shoichet, 2009).

Although osteoblasts are quite sensitive to mechanical changes in their external environment to date; however, no long term 3D osteogenic studies have examined this

relationship of osteoblasts to substrate stiffness for bone tissue engineering.

4.1.2 Scaffold contraction

An important property of the CG scaffold is its ability to contract during *in vitro* culture (Freyman *et al.*, 2001b; Menard *et al.*, 2000; Schulz Torres *et al.*, 2000; Spilker *et al.*, 2001). Contraction is often clinically viewed as a potential limitation of the properties of a scaffold, linked with structural instability within a defect. However, *in vitro* contraction has been linked with the production and maintenance of ECM and may benefit tissue formation (Menard *et al.*, 2000). As a result, these contractible CG scaffolds are mechanoactive and have been shown to be beneficial for chondrogenesis and nerve wound healing (Lee *et al.*, 2001; Spilker *et al.*, 2001; Zaleskas *et al.* 2004).

Contraction may occur naturally in a biomaterial or by the effort exerted by contractile cells. Cell mediated contraction is often linked with the expression of the contractile protein smooth muscle actin–α (SMA-α). SMA-α has been clinically demonstrated to be the leading cause for fibrous contracture of the hand (Dupuytren's contracture) (Hartwig *et al.*, 1995). Typically SMA-α is associated with contractile cells like myocytes however, it has been reported over the past three decades to be expressed in fibroblasts (Kinner *et al.*, 2002a) and more recently in osteoblasts, chondroblasts and MSCs (Kinner *et al.*, 2002b). Research is now focusing more on the reason why these typically non-contractile cells express contractile proteins.

Studies have imaged fibroblast contraction of a CG strut (Corin and Gibson, 2010; Freyman *et al.*, 2001b) (Fig 4.2). Freyman *et al.* (2001) showed that fibroblasts buckle struts of collagen by applying 10% strain the force of which was calculated to be 20-45nN for a CG scaffold with a pore size of 96-151μm according to findings by Harley *et al.* (2008). Using a finite element computational model Corin and Gibson (2010) found fibroblast contraction of CG scaffolds was effected by varying pore sizes and cell density where fewer cells resulted in a lower buckling force exerted.

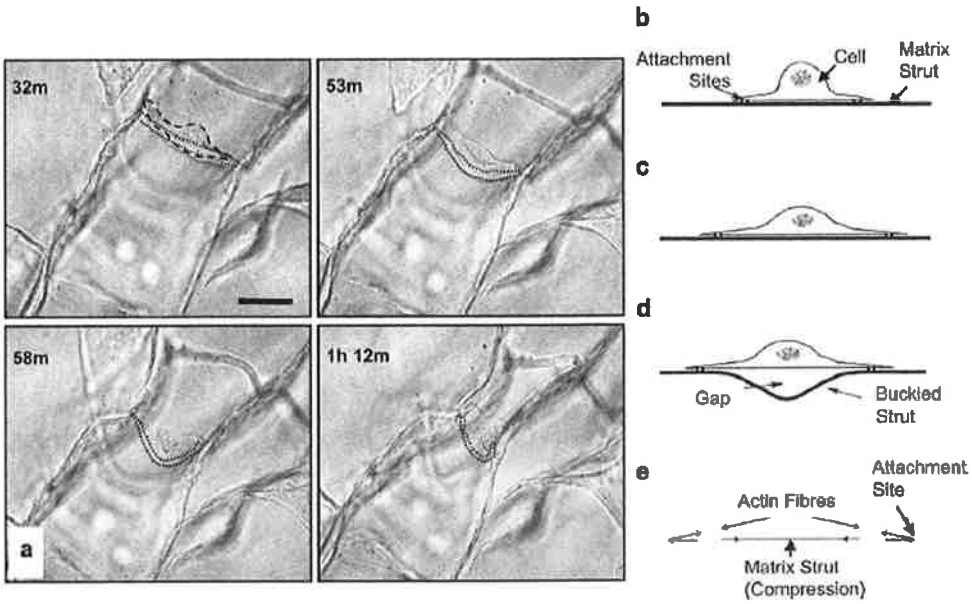


Fig 4.2 (a) Micrographs of dermal fibroblasts buckling a strut in a CG scaffold; Scale bar 50μm. (b-e) Schematic of cell buckling the scaffold strut (Corin and Gibson, 2010; Freyman *et al.*, 2001b).

4.1.3 Effect of cross-linking on CG scaffold stiffness

A CG scaffold's stiffness and its ability to contract can be controlled by crosslinking the material (Dado and Levenberg, 2009; Engler *et al.*, 2004a; 2004b) (see also section 1.7.2). Crosslinking strengthens substrates by introducing extra molecular bonds; in collagen for example this can increase stiffness by restricting collagen fibres from sliding over each other during mechanical loading. Different crosslinking methods exist and are classified into being either physical or chemical treatments. Dehydrothermal (DHT) treatment is a physical cross-linking process which removes water molecules and creates cross-links by esterification and amide formation (Weadock *et al.*, 1983). Glutaraldehyde (GLUT) is chemical crosslinker which has traditionally been used for reducing the antigenicity of bioprosthesis since the late 1960s and has been shown to be an effective cross-linker of collagen (Lynn *et al.*, 2004; Olde Damink *et al.*, 1996). It crosslinks collagen by bridging amine groups between adjacent polypeptide chains and has been shown to provide greater fibroblast infiltration and vascularisation (Wu *et al.*,

2004). GLUT-treated materials often have a tendency to calcify. Furthermore residual GLUT may also restrict cell growth and viability (Lee *et al.*, 2001). EDAC is a carbodiimide which acts as a catalyst in the presence of N-hydroxysuccinimide (NHS) and forms bonds between collagen fibres and glycosaminoglycans in CG scaffolds, it is not incorporated itself into the collagen scaffold but is converted to 1-ethyl-3-(3-dimethyl aminopropyl)-urea. Increased substrate stiffness can also slow down the overall degradation of a material thus improving the structural integrity of the scaffold (Pieper *et al.*, 2000).

Research question

To date, it is not yet known how substrate stiffness and the ability of a scaffold to contract may affect *in vitro* bone formation and osteogenesis. Therefore, the objective of this study will be to compare the effects of substrate stiffness and contractile behaviour on osteoblast cells within 3D CG scaffolds. This study will examine the effect of DHT, EDAC and GLUT-treated CG scaffolds on long term osteogenesis and mineralization using the mechanosensitive MC3T3 pre-osteoblast which have been shown to develop into mature osteogenic mineralizing osteoblasts *in vitro* (see Appendix A) and recently, to express the contractile protein smooth muscle actin-α (SMA-α) and to contract collagen matrices (Fratzl-Zelman *et al.*, 1998; Spilker *et al.*, 2001). The MC3T3 cell line was chosen to coincide with preliminary studies within our research group; it is intended to apply the information obtained from this study to further experiments using human cells like hFOBs.

An investigation of MC3T3 cells on CG scaffolds with varying substrate stiffness will be carried out by examining osteoblast growth and differentiation identified by analyzing early, mid and late stage bone formation markers and the cell contractile related gene expression.

4.2 Materials and methods

4.2 Effects of substrate stiffness and contraction in a CG scaffold on osteogenesis

4.2.1 CG Scaffold fabrication and scaffold cross-linking

Scaffolds were fabricated as per section 2.2.2.1. Following fabrication CG scaffolds were aseptically cut to size (10x10mm). Three different crosslinking techniques were used:

A) Dehydrothermal (DHT) physical crosslinking treatment. Briefly scaffolds were placed in an aluminium foil bag in a vacuum oven (Fisher IsoTemp 201, Fisher Scientific, Boston, MA) at 50mTorr for 24 hours at 105°C (Chamberlain *et al.*, 1998b; Yannas *et al.*, 1989).

B) 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDAC) cross-linking was carried out by first pre-hydrating scaffolds PBS at 4°C for 24 hours. Then a 2ml filtered solution containing 6mM EDAC per gram of collagen and 2mol N-hydroxysuccinimide (NHS) (Sigma-Aldrich, Germany) in distilled H_2O was added and scaffolds were incubated for 2 hours at room temperature. Scaffolds were rinsed several times with sterile PBS prior to cell seeding (Engler *et al.*, 2004b).

C) Glutaraldehyde (GLUT) chemical crosslinking was carried out by first pre-hydrating scaffolds in PBS at 4°C for 24 hours. Each pre-hydrated scaffold was immersed in a 2ml PBS solution containing 0.125g GLUT (Sigma-Aldrich, Germany) per gram of collagen and crosslinked for 24 hours at room temperature, scaffolds were rinsed several times using sterile PBS to remove residual GLUT.

4.2.2 Cell culture and cell seeding on CG scaffold

MC3T3 (ATCC, MA) pre-osteoblastic cells were cultured under standard conditions (5% CO_2; at 37°C). Cells were routinely grown to ~80% confluency in T175 culture flasks (Sarstedt, Germany) containing culture media; 89% α-MEM (Gibco, UK), 10% FBS

(Sigma-Aldrich, Germany), 1% (10mg/ml) penicillin/streptomycin (Sigma-Aldrich, Germany). Once confluent $1x10^6$ MC3T3 pre-osteoblasts were seeded on both sides of DHT, EDAC and GLUT-treated scaffolds. Constructs were cultured under osteogenic conditions i.e. standard MC3T3 media supplemented with 50μg/ml ascorbic acid and 10mM β-glycerolphosphate (Sigma-Aldrich, Germany) for 2, 4 or 6 weeks. Constructs were placed into 6-well culture plates with 5ml osteogenic media. 2/3 of the media was replenished every 3 days.

4.2.3 Analysis of mechanical testing and scaffold contraction on CG scaffold

Cell seeded constructs were mechanically tested as per section 3.2.2.6 at 2, 4 and 6 weeks of culture (n=6). Scaffold diameter was measured at 2, 4 and 6 weeks of culture post incubation and compared to the original diameter as per section 3.2.2.5 (n=3).

4.2.4 Analysis of cell viability for cells on CG scaffold:

Cell number was assessed using the Hoechst 33258 DNA assay as per section (n=3) 3.2.2.2. Metabolic activity was assessed for percentage reduced dye at 2, 4 and 6 weeks as per section 2.2.5.2 (n=6).

4.2.5 Analysis of histological and mineralisation staining of cells on CG scaffold

Hematoxylin & Eosin (H&E) staining was carried out post fixation, tissue processing, wax embedding and sample sectioning (section 2.2.1.6) at 2, 4 and 6 weeks as per section 2.2.1.9 (n=3). Alizarin red staining was carried out post fixation, tissue processing, wax embedding and sample sectioning at 2, 4 and 6 weeks as per section 3.2.1.3 (n=3).

4.2.6 Analysis of gene expression

Constructs were flash frozen in liquid nitrogen at each time point and stored at -80°C. RNA was isolated and quantified from cell seeded constructs prior to reverse transcription polymerase chain reaction for gene expression analysis as per section

3.2.2.4 (n=3). Analysis of expression of mouse bone formation markers was carried out, specifically, for early markers collagen type I and alkaline phosphatase, mid/late stage markers, osteopontin and osteocalcin and the contractile marker smooth muscle actin-α (SMA-α). Expression levels were assessed using the relative quantification ΔΔCt method. 18S acted as a house keeping control.

4.2.7 Statistical analysis

Statistical analysis was determined using sigma statistical software package SigmaStat 3.0. The statistical differences between 2 groups were calculated using the Students t test and multiple groups were calculated using Kruskal-Wallis One Way Analysis of Variance on Ranks (ANOVA). Statistical significance was declared at $p<0.05$.

4.3 Results

4.3.1 Biomechanical analysis of CG scaffold

Following initial crosslinking an increase in the mechanical stiffness of all CG scaffolds was observed compared to the non-crosslinked control (0.4 kPa). Both EDAC and GLUT displayed similar levels of stiffness (EDAC 1.07 ± 0.89 kPa; and GLUT 1.29 ± 1.5 kPa) showing over a 2-fold significant increase over DHT treated scaffolds (0.48 ± 0.45 kPa) ($p<0.01$) (Fig. 4.3). There was a significant 2.65-fold increase in the stiffness of the DHT scaffold over the 6 week culture period ($p=0.01$); this increase was not observed in either EDAC or GLUT treated CG scaffolds.

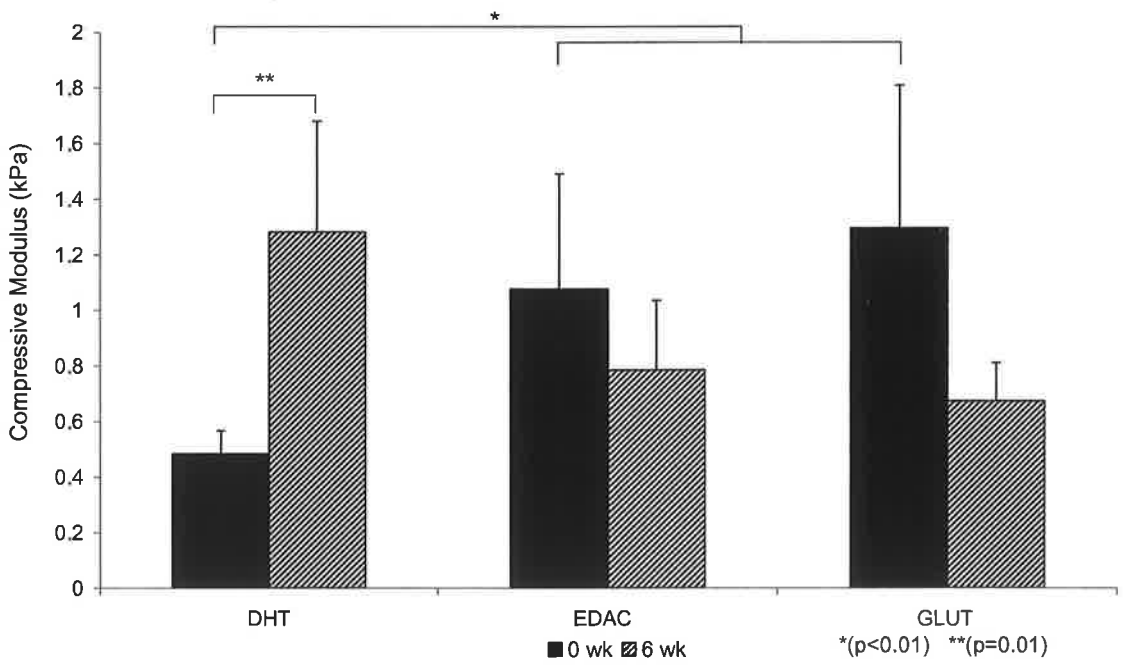


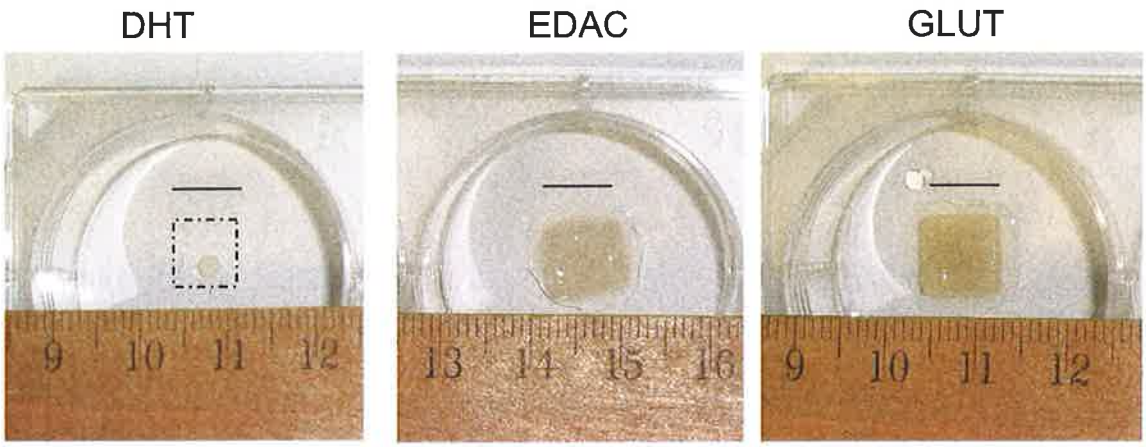
Fig. 4.3 Compressive modulus of CG scaffolds crosslinked with DHT, EDAC and GLUT treatments. Crosslinking increased CG scaffold mechanical stiffness. Following culture no difference was observed in EDAC or GLUT however a 2.65 fold increase occurred in DHT groups (n=6).

4.3.2 Analysis of CG scaffold contraction

The stiffer EDAC and GLUT treated constructs resisted cell-mediated contraction; retaining their shape and size after 6 weeks of culture with construct contraction

observed only in the DHT groups (Fig. 4.4 a). DHT constructs contracted by 50% (from 10mm to 5mm) within 2 weeks of culture and by 6 weeks constructs had contracted by 70% (3mm) ($p<0.01$) (Fig. 4.4 b). A statistically significant difference was observed between DHT treatments and GLUT or EDAC treatments at 2, 4 and 6 weeks ($p<0.01$).

a



b

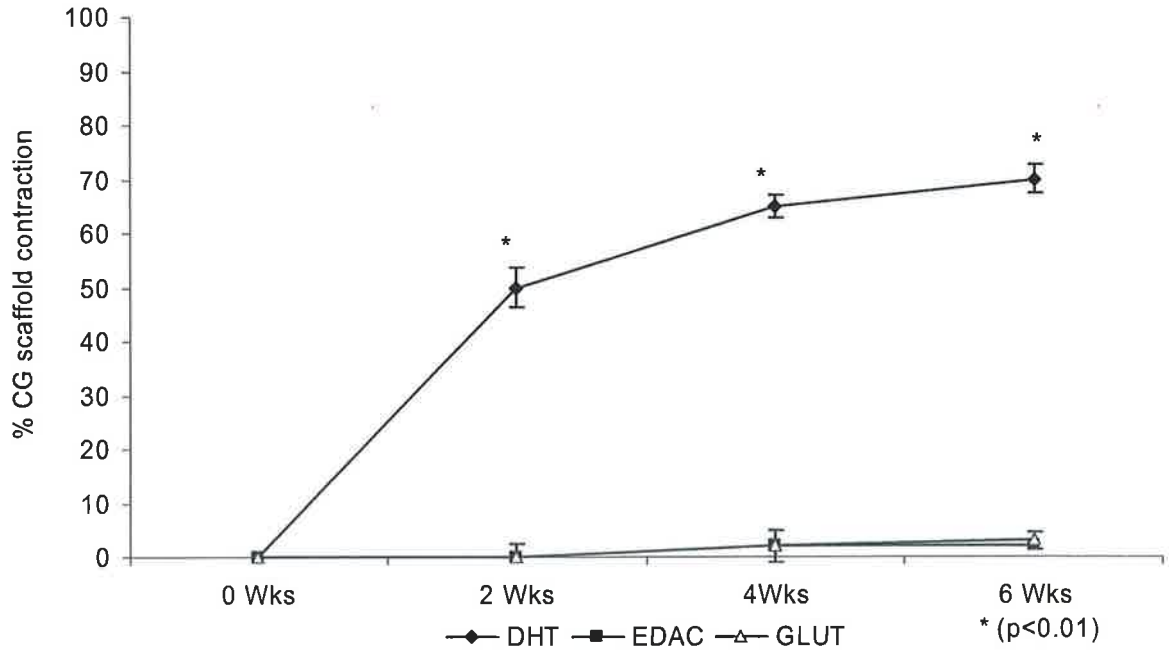


Fig. 4.4 (a-b) Percentage change of diameter from the original size over time of cell seeded CG construct contraction (n=6). (a) Photographs of construct contraction of different crosslinked CG construct seeded with MC3T3 cells following 6 weeks of culture (n=6). DHT scaffolds permitted contraction where EDAC and

GLUT resisted contraction over time. Scale in cm. (b) Graphical representation of percentage CG scaffold contraction

4.3.3 Analysis of cell viability on the CG scaffold

Cells remained viable on the scaffolds at all time points irrespective of crosslinking treatment. DNA analysis found a lower cell number in DHT constructs than in EDAC or GLUT groups ($p<0.003$) (Fig. 4.5a). Metabolic viability reflected cell number where all constructs were viable up to 6 weeks and EDAC and GLUT groups yielded a 2 fold greater viability than DHT scaffolds ($p<0.001$) (Fig. 4.5b).

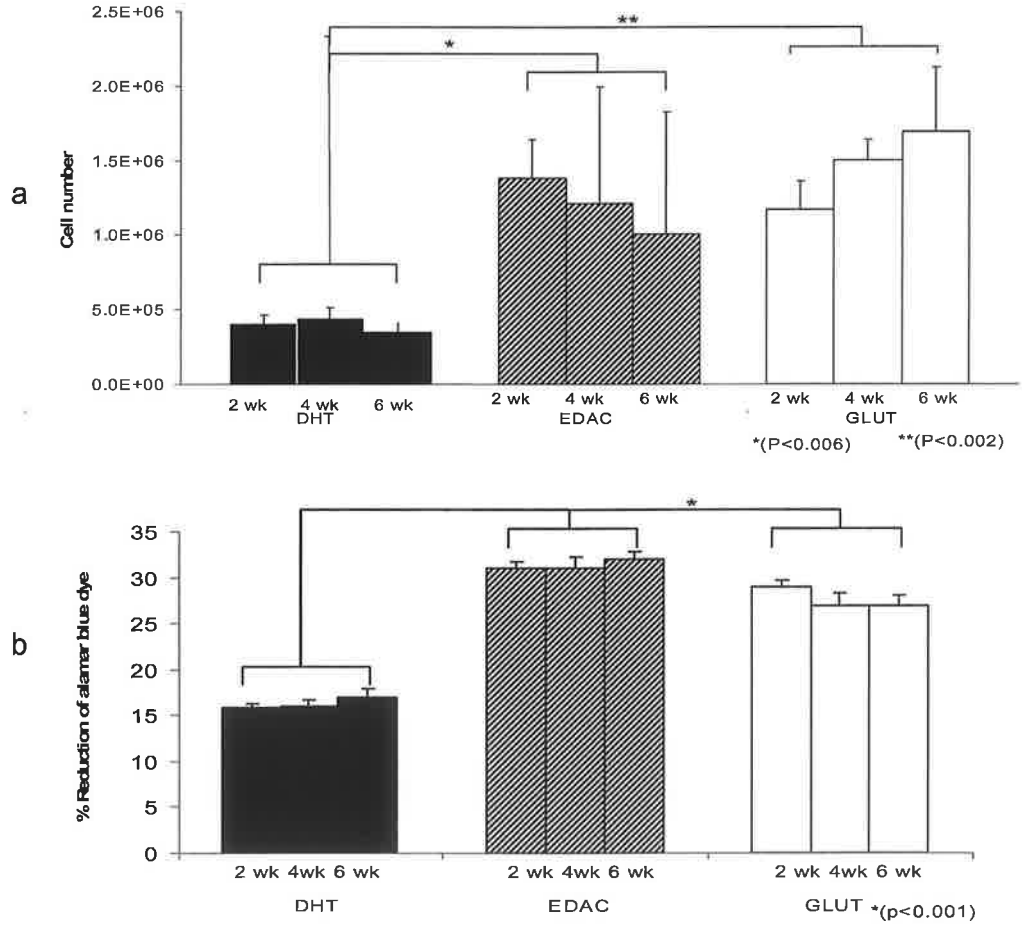


Fig. 4.5(a-b) Shows cell viability on CG scaffolds with varying crosslinking methods; (a) illustrates Hoechst DNA analysis of cell number on CG scaffold over time (n=3) (b) metabolic viability using alamar blue (n=6). Both cell number and metabolic activity remained constant over time between groups however, a 2 fold increase was noted in EDAC and GLUT over DHT scaffolds.

4.3.4 Histological analysis and cell distribution of CG scaffolds

All CG constructs provided for cell attachment and infiltration with time (Fig. 4.6 a-i). At 2 weeks, histological analysis showed a similar level of infiltration for all construct types; cells resided mainly on the periphery of the constructs with gradual migration evident (Fig. 4.6 a-c). The DHT constructs at 4 weeks showed a noticeable visual reduction in porosity as a result of cell mediated contraction and increased levels of matrix production (Fig. 4.6d). At 4 weeks, the EDAC and GLUT treated constructs showed cells predominantly near the surface of the constructs; these groups retained a highly porous scaffold structure (Fig. 4.6 e-f). At 6 weeks, cells were distributed throughout all scaffold types.

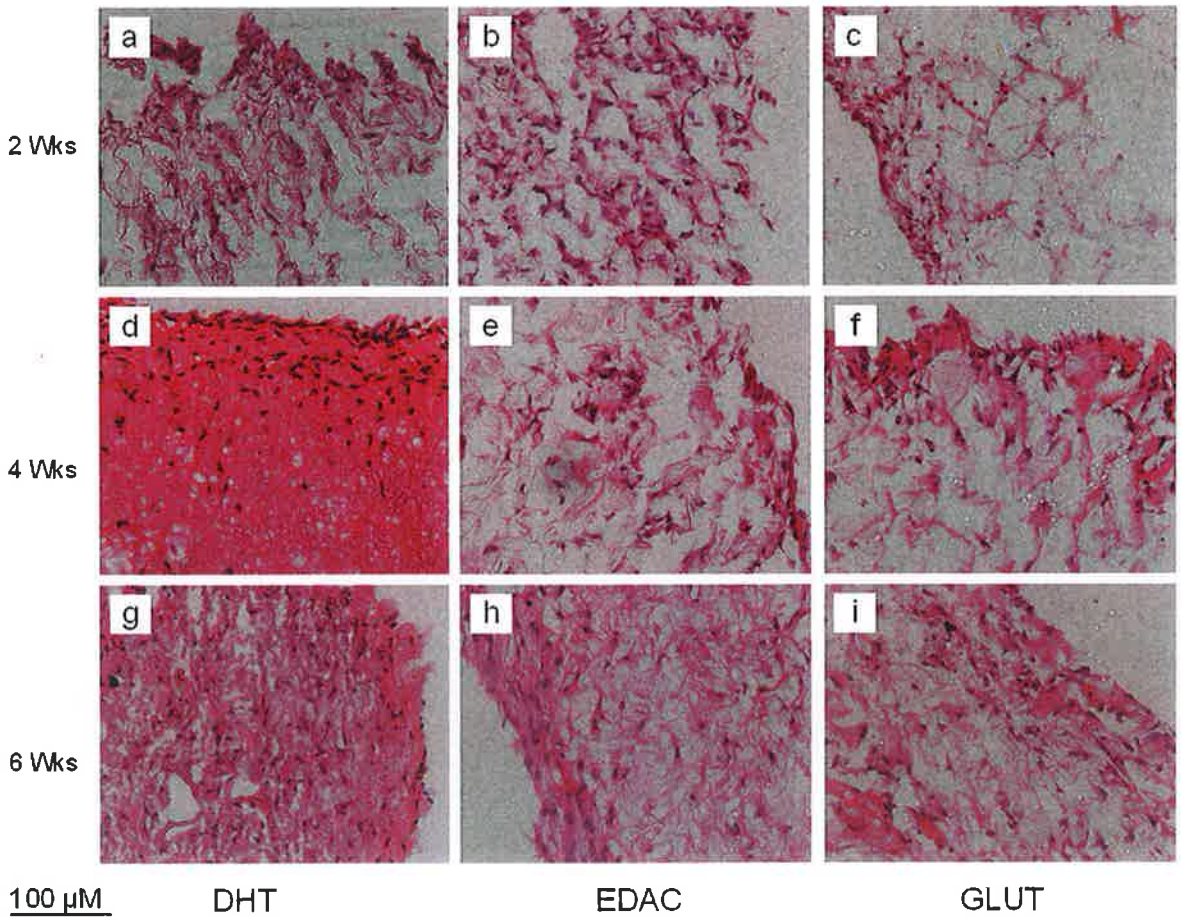


Fig. 4.6 H&E staining histological analysis of cells on scaffolds showed attachment and infiltration of MC3T3 cells throughout all scaffold treatments; DHT scaffolds (a, d, g) EDAC (b, e, h) and GLUT (c, f, i). All sections were taken ~40-50% into the scaffold; sections of 1 scaffold representative of n=3, 100µm scale bar.

Alizarin red staining for mineralization was positive for all constructs. Staining reflected cellular distribution; EDAC and GLUT scaffolds showed positive staining at the periphery which increased with time (Fig. 4.7e, h & f, i). The most intense staining was observed in the DHT treated groups evident after 2 weeks and peaking at 4 weeks and maintained at 6 weeks (Fig. 4.7 a, d and g).

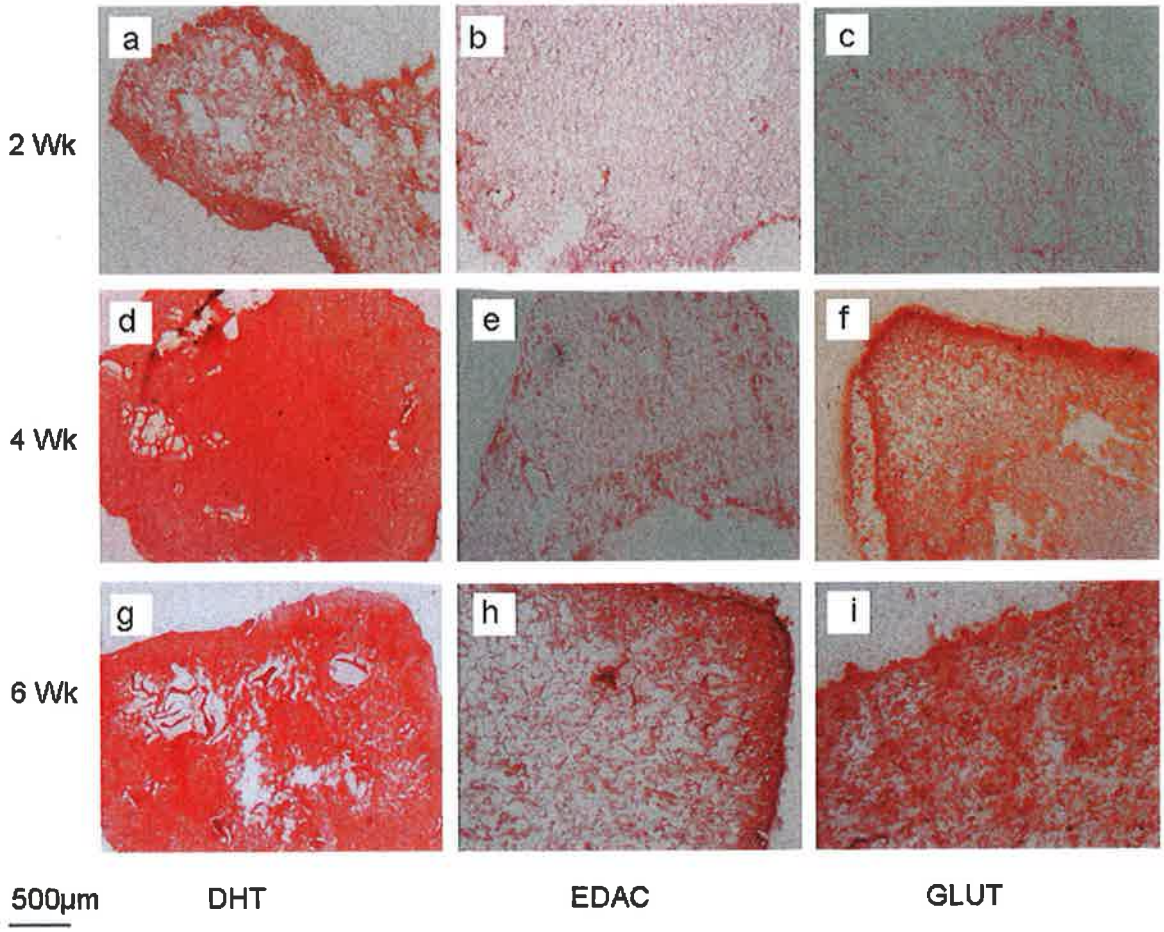


Fig. 4.7 Alizarin red staining for mineralization of cells seeded on CG scaffold; DHT (a, d, g) EDAC (b, e, h) and GLUT (c, f, i) treated groups. All sections were taken ~40-50% into the scaffold; sections of 1 scaffold representative of n=3, 500µm scale bar.

4.3.5 Gene expression analysis

Expression of osteogenic markers was detected in all constructs at all time points. Levels of the early bone formation marker alkaline phosphatase increased with time, with all scaffold treatments showing greatest expression at 6 weeks (Fig. 4.8 a&b). This

increase was 3-fold higher at 6 weeks than at 2 weeks for both EDAC and GLUT groups ($p<0.005$). Another early stage bone formation marker, collagen type-1 showed no significant difference in expression levels over time; however, DHT scaffolds showed a decrease between 2 and 6 weeks. The mid/late stage marker osteopontin increased significantly in DHT-treated scaffolds at 4 weeks ($p<0.001$); this increase was 24 fold higher than EDAC or GLUT at this time point (Fig. 4.9 a&b). Similarly, the late stage marker osteocalcin increased 6 fold in expression level at 4 weeks for DHT ($p<0.001$). DHT scaffolds produced significantly higher levels of osteocalcin than GLUT and EDAC at 4 and 6 weeks ($p<0.05$).

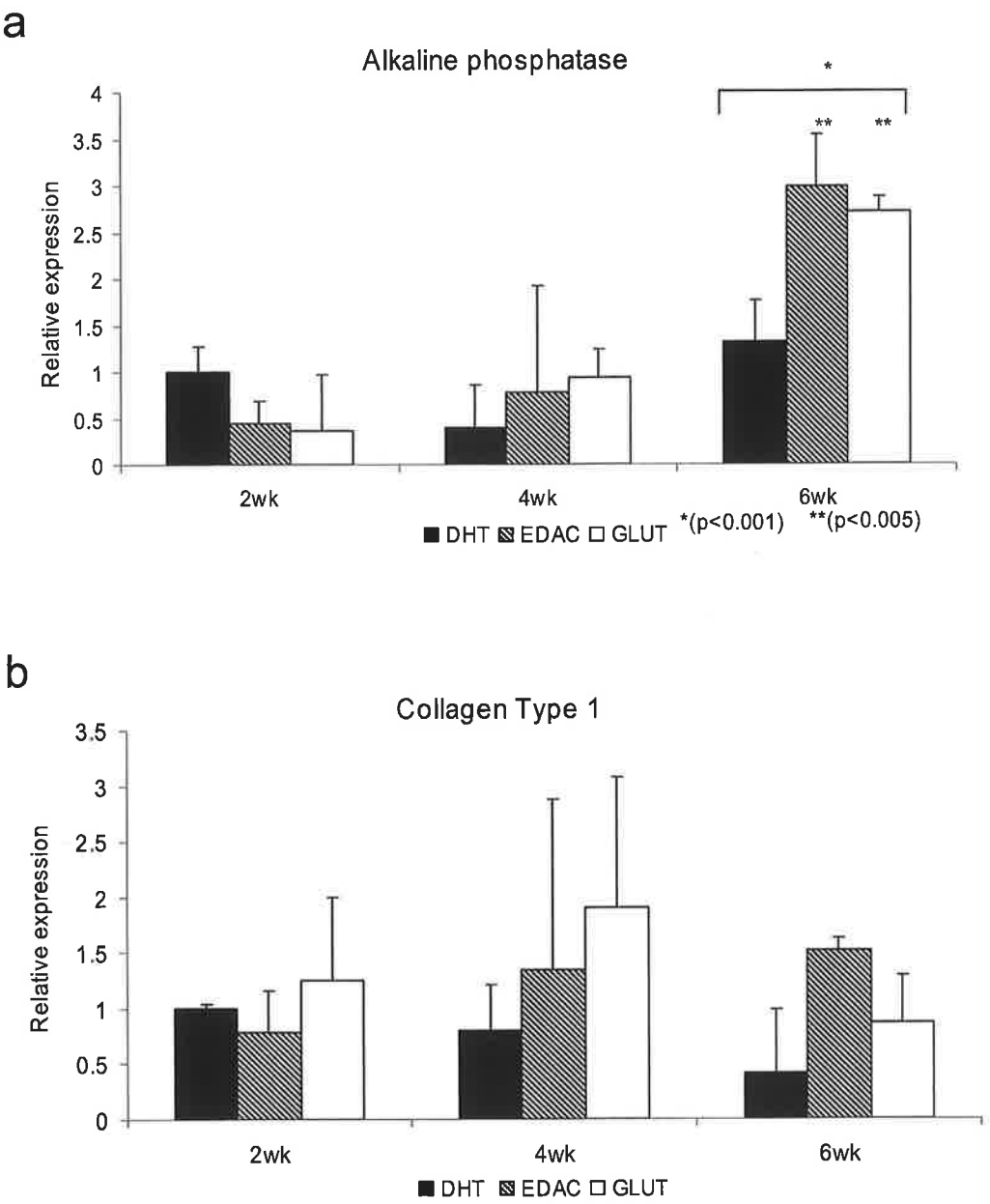


Fig. 4.8 (a&b) Gene expression levels of early stage osteogenic markers for constructs with varying stiffness (a) alkaline phosphatase levels increased 3 fold in EDAC and GLUT constructs over DHT (b) collagen type I expression showed no difference between the groups (n=3).

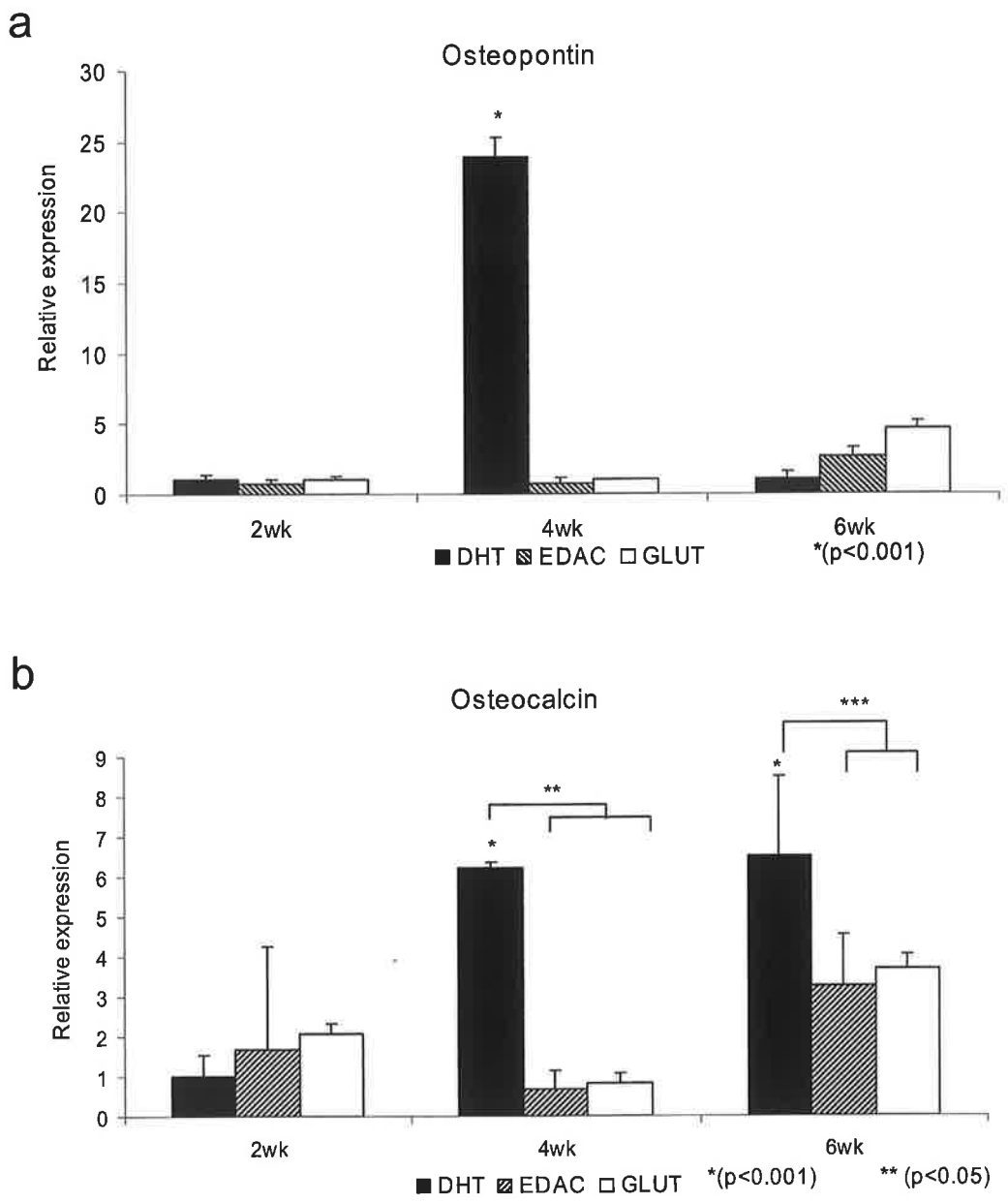


Fig. 4.9 (a&b) Gene expression levels of mid to late stage osteogenic markers for constructs with varying stiffness (a) osteopontin levels increased in DHT groups by 24 fold (b) A 6 fold increase was observed in for osteocalcin expression at 4 and 6 weeks (n=3).

A 3.8 fold decrease in the expression levels of the contractile related SMA-α gene was observed from 2 to 4 weeks in DHT groups ($p<0.05$), whereas the non contractile EDAC and GLUT scaffolds maintained higher levels of SMA-α up to 6 weeks (Fig. 4.10). At both 4 and 6 weeks DHT maintained the lowest expression levels in comparison with EDAC or GLUT which resulted in a 1.2 fold increase and 2.1 fold increase respectively.

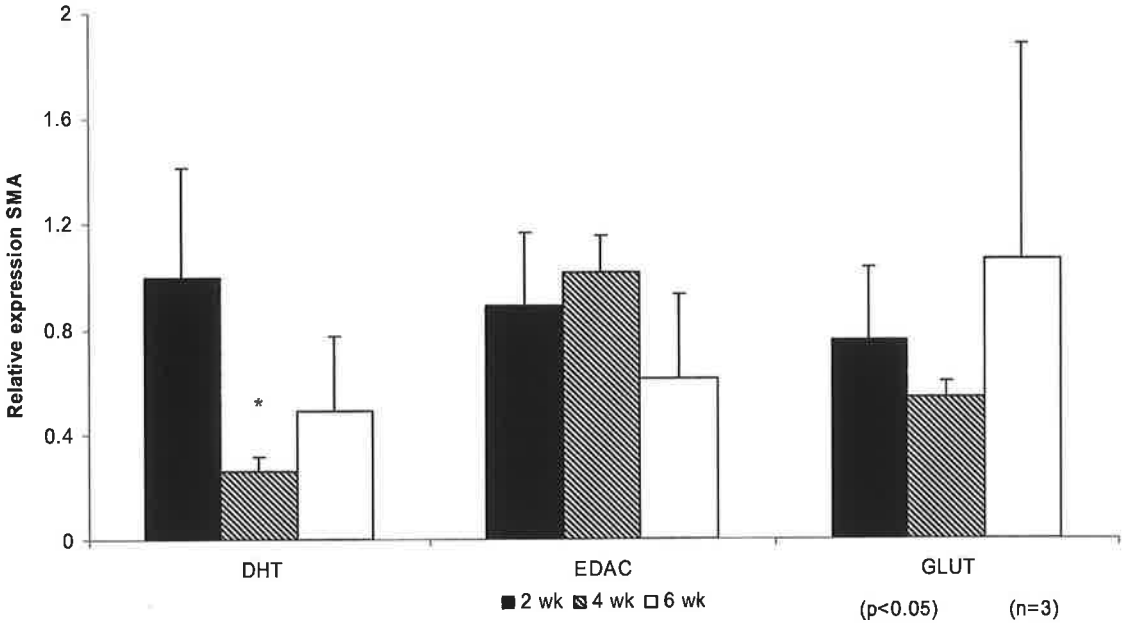


Fig. 4.10 SMA-α gene expression for cell seeded constructs of varying stiffness; SMA levels reduced significantly in DHT groups at 4 weeks (n=3).

4.4 Discussion

Recent studies have shown that cells can sense differences in 2D matrix elasticity by sensing the effort required to pull against a matrix using stimulated cellular mechano-transducers (Engler *et al.*, 2006). Although several forces such as fluid flow, hydrostatic pressures, compression, tension and cell shape are all important in activating mechanical cues for differentiation, the actual mechanisms involved in activating mechano-transducers to direct a cell towards osteogenesis remains under discussion. What is more certain is that properties of a scaffold influence cell behaviour (Kelly and Christopher, 2010; Reilly and Engler, 2010). This study examined the correlation between substrate stiffness, cell-mediated contraction and long term osteogenesis of MC3T3 pre-osteoblasts.

By comparing the effects of three established crosslinking methods, DHT, EDAC and GLUT on CG scaffolds it was revealed that mechanically compliant DHT treated CG scaffolds which allowed for cell-mediated contraction, supported a greater osteogenic maturation of MC3T3 cells; while mechanically stiffer non-contractible EDAC and GLUT scaffolds displayed a lower osteogenic cell phenotype but higher cell numbers. The results demonstrated a significant 2.65-fold increase in the mechanical stiffness of the DHT construct over the 6 week culture period. This is likely due to the increased density (reduced porosity) which results from a combination of construct contraction and increased osteogenesis (ECM deposition & mineralization) in the DHT group. This is validated by the fact that no significant change in stiffness occurs in the EDAC and GLUT scaffolds over the culture period where lower levels of contraction and osteogenesis were found.

2D substrate studies have shown that matrix elasticity can direct cell lineage; with soft tissue like neuronal tissue cells preferring soft substrates and hard tissues like bone preferring stiffer substrates (Engler *et al.*, 2004a; Pek *et al.*,.2010; Reilly and Engler 2010). The results from this study suggest that although substrate stiffness influences

cell phenotype and differentiation; a compliant mechanically active scaffold can also direct the maturity of osteoblasts to a well defined mineralizing phenotype.

In this study, non-contractible EDAC and GLUT scaffolds resulted in a 2-fold greater cell number and metabolic viability. This may be attributed to higher scaffold porosity which allowed for greater diffusion of nutrients and waste as well as providing a greater scaffold surface area and therefore higher ligand density for cell adhesion than to DHT treated scaffolds over time (O' Brien *et al.*, 2007). Previous studies have also shown the importance of pore structure and how pore size can directly regulate osteoblast adhesion and growth (Byrne *et al.*, 2008; Murphy *et al.*, 2010; O' Brien *et al.*, 2005). This study demonstrated that as the pore structure was not altered by contraction, there was a greater surface area available for cell proliferation and cell infiltration. As a result cells appeared to have retained a proliferative state for longer thus increased in greater numbers. Similar findings were reported by Lee et al. (2001) where it was shown that chondroblasts remained in a proliferative state on stiffer CG scaffolds.

On the other hand, scaffold contraction causes changes in pore size and void space; this results in new mechanical environments which can affect cellular behaviour (Dado and Levenberg, 2009). Similarly, as the constructs contract they can increase cell-cell contact and cell confluency within the constructs pores; an important factor which has been shown to enhance cellular differentiation (Tang *et al.* 2010). Although contraction did result in a reduced porosity for cell proliferation within DHT constructs (hence lower cell numbers), there were still sufficient cells present to maintain biological activity and osteoblast maturation; as observed by the greater levels of the mid to late stage bone formation markers osteopontin (24 fold increase) and osteocalcin (6 fold increase) coupled with greater levels mineralization as observed after 4 weeks of culture.

Of particular note was MC3T3 SMA-α expression levels. SMA-α has been shown to be associated with cellular contraction of CG matrices (Schulz Torres *et al.*, 2000). SMA-

α levels were maintained in both EDAC and GLUT constructs however, they decreased in DHT constructs following a contraction phase. Therefore, constructs which resisted contraction had consistent levels of SMA-α expression as these cells were still trying to contact the scaffold. In the DHT treated scaffolds, once contraction had occurred and ceased, a decrease in SMA-α expression was observed. This down-regulation in SMA-α following a contraction phase in culture has been previously identified in CG scaffolds (Menard *et al.*, 2000). Reduced expression of SMA-α coincided with the end of substrate contraction and an increase in the expression levels of late stage osteogenic markers and mineralization. As crosslinking the scaffold does not change the composition of the scaffold therefore it appears that the greater levels of osteogenesis observed in DHT treatments was a result of cell-mediated contraction.

Recent studies have demonstrated the importance of mechano-active poly(D,L-lactic-co-glycolic acid) matrices for tendon remodelling (Joseph and Roberto, 2005; Spalazzi *et al.*, 2008). Physical properties of scaffolds can exert forces on cells which respond via mechano-sensitive pathways (Reilly and Engler, 2010). The ability of the scaffold to allow for cellular contraction may be beneficial for differentiation in 3D structures by providing greater cell-cell interactions as well as inducing mechanostimulation (Byrne *et al.*, 2007; Tang *et al.*, 2010). Contractile scaffolds may act as alternative option other than using bioreactors to provide biophysical cell responses on scaffolds during the cultivation of a construct in vitro. Similarly, they may also be more beneficial than using bioactive (growth factor loaded) scaffolds which may be limited in chemical release due to temporal degradation (Wiesmann *et al.*, 2004b; Yang *et al.*, 2002).

Cell-scaffold contraction can have a negative connotation, often linked with collapsed pores and limited nutrient/waste diffusion *in vitro*. However, this study showed that although contraction changed the pore architecture dramatically coupled with a lower cell number, it did not prevent cellular viability or osteogenesis. In the

clinical setting contraction may result in gap formation at a defect site. However, although DHT scaffolds contracted greatly, 50% of the contraction occurred in the first 2 weeks, after which there was only a 20% change in scaffold size. This limit in contraction was reached following osteogenesis and could be a more appropriate time for implantation into a defect at this point.

On the other hand the structurally more stable EDAC and GLUT scaffolds allowed for osteogenesis to occur; retained a higher cell number and viability and supported osteogenesis albeit at an earlier stage. Therefore further analysis into enhancing this level of osteogenesis on these scaffolds may provide more benefit from a clinical point of view.

4.5 Conclusion

In conclusion, the results from this study demonstrate that different crosslinking techniques can be used to produce a scaffold of varying stiffness; these can modulate the functional maturation of osteoblasts on a CG scaffold. The stiffness of the CG scaffold and its ability to contract displayed a converse effect on cell number and osteoblast differentiation; where the more compliant contractible DHT CG constructs displayed a greater osteogenic maturation while the stiffer, non contractible EDAC and GLUT constructs resulted in a lower osteogenic phenotype but higher cell numbers. Therefore, stiffness has different effects on differentiation and proliferation, whereby increased cell-mediated construct contraction facilitated by the less stiff DHT scaffolds positively modulates osteoblast differentiation while reducing cell numbers contrary to stiffer non-contractible EDAC and GLUT CG scaffolds.

CHAPTER 5

Effect of a flow perfusion bioreactor on osteogenesis of human cells within a collagen GAG scaffold

5.1 Introduction

In the previous chapter the collagen glycosaminoglycan (CG) scaffold was shown to provide enhanced levels of osteogenesis in substrates which allowed for contraction over more structurally stable scaffolds. This increase may be contributed to the mechanostimulatory effects of the scaffold coupled with increased cell-cell contact throughout the construct. A limitation with contraction is that the homogenous pore structure is lost as observed in Chapter 3 (section 3.7). Retaining scaffold porosity is an important factor when considering angiogenesis and preventing construct core degradation following implantation. As a result the focus of this study is to enhance the osteogenic ability of the mechanically stiffer EDAC crosslinked scaffold from Chapter 4 by providing an improved homogenous cell distribution coupled with mechanical stimulation using a fluid flow bioreactor.

Bioreactors have been used to improve *in vitro* tissue formation of several organs including cartilage prostate, gut, heart and liver formation (Kim *et al.*, 2007; Margolis *et al.*, 1999; Syedain and Tranquillo, 2009; Yates *et al.*, 2007). The design of a bioreactor intends to improve on traditional static culturing methods by providing improved mass transfer to enable efficient nutrient delivery and waste removal under controlled settings. In Chapter 3 a cellular capsule developed along the construct periphery inhibiting mass transfer of nutrients and waste removal resulting in the development of

an acellular necrotic core region. Bioreactors have previously demonstrated their ability to improve cell distribution and reduce cell encapsulation on scaffolds (Martin *et al.*, 2004).

As well as improving mass transfer bioreactors provide mechanical stimuli to cells which can be beneficial for improving cell activity and maturation of cells in both 2D and 3D cultures (Owan *et al.*, 1997; Zhu *et al* 2009.). Although several types of bioreactor exist, this study, examines the use of a flow perfusion bioreactor as they have shown most promise for bone tissue engineering with previous studies reporting improved cell seeding and distribution within 3D biomaterials (Glowacki *et al.*, 1998; Goldstein *et al.*, 2001; Janssen *et al.*, 2006).

5.1.2 Flow perfusion bioreactor culture

This study uses the flow perfusion bioreactor which acts by forcing media through the interior of a cell seeded scaffold (Fig 5.1). As flow perfusion bioreactors provide the best fluid flow throughout a construct compared to other bioreactors, they are an ideal choice to mechanically stimulate cells such as osteoblasts and for bone tissue engineering, the flow perfusion bioreactor has been shown to be beneficial for enhancing osteoblast growth, differentiation and mineralisation (Goldstein *et al.*, 2001; Bancroft *et al.*, 2002).

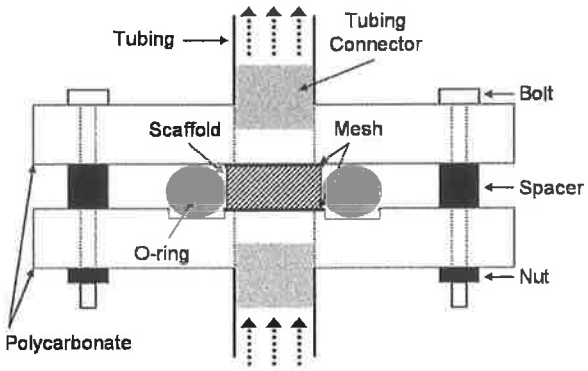


Fig 5.1 Illustration of the scaffold chamber of the flow perfusion bioreactor; arrows indicate flow through the scaffold (Jaasma *et al.*, 2008).

Different versions of flow perfusion reactors exist for bone tissue engineering; these are specifically developed for either stiffer scaffolds such as titanium mesh (Bancroft *et al.*, 2002) or for compliant scaffolds such as the CG scaffold. An important consideration when using a bioreactor on a cell seeded construct is to use a scaffold stable enough to resist being compressed by the effect of the bioreactor fluid flow shear stress caused by medium perfusion (Janssen *et al.*, 2006) thus design and validation is therefore a fundamental consideration. In our laboratory, a flow perfusion bioreactor has been previously designed and optimised for use with the CG scaffold (Jaasma *et al.*, 2008; Plunkett *et al.*, 2010). Previous flow perfusion bioreactor studies on the CG scaffold have only focused on short term culture periods of up to 14 days using non-human mammalian cell lines (Jaasma *et al.*, 2008; Plunkett *et al.*, 2008, 2010). These studies have shown a decrease in cell number during long term bioreactor culture (Plunkett *et al.*, 2008).Therefore, the focus of this study is to stimulate pre-osteoblasts on CG scaffolds for 24 hours and further culture under static conditions for 28 days.

Other than stimulating cells, fluid flow has shown to be beneficial for improving cell distribution throughout a scaffold. Uniform seeding is important to enhance cell migration and overall functionality of the construct (Bueno *et al.*, 2007). In Chapter 4 it was observed that it took as long as 6 weeks for EDAC scaffolds to become fully infiltrated into the centre of the constructs. From a clinical point of view having a shorter *in vitro* cultivation duration is always preferred; therefore the bioreactor could be used to re-distribute cells from the periphery into the centre of the scaffold rather than relying on cell migration alone. Thus it may be possible to develop a confluent construct shorter culture duration.

In vivo bone tissue often experiences mechanical loading and fluid flow shear stresses. When bone tissues are loaded in compression, the fluid in the canaliculi is forced out however, when the load is removed, the fluid can flow back into the canaliculi, over osteocyte/bone lining osteoblast membranes. This fluid shear stress

caused by mechanical loading in bone tissue has been shown to be important for both the structure and function of bone through its effects on osteocytes and osteoblasts. *In vitro* studies have previously investigated the effects of fluid flow on these cells; these studies tend to be carried out in 2D using plate flow chambers (Jacobs *et al.*, 1998). Flow chambers use fluid flow to stimulate cells similar to flow perfusion bioreactors, but in plate flow chambers, the flow is directed over the surface of the cells rather than through a scaffold on which they are attached. Less is known about fluid flow on cells in 3D constructs; however, recently, a few studies have investigated mechanical stimulation using fluid flow in 3D bone cell cultures (Partap *et al.*, 2009; Zhu *et al.*, 2009). Ignatius *et al.* (2005) demonstrated how fluid flow could affect hFOB cells in collagen matrices up to 3 weeks. In this study they mechanically stretched the cell seeded gels and found cyclic stretching caused a slight increase for both cell proliferation and differentiation over static cultures (Ignatius *et al.*, 2005).

Different types of fluid flow exist; these may be pulsatile, oscillatory and steady flow. Pulsatile is a flow regimen shown to be beneficial in 2D assays; it comprises of timed pulse flow followed by intermittent rests of static culture (Jacobs *et al.*, 1998). Oscillatory flow is fluid flow that occurs in an oscilitory direction, this type of flow thought to most resemble *in vivo* conditions but has not proven to be very effective for stimulating cells using on a CG scaffold (Jaasma *et al.*, 2008). Steady flow as the name suggests is a continuous flow of media throughout the scaffold. This is beneficial for complete perfusion of nutrients and waste through the construct. In this study the optimised steady flow conditions as determined by Jaasma *et al.* (2008) will be used.

Research question:

The aim of this chapter is to combine findings from previous chapters; (i.e. combining long term viability growth of hFOB cells on CG scaffolds in the presence of high TGF osteogenic treatment with a mechanically stable EDAC crosslinked scaffold) and to examine the response of short term fluid flow on osteogenesis of human cells within a CG scaffold. Specifically, it is intended to use the mechanically stable EDAC treated CG scaffold with hFOB cells and to expose these to fluid flow from a perfusion bioreactor in order to 1) improve initial cell distribution throughout the CG scaffold and 2) to examine the effects of short term fluid flow on human osteoblast differentiation.

The study will use a flow perfusion bioreactor which has been previously developed in our laboratory for use on an EDAC crosslinked CG scaffold (Jaasma *et al.*, 2008). Using the optimised flow conditions as reported by Jaasma *et al.* (2008) this study will examine the effects of exposing hFOB cells to 24 hours of bioreactor culture followed by a static culture period up to 28 days. The effects of the bioreactor culture will be compared to that of a static standard control group.

5.2 Materials and methods

5.2.1 Bioreactor design

A validated flow perfusion bioreactor system was used as described by Jaasma et al. (2008). It consisted of a syringe pump, a polycarbonate scaffold chamber and a media reservoir connected with gas permeable silicon tubing and quick release tubing connections (Cole-Parmer, Vernon Hills, IL). The whole bioreactor system was placed in a standard incubator. Sterility was maintained using gas-permeable stoppers with the bottom dispensing media reservoir (AGB, Ireland). This enhanced O_2/CO_2 gas exchange during bioreactor culture. 12.7 mm diameter scaffolds were held in the scaffold chamber using silicone O-rings. The syringe pump held six 50ml syringes to allow simultaneous culture of 6 scaffolds per run (Fig 5.2).

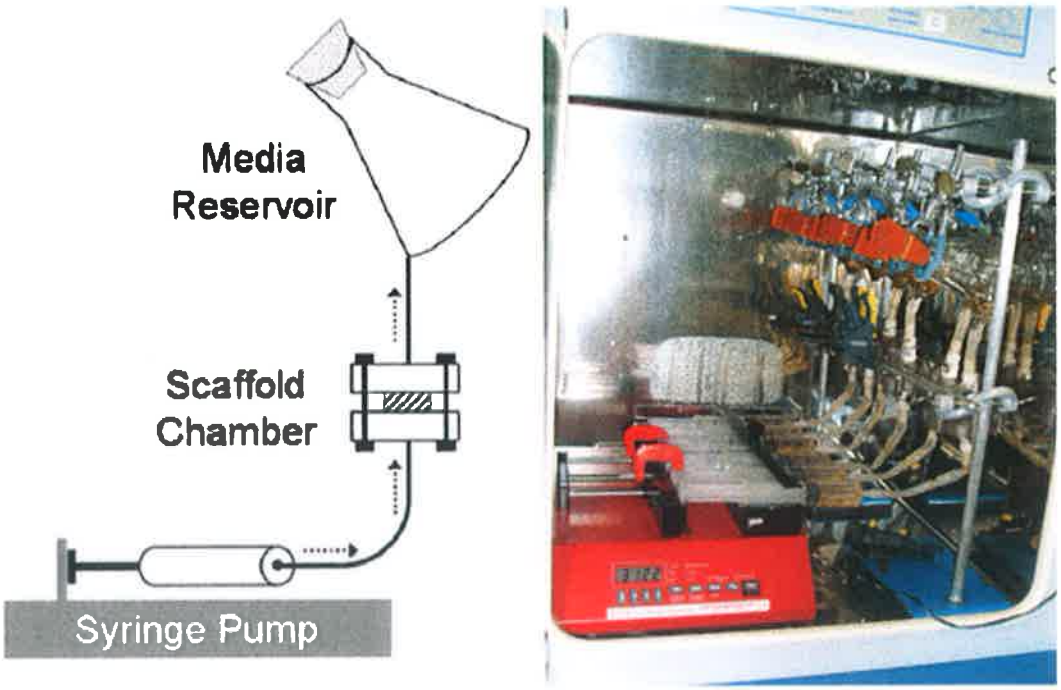


Fig. 5.2 Flow perfusion bioreactor setup: Left hand side shows a schematic of the syringe infusing the scaffold chamber into a media reservoir; adapted from Jaasma *et al.* (2008). The photograph on the right shows the set up of an operational 6-syringe bioreactor inside a tissue culture incubator.

5.2.2 Scaffold fabrication and crosslinking

Scaffolds were fabricated as per section 2.2.2.7. CG sheets (thickness = 3.5mm; mean pore diameter = 96μm; porosity = 99.5%) were aseptically cut to 12.7 mm diameter to accommodate the bioreactor chamber. Scaffolds were cross-linked with an aqueous based filtered solution of 6mM 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDAC) and 2mol N-hydroxysuccinimide (NHS) in distilled water for 2 hours at room temperature. Scaffolds were rinsed several times in sterile PBS prior to cell seeding (A. Engler *et al.*, 2004a).

5.2.3 Cell seeding and bioreactor conditions

$2x10^6$ hFOB pre-osteoblasts were seeded dropwise on each side of the CG scaffolds (total of $4x10^6$ cells per construct) as per section 3.2.2.1. Cell seeded constructs were pre-cultured in standard media for 6 days to allow for cell attachment (34°C). Constructs were then placed in the flow perfusion bioreactor and exposed to 3 x 1hr bouts of steady flow (1ml/min) with each bout being followed by 7 hrs of no flow (to prevent cellular desensitization) for 25 hrs (bioreactor groups). Once removed from the bioreactor, the constructs were cultured statically in osteogenic conditions for a further 28 days (39°C). Osteogenic media consisted of standard media supplemented with 50μg/ml ascorbic acid, and 10mM β-glycerolphosphate, 10nM vitamin D_3, 50nM vitamin K_3, 10ng/ml transforming growth factor-β_1 (TGF-β_1) for 7 days, reduced to 0.2ng/ml thereafter (Sigma-Aldrich, Germany). This was identical to the optimised 'high TGF' formulation from Chapter 3. The control group consisted of constructs that were placed in osteogenic 5ml media after the 6 day pre-culture period in static conditions (static group). Two thirds of the spent media was replenished every 3 days.

5.2.4 Cell viability analysis of cells on CG scaffold:

Cell number was assessed using the Hoechst 33258 DNA assay as per section 3.2.2.2 (n=3). Metabolic activity was assessed for percentage alamar blue reduced dye at 1, 4, 7,

14, 21 and 28 days as per section 2.2.4.1 (n=6).

5.2.5 Histological staining for distribution and of mineralisation of cells on CG scaffold

Hematoxylin & Eosin (H&E) staining was carried out on constructs post 1, 4, 7, 14, 21 and 28 days of culture as per sections 2.2.1.6 (n=3). Alizarin red staining was carried out on constructs at 1, 4, 7, 14, 21 and 28 days as per section 3.2.1.3 (n=3).

Alizarin red quantification was carried out by adding 200µl of a prewarmed 10% cetyl-pyridine chloride (Sigma-Aldrich, UK) aqueous solution to each section of alizarin red stained constructs at days 1, 4, 7, 14, 21 and 28 and incubated for 2 minutes. 100µl samples were taken and read at 570nm on a spectrophotometer.

5.2.6 Analysis of mechanical testing on CG scaffold

Cell seeded constructs were mechanically tested as per section 3.2.2.6 (n=3).

5.2.7 Gene expression analyses on CG scaffold

Constructs were flash frozen in liquid nitrogen at each time point and stored at -80°C. RNA was isolated and quantified from cell seeded constructs prior to reverse transcription polymerase chain reaction for gene expression analysis as per section 3.2.2.4 (n=3). Analysis of the human bone formation markers were carried out, specifically, for alkaline phosphatase, osteonectin, osteopontin and osteocalcin. Expression levels were assessed using the relative quantification ΔΔCt method. β-actin acted as a house keeping control.

5.2.8 Statistical analysis

Statistical analysis was determined using Sigma statistical software package SigmaStat 3.0. The statistical differences between 2 groups were calculated using the Students t test and multiple groups were calculated using Kruskal-Wallis One Way Analysis of Variance on Ranks (ANOVA). Statistical significance was declared at $p<0.05$.

5.3 Results

hFOB cell number increased on the CG scaffold up to 21 days in both the static and bioreactor groups with no difference between groups or between time points (Fig. 5.3). A similar trend was observed for metabolic activity; here viability was maintained over time, again with no difference between the groups (Fig. 5.4).

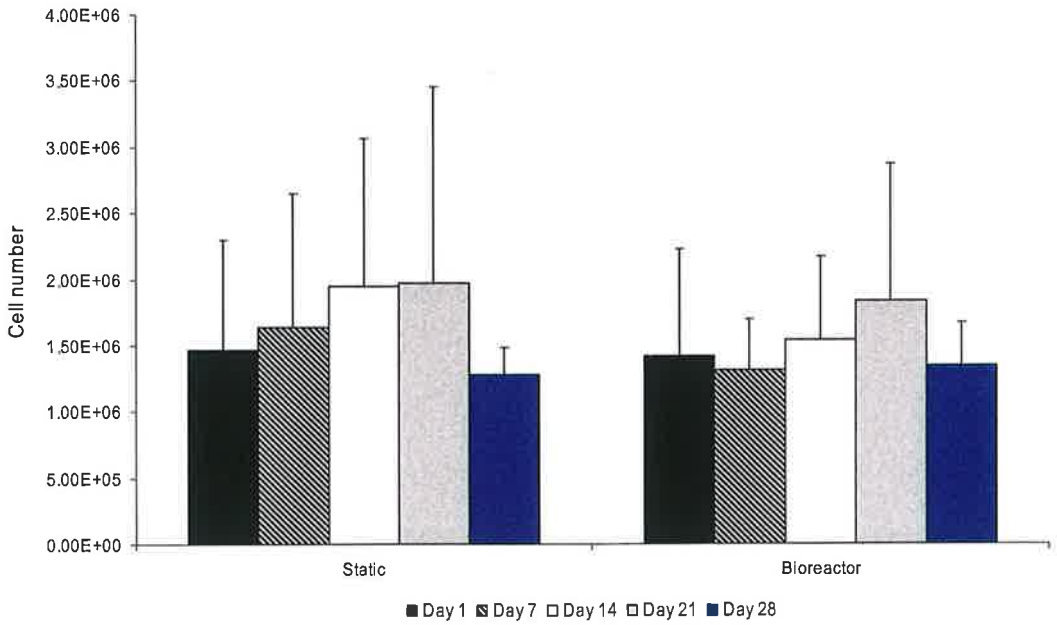


Fig. 5.3 Number of hFOB cells on CG scaffold following static and bioreactor culture groups in static osteogenic culture up to 28 days. No difference in cell number was observer between groups (n=3).

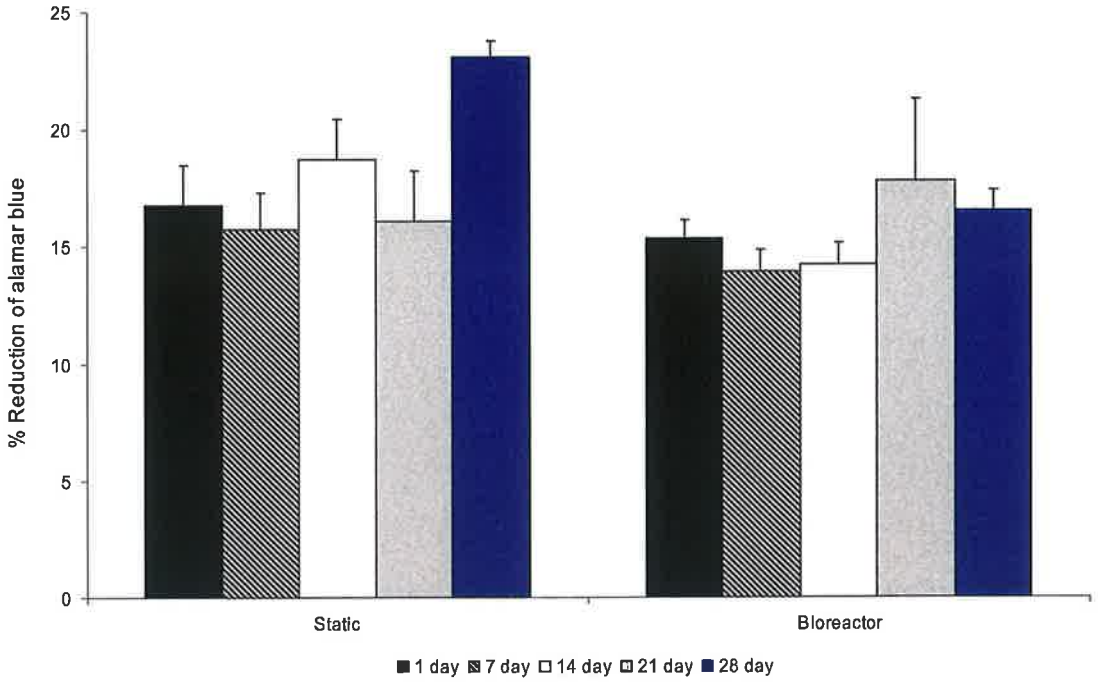


Fig 5.4 Metabolic activity of hFOB cells on CG scaffold, for both static and bioreactor groups. Cells remained viable on CG constructs at all time points with no statistical difference between groups (n=6).

Histological analysis showed that cells infiltrated the constructs in both the static and bioreactor groups up to 28 days (Fig. 5.5), although, differences in cell distribution were observed. Cells in the constructs, following bioreactor culture, appeared in clusters which increased in number and distribution over time. However, cell clusters were gone by 14 days and cells were observed in the centre of the bioreactor constructs after 21 days. The static groups demonstrated increased proliferation and cell number along the edges of the construct resulting in a heterogonous distribution and a cellular encapsulation effect by 14 days which increased with time. Cells were observed at the centre of the construct by both groups at 28 days.

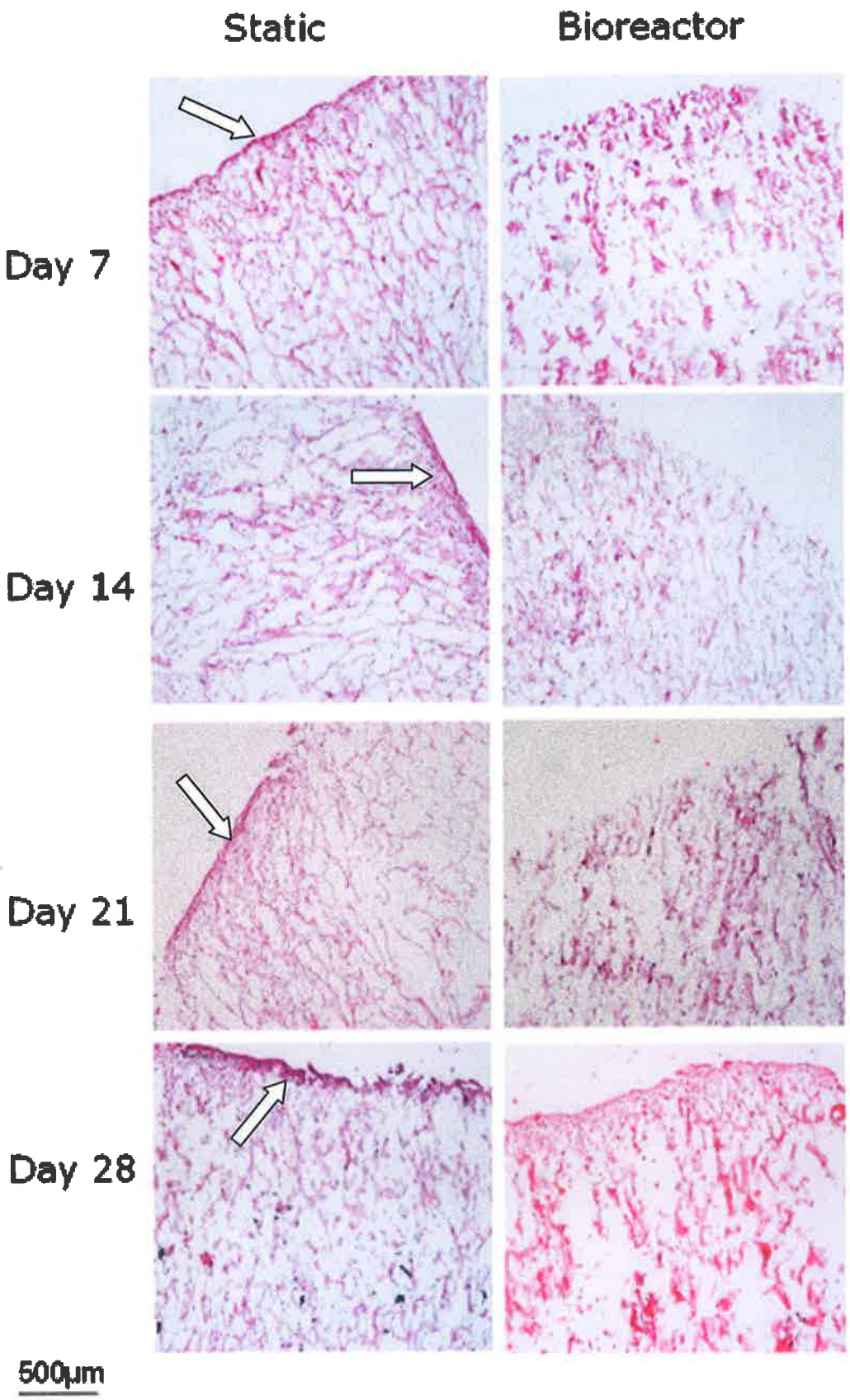


Fig. 5.5 Histological analysis of hFOB cells on CG scaffold following static and bioreactor culture up to 28 days in osteogenic media. White arrow indicates the development of a cell capsule in static culture groups.

Mineralisation reflected cell distribution; greatest staining was observed in static groups; predominantly along the edges of the scaffold at the cellular capsule from 14 days (Fig. 5.6).

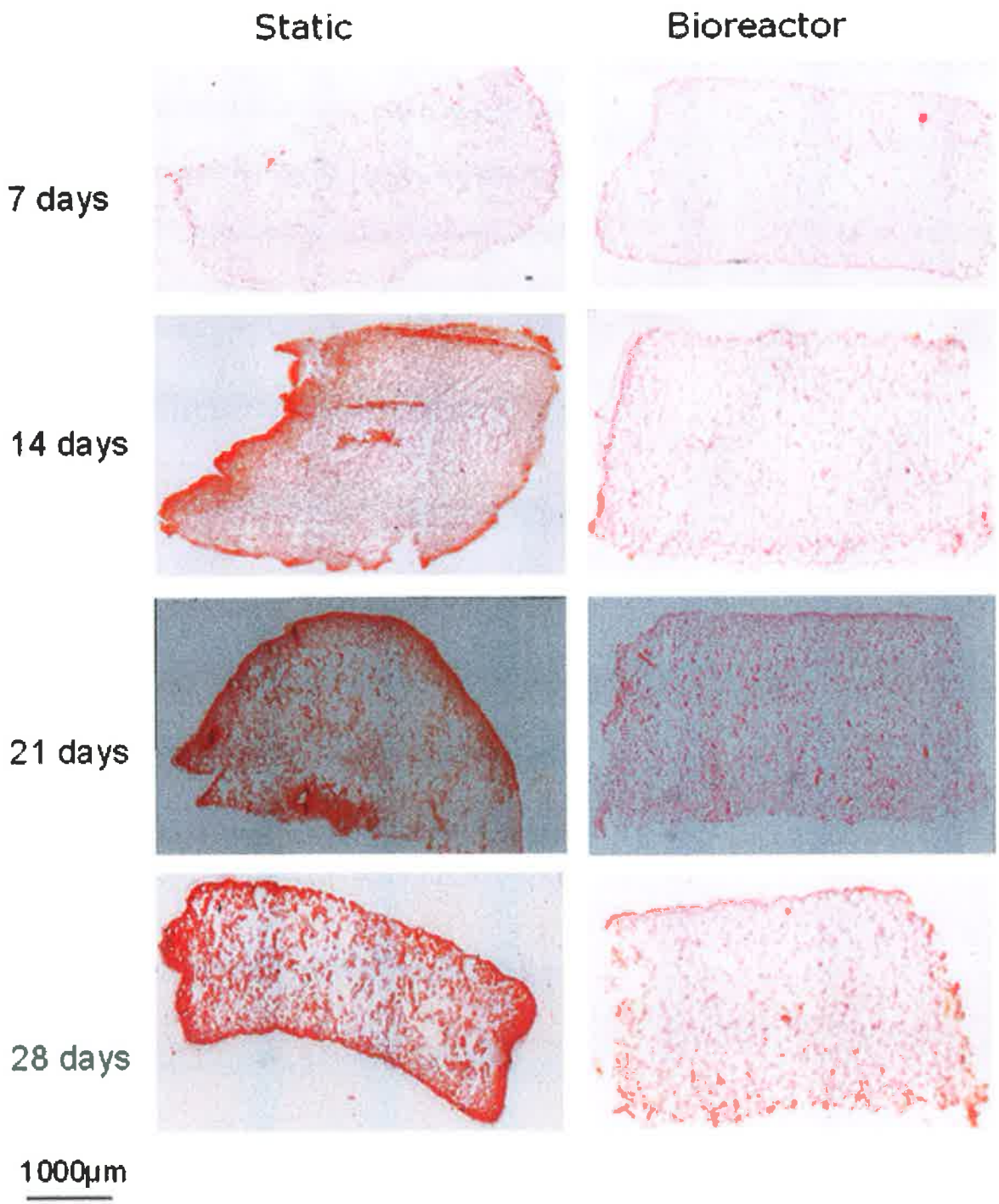


Fig. 5.6 Alizarin red staining for mineralisation of hFOB cells on CG scaffold following bioreactor and static cultures up to 28 days.

The bioreactor groups displayed lower levels of mineral staining although a gradual increase was observed over time. Quantified mineralisation for static groups levels showed a 3.8 fold increase at 28 days over the bioreactor group ($p<0.007$) (Fig. 5.7).

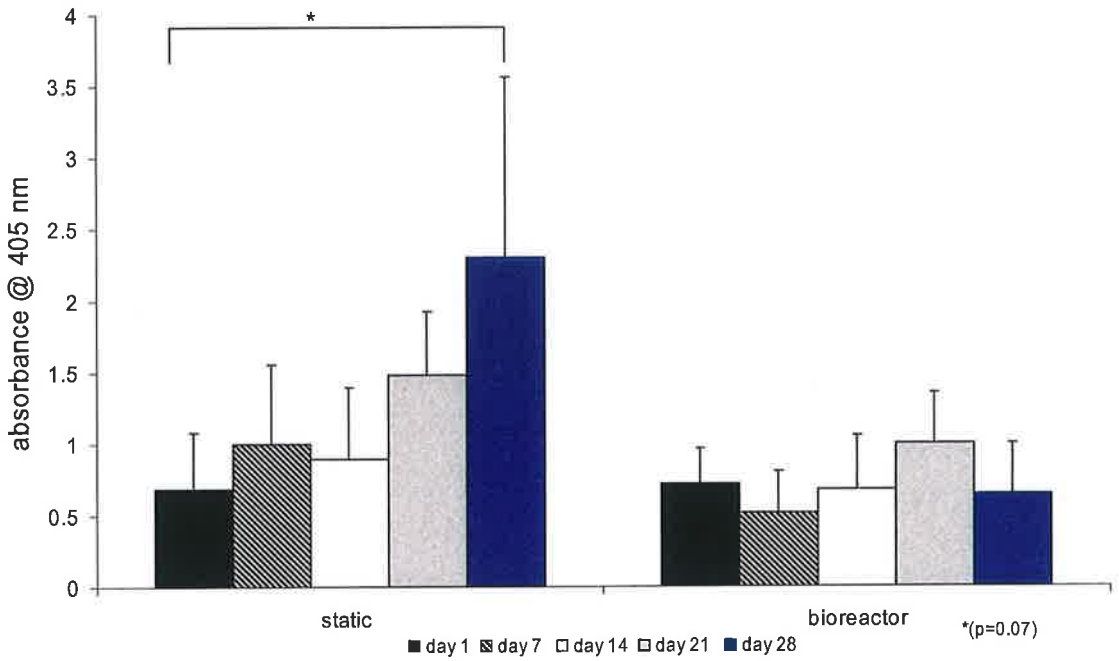


Fig. 5.7 Quantified alizarin red staining for mineralisation on hFOB seeded CG scaffold following bioreactor and static culture up to 28 days.

Osteogenesis was supported in both static and bioreactor groups on the CG scaffold. Gene expression results show that the bioreactor generated a greater osteogenic response over static culture (Fig. 5.8). The early bone formation marker alkaline phosphatase displayed a 3 fold increase in its expression in the bioreactor group versus the static group at 21 days. The mid stage markers osteopontin and osteonectin showed similar trends to each other, with bioreactor groups providing higher expression levels earlier than the static groups; osteopontin increased by 1.6 fold after 1 day post bioreactor whereas the maximum peak of static groups occurred after 14 days. Similarly, osteonectin peaked after 14 days with a 1.7 fold increase between groups ($p<0.01$); static cultures peaked later at 21 days with equivalent expression levels to bioreactor groups. The late stage marker of bone formation, osteocalcin gave a 2 fold

increase after 1 day of culture over static ($p<0.01$). Levels of osteocalcin peaked for both static and bioreactor groups at 21 days, within that period, static cultures increased 8 fold whilst bioreactor groups displayed a 12 fold increase ($p<0.001$).

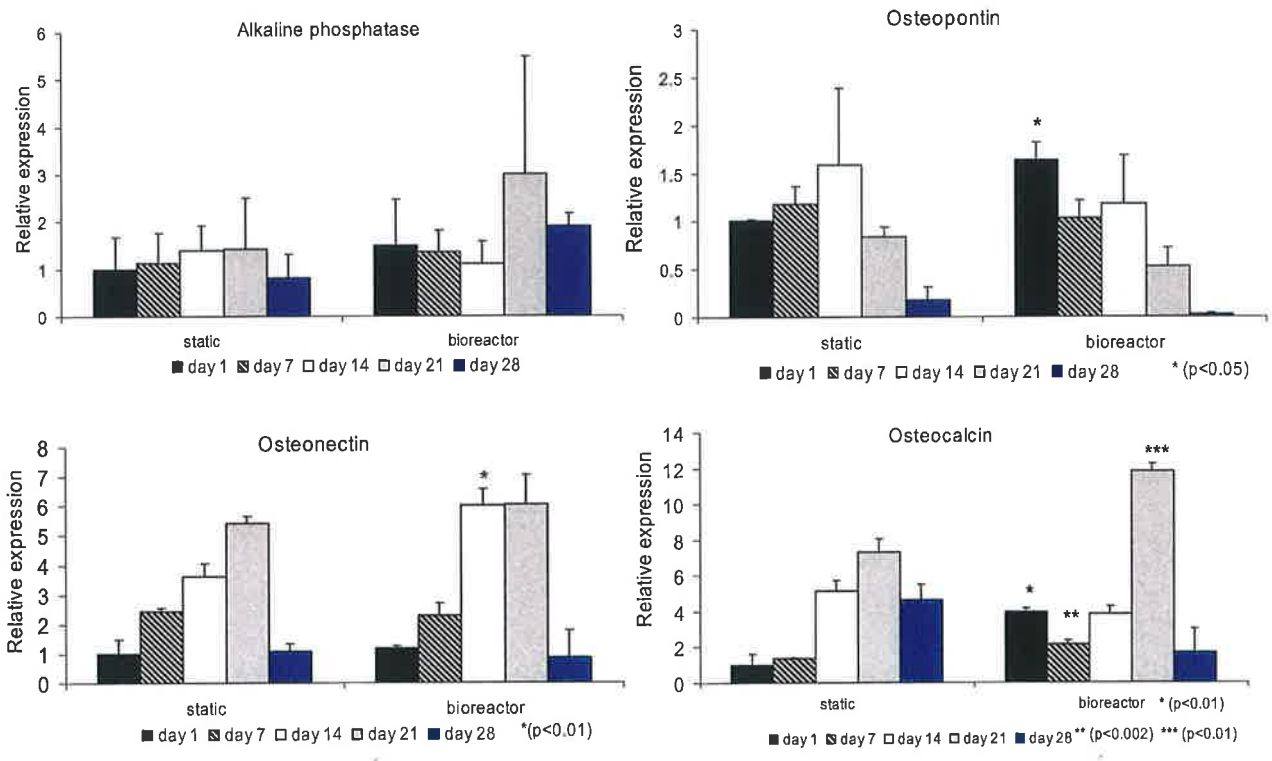


Fig. 5.8 Osteogenic gene expression of hFOB cells on CG scaffold following static and bioreactor cultures up to 28 days. One day of bioreactor exposure resulted in the bioreactor group expressed greater levels of alkaline phosphatase, osteopontin, osteonectin and osteocalcin over static cultures .

An increase in mineralisation in the static group did not lead to an increase in mechanical properties (Fig. 5.9). Mechanical stiffness did increase in both groups with time where greatest mechanical stiffness was observed at 28 days following exposure of cell seeded constructs to fluid flow in the bioreactor group. An increase in stiffness was observed in cell seeded constructs versus unseeded controls.

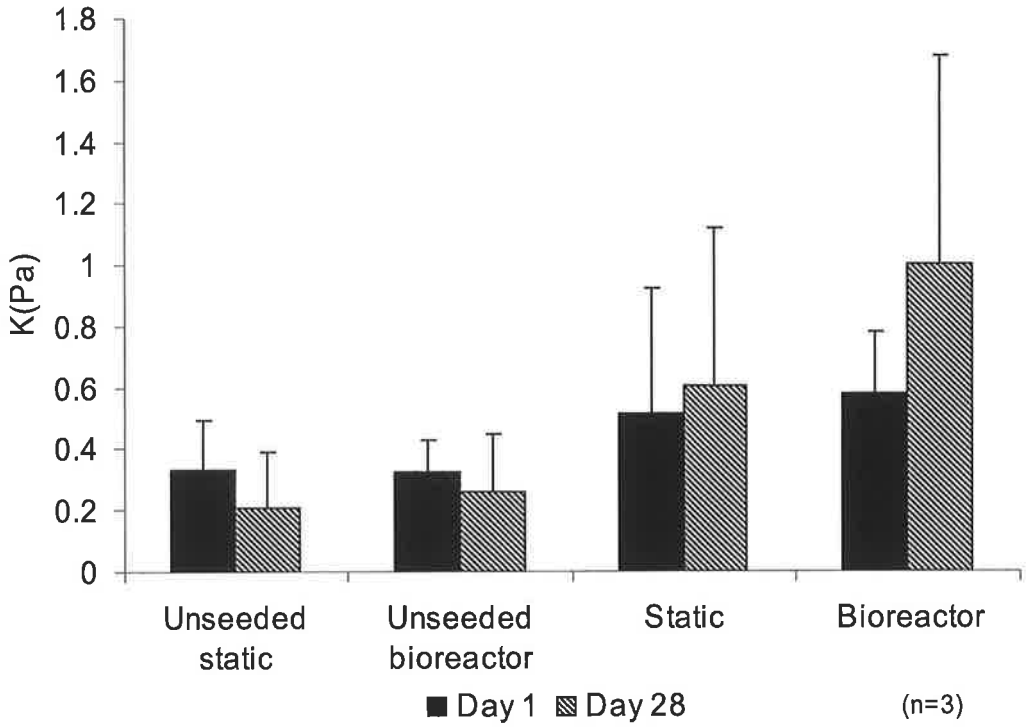


Fig. 5.9 Biomechanical analysis cell free and cell seeded CG constructs following bioreactor and static culture up to 28 days. Cell seeded constructs were stiffer than unseeded scaffolds however no difference was observed between static or bioreactor groups,

5.4 Discussion

Long term static culturing conditions can lead to a heterogeneous cell distribution, often resulting in the development of a cellular peripheral capsule which may result in the development of a necrotic/acellular central core caused by a reduction in mass transfer conditions. Bioreactors can improve mass transfer of media and wastes via fluid flow and may be used to encourage cell growth and maturation (Bjerre *et al.*, 2008; Yu *et al.*, 2004). This study investigated the use of a previously designed and validated flow perfusion bioreactor system (Jaasma *et al.*, 2008) to enhance human hFOB cell distribution and osteogenic activity in a CG scaffold.

For this study the EDAC crosslinked CG scaffold was chosen from Chapter 4 as it was mechanically more stable than the DHT scaffolds. Following the results in Chapter 4 which showed that the CG scaffold supported growth and differentiation of MC3T3 cells; it was intended to apply these findings to examine the human osteoblast response using hFOB cells coupled with the optimised conditions for growth and differentiation of high TGF exposure as determined in Chapter 3.

Results of this study found that both static culture and short term exposure to bioreactor conditions for 24 hours provided similar levels of cell number, and metabolic activity within the CG constructs; this is contrary to some studies which have reported a decrease in cell number following bioreactor cultures using flow perfusion methods (Plunkett *et al.*, 2010). Plunkett *et al.* (2010) demonstrated a reduced cell number in bioreactor culture groups exposed to bioreactor cultures for longer than 1 day; however, no difference was observed in cell number following 1 day bioreactor exposure, a similar result to this study.

Bioreactor groups displayed more clusters of cells than the static groups. The clusters appear to be due to the spatial distribution of the hFOB cells caused by bioreactor fluid flow through the scaffold redistributing cells from the periphery

towards the centre of the construct. In order to do this the flow rate is required to be higher than that required for mass transfer of nutrients alone. These fluid flow channels have been reported previously by Zhao *et al.* (2005) who hypothesised that they could be problematic as they may affect the functionality of the overall construct, however in this study construct functionality was not inhibited nor was cell number when compared to static conditions (Zhao and Ma, 2005).

A pronounced histological difference occurred along the CG construct periphery between static and bioreactor groups. The bioreactor discouraged the formation of a cell based capsule as observed in static culture conditions. Cell capsules have been observed in other scaffold studies and can inhibit nutrient and waste mass transfer resulting in necrotic core regiond. This study showed that static cultures developed a capsule after 14 days incubation, a result that is consistent with other observations (Goldstein *et al.*, 2001; Glowacki *et al.*, 1998; Jaasma *et al.*, 2008; Janssen *et al.*, 2006; Yu *et al.*, 2004). As the cell number was not significantly lower in the bioreactor group than the static group and cells were in the centre of the construct by 21 days using the bioreactor but not until 28 days in static cultures, it is therefore clear that the bioreactor was beneficial for improving cell distribution within the scaffold.

Similarly, levels of mineralization, reflected cell distribution with greatest levels identified along the construct periphery of static cultures where cell aggregation occurred, a finding similar to static MC3T3 cultures in Chapter 4 and previous reports using rat MSCs by Farrell et al. (2006). Although an ability to produce large levels of calcified tissue is of interest, it is also important to be able to produce a construct with homogenously distributed cells primed for osteogenesis *in vitro*, which will not restrict mass transfer *in vivo* after implantation into a defect.

This osteogenic priming may be observed from gene expression results which showed that the bioreactor provided an enhanced hFOB osteogenic response on the CG scaffold than that of static alone. Static conditions resulted in less osteogenic priming at

a gene expression level than constructs exposed to 1 day of bioreactor flow. Previous studies have found bioreactors down-regulate many of the osteogenic formation markers with only osteopontin[14] increasing (Plunkett *et al.*, 2010) This study showed an increase in all early, mid and late stage bone formation markers investigated following bioreactor based culture over static conditions. Specifically, the levels of the early bone formation marker alkaline phosphatase increased over time with greatest expression observed at 21 days in the bioreactor group. The mid stage marker osteonectin showed similar trends between static and bioreactor groups, it increased up to 21 days although greatest expression occurred at 14 days in bioreactor groups ($p<0.01$). In the bioreactor group, levels of osteopontin increased following 1 day of flow and declined thereafter. The late stage marker for osteogenesis osteocalcin increased 8 fold in static cultures up to 21 days, but greatest expression (1.3 fold higher) was observed in bioreactor groups ($p<0.01$). As the only difference between the culture groups was the effects of the bioreactor this mechanostimulatory based response on gene expression was a direct result of 1 day of perfusion fluid flow.

There was also no effect observed on the biomechanical properties of the compliant CG scaffold exposed to bioreactor culture or static culture. This is an important finding as the function of the bioreactor was intended to improve cell distribution and not to interfere with the scaffolds structural integrity. Mineralisation therefore did not benefit mechanical behaviour and although the static groups provided greater levels of mineralisation, this mineral was inhomogeneous, predominantly detected along the periphery of the scaffold, at the cell capsule. Cell seeded constructs were 3 fold stiffer than the unseeded scaffolds. This is likely due to cell based matrix deposition during the culture for both static and bioreactor groups.

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[14] An increase in osteopontin following fluid flow based mechanostimulation is likely to be due to its role in substrate adhesion (Carvalho *et al.*, 2003).

These findings suggest that the bioreactor is advantageous for improving cell distribution and osteogenic priming throughout the CG scaffold.

5.5 Conclusion

The results from this study show that flow perfusion bioreactor culture leads to improved cell distribution and osteogenic priming of hFOB pre-osteoblasts. No difference was observed in levels of cell number or metabolic activity using the bioreactor over static culture. Cell distribution improved following bioreactor culture becoming more homogenous throughout the construct and avoiding the formation of a peripheral cell capsule forming. As a result of this cell distribution, the bioreactor displayed lower levels of mineralisation than static cultures. However, gene expression analysis showed that bioreactor cultured constructs gave higher cellular expression levels of bone formation markers than static culture alone. This suggests that the bioreactor can be beneficial for improving cell distribution and osteogenic priming of cells seeded onto CG scaffolds.

CHAPTER 6

General discussion

6.1 Introduction

Tissue engineering aims to replace or restore the structure and functional properties of tissue that has been damaged through trauma, disease or congenital defects (Vacanti, 2010). Tissue engineering is based on a biological triad and consists of (1) scaffolds, (2) cells and (3) signaling mechanisms which aims to mimic the structure and function of native tissue which consist of cells, signalling mechanisms and extracellular matrix. In this work the role of each of the components of the biological triad was examined. Specifically, this thesis examined (1) the ability of a collagen glycosaminoglycan (CG) scaffold to support growth viability and maturation of human fetal osteoblast cells (hFOB) (Chapters 2&3), (2) the effects of CG substrate stiffness on osteogenesis (Chapter 4) and (3) the use of a flow perfusion bioreactor to improve cell distribution and osteogenic stimulation for cells within a CG construct (Chapter 5), thus, developing a tissue engineered construct with enhanced osteogenic capabilities populated by a homogeneous distribution of stimulated cells.

6.2 CG scaffolds support human cell proliferation, growth, viability and osteogenesis in vitro

Porous CG scaffolds have been shown to be successful for supporting the growth of several cell types such as fibroblasts, mesenchymal stem cells (MSCs), chondrocytes, myofibroblasts and pre-osteoblasts (Schulz Torres *et al.*, 2000; Freyman *et al.*, 2001b;

Lee *et al.*, 2001; Farrell *et al.*, 2006; Tierney *et al.*, 2009). However, in this thesis we aim to investigate these CG scaffolds specifically, human bone cells. In Chapters 2&3, following initial cell-scaffold optimisation parameters, hFOB cells were shown to attach to the CG scaffold and migrate gradually from the construct periphery towards the centre within 14 days. Cell proliferation continued until the construct became fully confluent with a homogenous distribution of cells which were shown to express alkaline phosphatase (ALP) and osteocalcin (Oc), early and late stage markers of bone formation respectively, in non-osteogenic conditions by 35 days of culture.

The ability of the CG scaffold to support osteogenesis of hFOB cells was then analysed. Under osteogenic conditions constructs remained viable up to 49 days, and osteogenesis was confirmed by the sequential expression of major bone formation markers ALP, collagen type I, osteopontin (Op), biglycan and Oc coupled with positive alizarin red and von kossa staining for mineralisation.

Although Harris *et al.* (1995) described a media formulation for inducing osteogenesis of the hFOB cell line not all authors reported this success (Dhurjati *et al.*, 2006). Consequently, Chapter 3 begun by examining the hFOB osteogenesis in 2D, it was found that adding transforming growth factor-β1 (TGF-β) (the most common growth factor found in bone) to the Harris media formulation provided the greatest levels of osteogenesis and mineralisation. Several *in vivo* studies have demonstrated a role for TGF-β in bone formation; animal models which had significantly reduced levels of TGF in the hind limbs of rats have been shown to become osteopenic (Kim and Russell, 1995). Furthermore TGF-β has been shown to be beneficial for bone formation *in vitro* and coupled with vitamin D a synergistic effect on osteoblast differentiation occurs (Eichner *et al.*, 2002; Harris *et al.*, 1995; Nagel and Kumar, 2002). Previous studies; however, have reported various optimal concentrations during *in vitro* culture (Centrella *et al.*, 1987; Eichner *et al.*, 2002; Zhang *et al.*, 2003). Therefore, Chapter 3 examined the effects of exposing hFOB cells on CG scaffold to a continuous, low

concentration (0.2ng/ml) of TGF-β versus one initial high concentration (10ng/ml) for one week followed by a low continuous concentration of TGF-β. Results found high TGF to be provided the greatest levels of osteogenesis i.e lower levels of the collagen type 1 and ALP and greater levels of Oc were identified similar to a previous study (Sloan and Smith, 1999). Similarly, an initial exposure of high TGF was beneficial *in vitro* for mineralisation a finding supported by Zhang *et al.* (2003). High TGF exposure resulted in greater staining for mineralisation and expression levels of late bone formation marker Oc (0.4 fold increase between low and high TGF at 49 days).

Importantly, it was shown that CG constructs developed an acellular necrotic degraded core by the end of the culture period. Core degradation occurred as a result of peripheral encapsulation which limited diffusion of nutrients and wastes to and from the centre of the construct; as such it is regarded as a limiting factor in tissue engineering. A construct displaying core degradation would not be beneficial for bone regeneration as it could impede angiogenesis, be mechanically unstable and result in fibrosis between the interface of the defect and the construct. The compressive stiffness; however, increased 10 fold from 0.5 kPa to 5 kPa by the end of the 49 days in culture.

This study is the first to show the CG scaffold can support osteogenesis by human cells and that it is possible to provide enhanced levels of osteogenesis by varying the TGF-β dose.

6.3 Scaffold stiffness and contractile behaviour can modulate the functional maturation of osteoblasts on a CG scaffold

Long term culture of hFOB cells on CG scaffold in Chapter 3 showed that the combination of cell mediated contraction (70% contraction) coupled with peripheral cell encapsulation of the scaffold resulted in changes in scaffold shape, cell viability and mechanical properties after 49 days in culture. This change in the scaffold stiffness associated with cell-mediated contraction is a trend that has also been reported by

others (Schulz Torres *et al.*, 2000; Spilker *et al.*, 2001). We decided to focus on the stiffness of the scaffold and explore it further as the current 2D theory reported by Discher *et al.* (2005) states that stem cell differentiation could be directed toward a particular lineage by varying the stiffness of the substrate i.e. a soft substrate encouraged differentiation of soft tissues such as blood and brain, whereas stiffer substrates directed stem cells towards stiffer tissues such as muscle and bone (Discher *et al.*, 2005). However, less is known about how substrate stiffness affects cells in 3D models.

Thus, Chapter 4 examined the effects of varying the mechanical stiffness of the CG scaffold using the three different cross-linking techniques of dehydrothermal (DHT), 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDAC) and Glutaraldehyde (GLUT). The results show that stiffness can modulate the functional maturation of murine osteoblasts on a CG scaffold albeit this occurs in a slightly different manner than in 2D. The stiffness of the more compliant contractible DHT CG constructs displayed a greater osteogenic maturation while the stiffer, non contractible EDAC and GLUT constructs resulted in a lower osteogenic phenotype with higher cell numbers. Similar findings have also been reported previously using chondrocytes on CG scaffolds (Lee *et al.*, 2001).

Of particular interest is smooth muscle actin-α (SMA-α) which has been shown to be associated with cellular contraction of CG matrices (Schulz Torres *et al.*, 2000). In the DHT treated scaffolds, once contraction had occurred and ceased, a decrease in SMA-α expression was observed. This down-regulation in SMA-α following a contraction phase in culture has been previously identified in CG scaffolds (Menard *et al.*, 2000). Reduced expression of SMA-α coincided with the end of substrate contraction and an increase in the expression levels of late stage osteogenic markers and mineralization. As crosslinking the scaffold should not change the composition of the scaffold, it therefore appears that the greater levels of osteogenesis observed in DHT treated scaffolds was as

a result of cell-mediated contraction.

The mechanically stiffer EDAC and GLUT crosslinked CG scaffolds provided greater structural stability, a higher cell number and viability and supported osteogenesis albeit at an earlier level of osteogenesis than for the DHT scaffolds. Although GLUT crosslinking has been linked with inducing calcification (Vincentelli *et al.*, 1998) no difference was observed between GLUT and EDAC scaffolds To summarise, varying construct stiffness affects osteoblast cell growth and maturation in 3D in-vitro culture.

6.4 Flow perfusion bioreactor leads to improved cell distribution and osteogenic maturation of human cells on CG scaffolds

Long term static culturing conditions can lead to a heterogeneous cell distribution, often resulting in the development of a cellular peripheral capsule which may result in the development of a necrotic/acellular central core caused by a reduction in mass transfer conditions. This result was observed in Chapters 3 & 4 which is a similar finding to previous studies (Farrell *et al.*, 2006). Therefore, a flow perfusion bioreactor was used to improve cell distribution and to mechanically stimulate the hFOB cells seeded on a CG scaffold under osteogenic conditions (Jaasma *et al.*, 2008). Bioreactors have been shown to improve mass transport throughout cell seeded constructs (Plunkett *et al.*, 2010) and may also be used to encourage cell growth and maturation (Yu *et al.*, 2004; Bjerre *et al.*, 2008).

The results from this study show that a 24 hour exposure in a flow perfusion bioreactor led to improved cell distribution and osteogenic priming of hFOB pre-osteoblasts. No difference was observed in levels of cell number or metabolic activity or compressive stiffness following bioreactor culture versus static culture. However, cell distribution was more homogenous throughout the construct and the development of peripheral encapsulation was prevented. Consequently, those constructs cultured in the bioreactor displayed lower levels of mineralisation compared to those cultured

statically. Although an ability to produce large levels of calcified tissue is of interest, it is also important to be able to produce a construct with homogenously distributed cells primed for osteogenesis *in vitro*, which will not restrict mass transfer *in vivo* after implantation into a defect. Where mineralisation may have been lower in the bioreactor group interestingly, gene expression analysis showed that bioreactor cultured constructs gave higher cellular expression levels of bone formation markers than static culture alone. Suggesting that the bioreactor can be beneficial for improving cell distribution and osteogenic priming of cells seeded onto CG scaffolds.

Future work

- This work relies on the human and mouse cell lines respectively. As the CG scaffold is intended for human application it would be have been interesting to compare these studies with primary human osteoblasts or MSCs. On a similar note this research was all carried out *in vitro* therefore *in vivo* applications should follow.
- In Chapter 4 the effects of substrate stiffness and contractile behaviour on osteogenesis was analysed; further research in this area may include:
    - Examining the effect of substrate stiffness and contractile behaviour on osteogenesis using unstimulated media on osteoblasts. This would give more information on how substrate stiffness and contraction influence cell phenotype. For this it would be interesting to examine specific mechanotransduction cascades such as the ERK pathway as currently little is known about the 3D cell response to substrates.
    - Other crosslinking approaches such as using transglutaminase could be used to control scaffold contraction during *in vitro* culture. Transglutaminase is a microbial enzyme which can crosslink collagen without affecting cell viability. Therefore it would be interesting to examine CG contraction further by permitting some level of contraction to provide mechanical stimulation and then cease further contraction to retain an appropriate level of porosity within the construct.
    - In Chapter 4 the effects of contraction was examined. As SMA-α levels were assessed one caveat may have been the absence of a group containing an actin inhibitor. This would provide information on cell mediated contraction alone and the possible role of SMA-α during osteogenesis.
- In Chapter 5 the bioreactor was shown to aid cell distribution within the

constructs. It would be interesting to speed up the duration it takes for the constructs to become confluent. This may be carried out by introducing a proliferation step into for cells seeded onto CG scaffolds following bioreactor re-distribution. For example one approach may be to combine recent technology by Vabrema® Eindhoven, (the Netherlands) who have developed a bioreactor to enhance cell number by exposing cells to electromagnetic waves. This can increase cell number by 2 fold and it may be possible to use this technology following our flow perfusion bioreactor culture of cell seeded CG constructs to improve levels of confluency within the construct.

- It would be interesting to continue with the idea of a bioactive scaffold as described by Yannas *et al.* (2010). One route may be to incorporate TGF into the scaffold during fabrication. This approach is ongoing within our research group using a variety of scaffolds and growth factors.
- Further research into preventing core degradation would prove useful. One option is to examine angiogenesis within the construct during culture so that following implantation the construct could readily provide nutrients and remove wastes from the centre of the graft. Research into this area is currently ongoing in our laboratory.
- Using results from Chapter 3 another interesting application of the 3D set up used in this study would be to apply this 3D CG model of osteogenesis for *in vitro* drug analysis. The CG constructs could compare the effects of novel osteoblast stimulating compounds such as cytokines on osteogenesis.

Conclusions

- The CG scaffold successfully supports the attachment, infiltration, migration and viability of human hFOB pre-osteoblast cells up to 35 days, under standard growth conditions *in vitro.* Following initial optimisation of hFOB cells on CG scaffold, long term culture of hFOB cells on CG scaffold demonstrated that cells migrated to the centre of the scaffold by 14 days, resulting in a homogenous, confluent construct by 35 days which displayed both osteoconductive and osteoinductive qualities.
- The CG scaffold was successfully shown to support osteogenesis of human hFOB cells and form mineralised tissue *in vitro.* Osteogenesis of hFOB was shown to occur in a temporal sequential manner in both 2D and 3D environments. TGF-β was shown to be beneficial to enhance osteogenesis with greatest effect using an initially high exposure of 10ng/ml TGF for 7 days and reducing this to 0.2ng/ml thereafter for cells on scaffold.
- Different crosslinking techniques produced scaffold of varying stiffness; these can modulate the functional maturation of osteoblasts on a CG scaffold. The results demonstrate that the more compliant contractible DHT CG constructs displayed a greater osteogenic maturation while the stiffer, non contractible EDAC and GLUT constructs resulted in a lower osteogenic phenotype but higher cell numbers. The results demonstrate that scaffold stiffness and contractile behaviour can modulate the functional maturation of osteoblasts on a CG scaffold.
- Flow perfusion bioreactor culture leads to improved cell distribution and osteogenic priming of hFOB pre-osteoblasts. Cell distribution improved following bioreactor culture becoming more homogenous throughout the construct and avoiding the formation of a peripheral cell capsule forming. Bioreactor exposure displayed lower levels of mineralisation than static

cultures; however, gene expression analysis showed that bioreactor cultured constructs gave higher cellular expression levels of bone formation markers than static culture alone.

Appendix A

Hoechst 33258 DNA standard curve for hFOB 1.19 cells

Introduction

Hoechst 33258 is a fluorescent dye that can bind to double stranded DNA. The assay is an end-point assay therefore; it requires the destruction of the cells for quantification the amount of DNA in the sample however it is not affected by rates of diffusion or digestion as have been shown in Chapter 2 using almar blue and dispase digest assays. To calculate cell number firstly a standard curve was carried out using hFOB 1.19 cells.

Materials and methods

Hoechst 33258 DNA standard curve:

hFOB cells were grown in tissue culture plates (Sarstedt, Germany) until near confluency. Cells were tripsinised and re-suspended into two tubes containing $2x10^6$ cells pellets each. A 30µl of papain solution (Sigma-aldrich, UK) containing cell concentrations between $5x10^4$ and$1x10^6$ was left to digest for 4-6 hrs, vortexing every hour (n=3). A total of 600µl of Hoechst working dye solution (Sigma-aldrich, UK) was added to each eppendorf tube and vortexed thoroughly. 210µl of this sample solution was plated out into a 96-well plate and the fluorescence of the samples was measured at 460nm after excitation at 355nm in the Wallac Victor2™ 1420 multilabel counter (Perkin Elmer Life Sciences, Finland). Fluorescence readings were plotted against cell number to give a standard curve.

Results

Figure A1 show the hFOB standard curve from this the equation of the line was determined to be $y = 0.6489x + 3562.2$ with an $R^2 = 0.9819$.

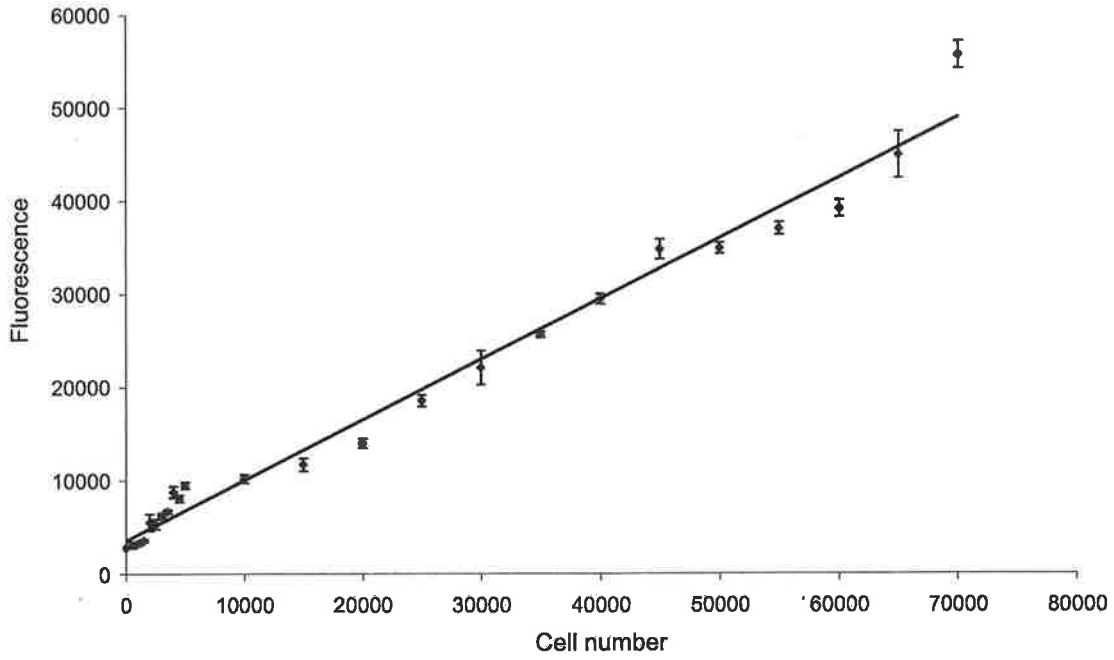

Fig A1 hFOB cell Hoechst DNA standard curve (cells alone)

Discussion/conclusion

Hoechst dye is a fluorescent nucleic acid stain for quantifying double-stranded DNA. Using the equation of the line obtained from the standard curve it is possible to quantify hFOB 1.19 cell number.

Appendix B

Analysis of MC3T3 2D osteogenesis

Introduction

Mouse clonal MC3T3 cells were assessed for their osteogenic potential prior to osteogenic studies on the CG scaffold. This calvarial derived cell line has been well reported in the literature for use in osteogenic analysis. They have been shown to mineralise in culture once exposed to ascorbate and a phosphate donor.

Materials and methods

MC3T3 seeding and osteogenic cell culture

MC3T3 cells were cultured routinely until near confluency in standard tissue culture flasks (37°C, 5% CO_2). 1x10^5 MC3T3 cells were seeded per well on a 12 well plate. 2ml of prewarmed standard media was added and incubated at 37°C until confluent; once confluent cultures were incubated at for a further 21 days in osteogenic media (i.e. standard media supplemented with 50μg/ml ascorbic acid, 10mM β-glycerophosphate). Two thirds of spent media was replenished every 3-4 days.

MC3T3 alkaline phosphatase expression and Alizarin red mineralisation analysis

Alkaline phosphatase analysis was carried by incubating 1ml a 10% solution of pNPP (Sigma-Aldrich, Germany) per pre-rinced cell confluent well at 7, 14 and 21 days (n=3). Staining for mineralisation was carried out as per section 3.2.2.3 and quantification was carried out as per section 5.2.5 at 7, 14 and 21 days (n=3).

Results

Expression levels of alkaline phosphatase increased with time up to 21 days (Fig B1). This increase was statistically significant between 7, 14 and 21 days ($p<0.001$). Between 7 and 14 days a 2- fold increase in expression was observed, this further doubled in expression between 14 and 21 days.

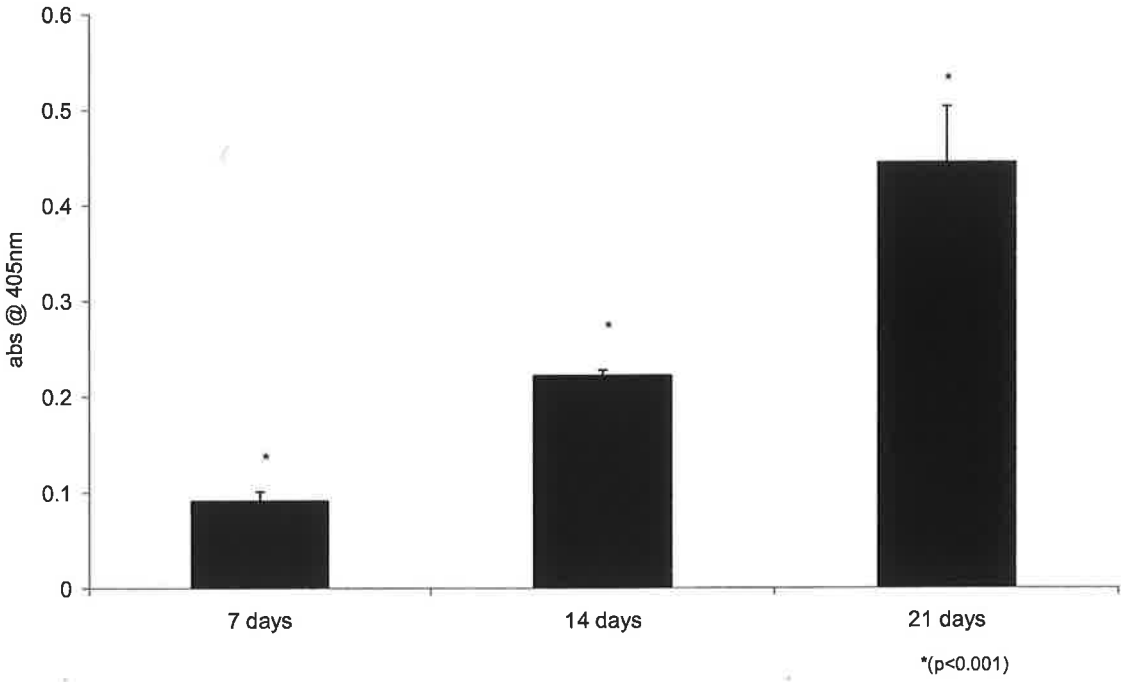

Fig. B1 Alkaline phosphatase expression for MC3T3s in osteogenic media up to 21 days (n=3).

Alizarin red staining showed an obvious increase in staining of calcium bound mineral up to 21 days (Fig. B2). Quantified results showed an increase in calcification with time (Fig. B3). The level of mineralisation increased by 2 fold between 7 and 14 days ($p<0.01$) and increased further by 8-fold over day 7 at 21 days ($p<0.01$).

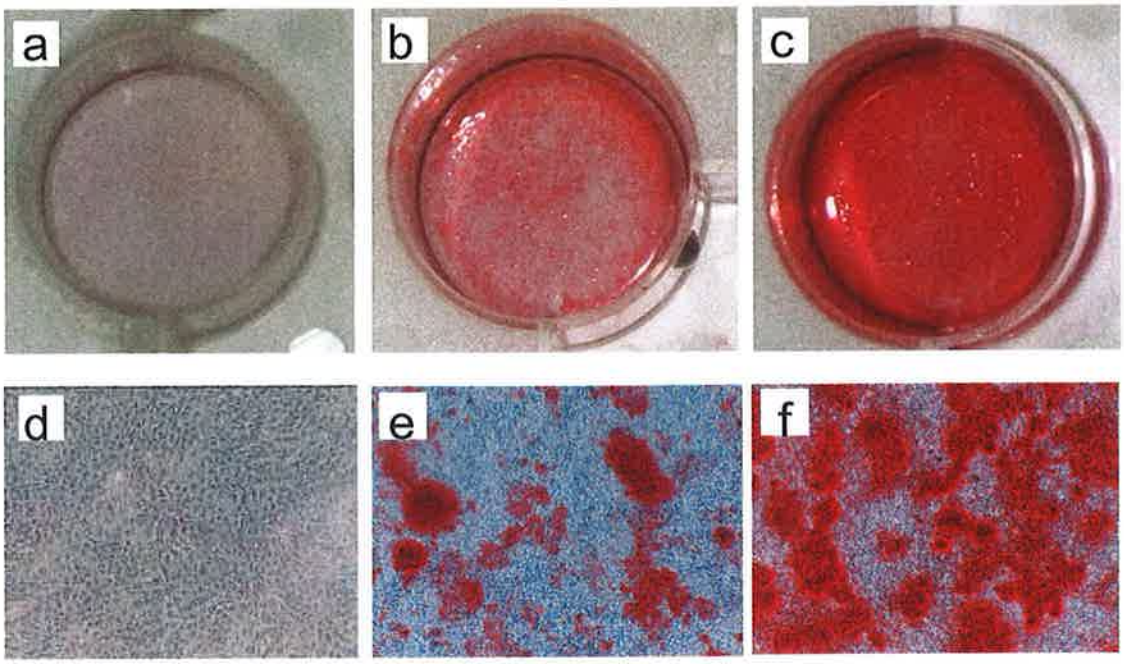

Fig. B2 Alizarin red staining for mineralisation in osteogenic media up to 21 days (n=3); (a-c) photograph of day 7, 14 and 21 days; (d-e-f) day 7, 14 and 21 under low power (4X magnification).

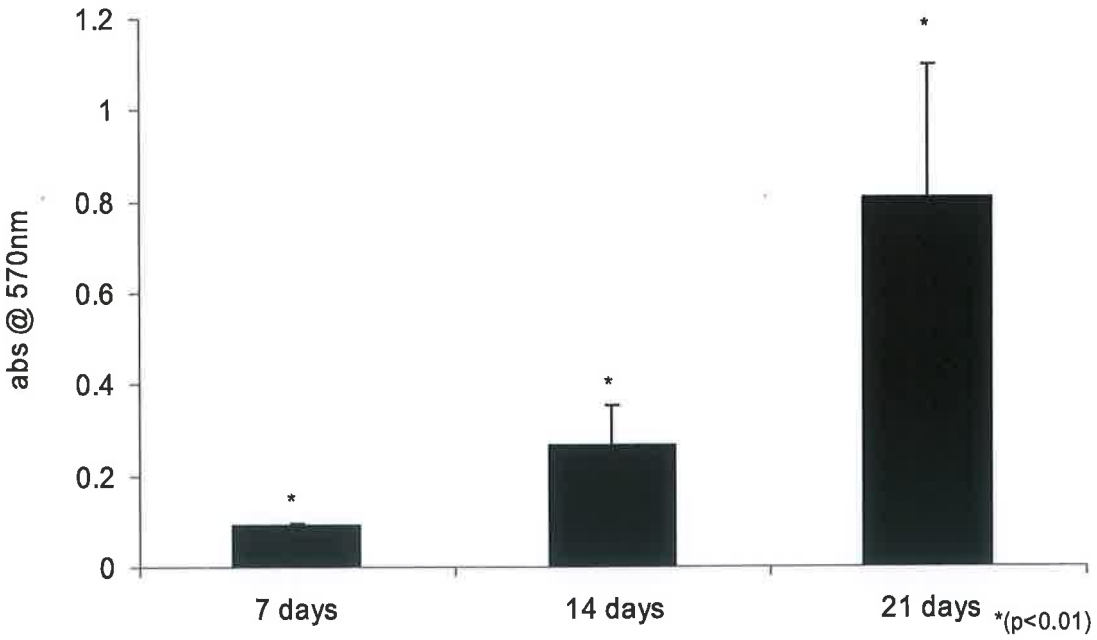

Fig. B3 Quantification of alizarin red staining for mineralisation in osteogenic media up to 21 days (n=3).

Discussion/conclusion

The MC3T3 cell line can be stimulated using ascorbate and a phosphate donor to differentiate into a mature bone forming osteoblast. Alkaline phosphatase an early marker for bone formation was expressed at all time points (Fig. A1). The level of this expression increased with time ($p<0.001$) (Fig. A2). Alizarin red staining for mineralisation was positive after 14 days increasing over 2-fold by 21 days ($p<0.01$) (Fig. A3). In conclusion, MC3T3 cells differentiate and stimulate into mature osteoblasts in 2D.

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