Development of novel strategies for musculoskeletal tissue engineering

by

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“Two roads diverged in a wood, and I -I took the one less traveled by, and that has made all the difference” - robert frost

To my mom
Abstract

The drastic rise in the world’s population coupled to an ever increasing aging population poses a considerable challenge to the orthopaedic community to maintain healthy activity levels. The field of Tissue Engineering and Regenerative Medicine aims to tackle these challenges by implementing more biomimetic strategies to improve upon current treatments. The success of new therapeutic developments in musculoskeletal tissue engineering relies on our ability to study and understand the complex biological interactions between cells, materials, and native tissues so that we may subsequently guide neotissue formation. This thesis is focused on the development of novel, well-defined, and reproducible in-vitro tissue culture models to explore, characterise, and control cellular behaviour and differentiation for osteochondral regeneration. In particular, these models utilised combinations of polymeric biomaterials, differentiated osteoblasts, human periosteal stem cells, and physico-chemical cell signalling cues. In a commercial venture with PolyNovo Ltd (Melbourne, Australia), a novel two-component injectable polymer platform was synthesized and evaluated for uses as a biomaterial construct in orthopaedic applications. The second aspect of this thesis focuses on the harvest, isolation, expansion, and extensive characterisation of human periosteal cells in-vitro. The periosteum is a bi-layered membrane that covers the outside of cortical bone and has been recently identified as a potential stem cell source; with the ability to form osteogenic, chondrogenic, adipogenic, and myogenic tissue types. To detail the heterogeneous cellular features and behaviours of human periosteal cells in-vitro, cells were isolated from surgical explants, expanding in monolayer in the absence of differentiation supplements, and characterised for changes in morphology, growth rate, cell-cycle, gene expression, and phenotype. Additionally, enrichment techniques were designed to preferentially isolate distinct progenitor cell types identified in periosteal cell cultures. Most interestingly, a novel cell-sorting platform utilising droplet microfluidic approaches, was developed and evaluated for its ability to identify and separate periosteal progenitor cells. In the third part of this thesis, a 3-dimensional agarose culture model was created to control and monitor lineage specific human periosteal cell differentiation in various biomechanical and biochemical environments. The work presented herein further demonstrates the potential of human periosteal cells for osteochondral repair and more importantly provides critical information regarding human periosteal cell expansion, phenotype, and differentiation.
Preface

Some of the results presented in this thesis have been published and presented in various journals and conferences.

**Journal publications**


**Conference contributions**


**Articles submitted or in preparation**


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Chapter 1

Introduction

1.1 Background and motivation

In developed countries, rapidly growing populations are continuously driving the expectations for a longer and more comfortable life. A majority of these demands are being shouldered by the medical community, with developments in surgical techniques and advancements in technology leading to an increased quality of life. However, this situation has effectively become a “catch 22”. Medical technology has contributed to an increase in human longevity, which in turn, has created a greater need for the replacement of tissues. This is particularly important for the future, as we live in an increasingly aging population, where the number of people over the age of 60 is expected to rise by 50% (1;2). This desire to live a more active, healthy life-style will place particular pressure on the orthopaedic community to provide new treatments for sustained mobility and ambulation throughout one’s life. These challenges faced by the orthopaedic community have led The World Health Authority to decree that 2000-2010 will be the Bone and Joint Decade (1). A relatively new branch of medicine has emerged with the intent of circumventing issues surrounding the deterioration of tissues due to aging, and trauma. Aptly named Regenerative Medicine, it incorporates the fields of tissue engineering, biomaterials, stem cell technologies, and the study of associated human diseases to facilitate the repair, regeneration, and replacement of injured tissues (3).

In recent years, skeletal reconstruction due to joint disease and trauma has become an increasingly common and important procedure for the orthopaedic community. In the United States and United Kingdom, a combined 50 million people suffer from some form of arthritis, accounting for roughly half of all chronic conditions in people over 65 (4;5). These chronic degenerative joint diseases cause extreme pain and considerably limit mobility due to the destruction of joint structure and functional impairment. Yet, these problems are not strictly associated with the elderly. Trauma, a major cause of mortality and morbidity in industrialised countries, has become the leading cause of death in people aged 1-44 years (6). Of these trauma cases, a vast majority are musculoskeletal (bone, cartilage, and surrounding soft tissues) in nature. In the UK alone, there are approximately 200,000 hospitalisations due to fractures each year, with more than half of these fractures being to the lower extremities (7;8). Combined, these musculoskeletal disorders cost the UK society over £6 billion a year (7). These injuries due to joint disease and trauma may not always be severe, but
their management can be complex. Treatment often involves choices between non-operative management and different types of surgical intervention (9).

Conventional approaches in musculoskeletal tissue repair include treatments such as autografts, allografts, and artificial grafts. Autologous grafts are the current “gold standard” in tissue repair; however, they create additional surgical sites, have limited availability, increase operative and recovery time, and have a risk of donor site morbidity (10). Allografts, although more abundant than autografts, are hampered by risks of disease transmission, high cost, and variability of results (11). Artificial grafts and implants raise concerns regarding toxicity, fatigue, failure and need for revision surgery (12). This is the current challenge that faces the orthopaedic community to date; how do you overcome the limitations of the limited lifespan and continued failing of bioinert artificial implants, while still improving the quality of life for millions of patients each year? To address this concern, researchers introduce a number of “ideal” biomaterials each year for proposed uses in a wide variety of clinical settings. However, the successful marriage of both biocompatibility and optimal mechanical properties has proved to be an extremely difficult task.

Although there has been major progress in orthopaedic research in the last 20 years, current treatments of severe injuries related to bone and cartilage fail to achieve long-term stability and often led to secondary degeneration. Over the past decade, there has been a definite shift in research emphasis, from developing bioinert man-made materials to more biological approaches concerning the natural regeneration of tissues (13). This shift in research has led to the emergence of a new field, known generally as Tissue Engineering, where more biological regenerative therapies are designed to behave like autologous implants (“gold standard”). “Tissue engineering is an interdisciplinary field that applies the principles of engineering and the life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function” (13). Tissue engineering effectively seeks to bridge the gap between widely used engineering-based solutions (e.g. implantable metals (14;15), calcium phosphate bioceramics (16)) and limited biological treatments (e.g. autologous chondrocyte transplantation (17), osteochondral plugs (18)). Through the combination of knowledge from numerous fields, biologists, chemists, and engineers endeavour to produce “bioinspired” materials that not only serve as a structural tissue replacement, but also functionally integrate with the surrounding tissue. Tissue engineering relies on the execution of three distinct strategies:

- **Isolated cells:** This approach avoids complicated surgical intervention, and permits expansion and manipulation of the cells towards specialised phenotypes prior to infusion. Potential problems include loss of cell function and immune rejection.
- **Cell-seeded matrices**: This approach is broken down into two different systems. In a closed system, the cell seeded matrix is encapsulated in a semi-permeable membrane, therefore isolated from the outside biological environment, which allows for the transport of nutrients and wastes but prevents penetration and destruction by immune cells. These closed-system therapies can be used in pharmacological treatments to target tissue specific sites. In an open system, the cell seeded matrix is implanted directly into the patient, and is allowed to integrate with the surrounding tissue.

- **Tissue inducing cues**: The approach relies on the induction of appropriate physio-chemical signalling cues, such as growth factors, into the cellular environment and the mechanisms used to deliver these cues to the selected cells.

The tissue engineering approach to tissue repair and regeneration, shown schematically in Figure 1.1, relies on a unique combination of the above said strategies. In utilising these tissue engineering strategies in parallel, this thesis aims to design and characterise novel in-vitro therapeutic models with the ultimate goal of developing clinically relevant treatments for musculoskeletal disorders affecting bone and cartilage.

![Figure 1.1 Schematic representation of the methodology behind tissue engineering. (1) Cell isolation usually from a biopsy. (2) In-vitro cell expansion in tissue specific culture conditions. (3) The seeding of cells and exogenous growth factors into a three-dimensional porous structure called a scaffold. (4) Further culture of cell-scaffold construct to allow for infiltration and inhabitation, and finally (5) re-implantation of the cell-construct tailored for the specific physical environment found at the site of repair and the tissue type it is repairing. (picture by Julian George)
1.2 Literature Review

In this thesis, separate research projects were undertaken, each devoted towards a particular aspect of the tissue engineering triad; namely biomaterials, stem cells, and environmental stimuli. In this context, a multidisciplinary framework is applied, utilising specific techniques from cell and molecular biology, chemistry, materials science, and engineering. To design rational, “bio-inspired”, therapies for musculoskeletal tissues, a heightened understanding of the interactions between cells, materials, and surrounding biological environment is necessary. Thus, the following background section details the structural and biological properties of bone and cartilage and explores the recent developments and current challenges associated with bone and cartilage tissue engineering.

1.2.1 Cartilage and Bone Biology

Bone is a highly-ordered, hard, yet dynamic composite material (19); cartilage is a structured gel-like matrix of proteins, proteoglycans and glycosaminoglycans (20). Both are highly organised tissues that assemble from the nano- to macro-scales to produce complex structural networks (Figure 1.2). Understanding of the subtle interplay that underpins these complex and sophisticated molecular dynamics is both challenging and crucial in the design of model systems for the repair and regeneration of orthopaedic tissues

1.2.1.1 Cartilage

Cartilage has three forms: hyaline, elastic, and fibrocartilage. The type of cartilage with particular importance to the normal functioning of the skeletal system is hyaline cartilage. The role of hyaline cartilage is then to provide a low-friction surface, participate in lubrication of synovial joints, and to distribute applied forces to the underlying bones. For this purpose, hyaline cartilage consists of relatively low amounts of cells, chondrocytes, and an extensive extracellular matrix. The macromolecules that make up hyaline cartilage include collagen, mainly type II, proteoglycans, non-collagenous proteins, such as fibronectin, and glycoproteins (21). This highly networked ECM combined with the fact that cartilage contains 77.7% water gives hyaline cartilage its strong mechanical and frictionless biological properties (22). However, when damaged, cartilage exhibits a unique inability to heal. This is due to cartilage’s avascularity, low cell numbers, chondrocyte immobility, and limited capacity to proliferate (23). This inability to regenerate itself makes cartilage a prime candidate for developing alternative tissue engineered therapies to heal damaged tissue.
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Figure 1.2 Hierarchical structure of bone and cartilage. Bone and cartilage are highly organised over different length scales (adapted from (24)). Bone biomineralizes to form a calcified outer layer filled with osteons, or Haversian systems – each supplied by their own vasculature. The osteocytes within these systems are surrounded by a dense network of aligned collagen I fibres, which provide templates for the self-assembly of hydroxyapatite crystals. Cartilage, in contrast, is a non-vascular, turgid network, which supports chondrocytes spaced between 10-200μm apart. Hydrophilic proteoglycans, such as aggrecan, interwoven between a network of collagen II fibres, provide a shock-absorbing matrix.

1.2.1.2 Bone

The major functions of bone are to provide mechanical support of soft tissues and provide a site of attachment for muscles used in locomotion. Bones also serve as a major reservoir of calcium and phosphorus minerals, protect the central nervous system and organs from trauma, and house bone marrow, a primary source of adult stem cells in the body. Furthermore, bone is capable of maintaining optimal shape and structure by a process of continuous self-renewal, such that it can respond to changes in its mechanical environment by remodelling (known as Wolff’s law)(25). Bone is a dynamic and adaptable organ that plays an integral part in the total functioning of our skeletal system. Bone tissue consists of both cortical (compact) and trabecular (spongy) bone (Figure 1.2 and 1.3) containing 4 major cell types embedded in both organic and inorganic matrix components. The cell types include:

- Mesenchymal precursor cells- located in the periosteum and endosteum, connective tissue lining bones. These cells have the potential to differentiate into osteoblasts, fat cells, chondrocytes, or muscle cells (26).
- **Osteoblasts**- bone-forming cells that synthesize and secrete unmineralised matrix components.

- **Osteocytes**- mature bone cells that have effectively trapped themselves in the bone tissue they have secreted. Maintain bone health by influencing mineral content and regulating calcium release into the blood. Account for about 90% of the cells in the adult skeleton (27).

- **Osteoclasts**- multinuclear cells that enzymatically break down bone tissue.

The organic, osteoid, portion of the extracellular matrix consists of proteoglycans, glycoproteins, and collagen fibres, mainly type I, all secreted by the osteoblasts. The inorganic component consists of mineral salts known as hydroxyapatites, which are largely composed of calcium phosphates. The hydroxyapatite crystals attach to and surround the collagen fibres. These ECM components give bone its properties of being strong and durable without being brittle.

Cortical (compact) bone comprises the outer tubular shell of long bones, as well as the outer surface of the small and flat bones. It contains densely packed cylindrical units called osteons (see Figure 1.2 and 1.3), which are arranged for optimal resistance to mechanical stresses. Trabecular (spongy) bone is less dense than cortical bone, and is found in the interior of flat bones and the epiphyses of long bones. It is composed of an array of interconnected lamellae arranged in an irregular latticework of struts and rods, called trabeculae, with the spaces in between containing the bone marrow (28). This arrangement of the trabeculae is focused on resisting compressive stresses. Within these two types of bone there are thin networks of capillaries, known as Haversian and Volkmann’s Canals, where blood flow and nutrient and gas exchange occur.

### 1.2.1.3 Periosteum

The outer surface of long bone shafts is covered by a connective tissue, called the periosteum, which is composed of two distinct layers. A highly cellular thin inner, “cambium”, layer which abuts the cortical bone and consists of osteoblasts, fibroblasts, and mesenchymal progenitor cells, that have the capacity to differentiate into both osteoblasts and chondrocytes (29-32). The thicker outer, “fibrous”, layer consists of fibroblastic cells immersed in large amounts of collagen and elastin fibres. In addition to this, both layers are shown to have distinctive nerve and microvascular networks (Fig. 2)(33).
The periosteum is known to cover the surfaces of long bones, however this coverage is not contiguous. Periosteum is absent from articular surfaces, tendon insertions, and sesamoid bone surfaces and is present in locations of high-risk for fractures, such as femoral necks, distal radius, and vertebrae (34). Also, site specific differences in periosteum anatomy, mechanical properties, and bone formation rates are known to exist throughout the skeleton (35). In support of these findings, it is suspected that changes in local mechanical environment in different areas of the periosteum may be a contributing factor to the increase or decrease in growth during the bone development and remodelling (36;37).

The periosteum as a whole is anchored to the underlying bone by coarse bundles of collagen and elastic fibres, termed Sharpey’s fibres, penetrating from the outer fibrous layer (38). With maturity these fibres thicken and bundles enlarge, effectively strengthening the connection between bone and the periosteum. As this process occurs, the effective level of mechanical stress needed to stimulate the cambial layer is raised (39). Most importantly, the periosteum provides the interface between bone and muscle, and plays a vital role in remodelling of bone and cartilage tissue during developmental growth and fracture healing.

1.2.1.4 Development and healing

The developing long bone consists of three regions; diaphysis, epiphyses, and metaphyses. These regions, established in the embryonic stage, continuously change size and shape until skeletal
maturity. These developmental changes are due to an intricate sequential process of mesenchymal stem cell differentiation into chondrocytes (cartilage cells), which then undergo well-ordered and controlled phases of proliferation, hypertrophic differentiation, apoptosis, vascularisation, and finally cartilage ossification (40). This process, known as endochondral ossification, leads to longitudinal growth and bone formation at the region between the epiphyses-metaphyses growth plates, directly below the cartilaginous articulating surfaces of the bone. At the same time, there is radial growth of the diaphyseal and metaphyseal growth plates caused by new cortical bone being laid down by osteoblasts of the inner cambium of the periosteum. This process is known as intramembranous bone formation. These two processes rely on the delicate functional balance of osteoblasts, osteoclasts, and chondrocytes. This cell coordination is regulated by a plethora of factors, including intrinsic and extrinsic growth factors and hormones, and mechanical forces. For an extensive list and discussion of regulatory molecules see Forriol and Shapiro’s review of this topic (41).

During fracture repair in long bones, the periosteum is known to play a vital role in fracture callus formation, which promotes subsequent osteogenesis, and eventual ossification (42;43). Initially, the periosteum contributes to increased cell production, resulting in periosteal thickening. In this role the periosteum serves as a source of osteoprogenitor cells and extracellular fluids to nurture cells filling the fracture callus (44). In addition, the periosteum provides signalling cues to the repair cells to modulate and coordinate cellular processes. Bone formation in the callus includes initial intramembranous bone formation mediated, in part, by periosteal cells undergoing primary osteoblastic differentiation and secondary endochondral ossification by preceding chondrocytic differentiation of progenitor cells (45). The recruitment and differentiation of these progenitor cells seems to be regulated by numerous cytokines and the local mechanical environment, however, the specific effects and interactions in normal fracture healing are yet to be fully elucidated (46). This topic of cell differentiation control will be covered more completely in future sections.

Similarly, the periosteum is also known to modulate bone size throughout an individual’s lifespan, which is recognised as a critical determinant of bone strength and possibly of fracture resistance (47). This capacity to adapt can be seen during endocortical bone loss, which leads to higher stresses on the remaining bone, especially the periosteal surface where stresses are the highest in bending. This change in mechanical environment results in compensatory periosteal bone formation to normalise the stress (48).

The replication of these natural regeneration processes to recreate osteochondral tissues is the ultimate hope of musculoskeletal tissue engineering. This complex goal will require a deep understanding of the mechanisms behind these regeneration processes combined with an
appropriate tissue engineered construct. Osteochondral constructs should consist of osteogenic and/or chondrogenic cell sources combined with an optimal biomaterial cell scaffold.

### 1.2.2 Biomaterials

Important to the success of regenerative strategies, is the ability to develop materials that can interface with tissues structurally, mechanically and biofunctionally (49). Many acute and chronic musculoskeletal conditions, such as full-thickness and arthritic defects, require osteochondral substitutes to repair the damaged or diseased tissue. The chemical and biological properties of the substitutes can vary greatly depending on the types of materials used. For both cartilage and bone, it is advantageous to find versatile, implantable, biocompatible materials that direct tissue regeneration, whilst providing adequate structural support. Current research is focused on a wide variety of bioresponsive materials which can simultaneously provide support and guide tissue repair, including bioactive glasses, biodegradable polymers, and extracellular matrix proteins. Scaffolds can broadly be termed as a temporary or permanent three-dimensional structure to support cells and provide them with a tissue specific environment and architecture (50). An ideal scaffold for bone and cartilage tissue should possess these essential properties(13;19;51-54):

- **Biocompatible:** The scaffold should naturally integrate with the host tissue and should not illicit an immune response

- **Biodegradable:** Scaffold degradation rate should be in tune with the growth of neo-tissue, so that by the time the injury site is totally regenerated the scaffold has been completely remodelled and degraded (55).

- **Mechanical Support:** Scaffolds should have sufficient mechanical strength to support tissue ingrowth and provide a stable architecture for cell attachment. For in vivo implantation, the mechanical properties of the construct should ideally match those of the bone and/or cartilage tissue it is repairing to reduce the recovery time (55;56).

- **Optimal Porosity:** Scaffolds must have an open, interconnected porous structure with large surface area to volume ratios that will allow for nutrient and gas influx and the removal of waste. The microporosity (pores <10 µm) contributes to cell-scaffold interactions and the resorption and mechanical properties of the material. The macropores (pores >50 nm) allow for the in-growth and homogenous distribution of cells and facilitate neovascularisation (57). It is well accepted that for bone tissue engineering, macropores should be within the 150-900 µm range and minimum of 100-200 µm for macropore interconnectivity (10;56;58)
Osteoconductive (Bone): Osteoconduction is a physical effect whereby the scaffold forms a three-dimensional architecture onto which bone cells and capillaries, from the host, can grow into and form new bone (59). An osteoconductive material guides the repair of bone in a location where normal healing will occur if left untreated (60). The surface properties of the material, both chemical and topographical, can control and affect cellular adhesion and proliferation and their downstream signalling pathways (61).

Osteoinductive (Bone): Osteoinduction is a chemical process in which stem or progenitor cells are recruited to a bone healing site, and are stimulated to undergo osteogenic differentiation (59). This can be accomplished through the use of biomolecules, such as bone morphogenic protein (BMP), transforming growth factor (TGF-β), and fibroblast growth factor (FGF), incorporated within the material scaffold (62).

The aim of this section is to explore recent approaches that combine varying degrees of synthetic, natural, and biomimetic materials and different fabrication techniques to construct highly tailored environments for bone and cartilage regeneration. The following review of materials and material systems is not meant to be a complete overview of biomaterials to date, but rather, highlight exciting new advances in the field of musculoskeletal tissue engineering. For an extensive review of novel materials for bone and cartilage regeneration, please refer to my review in Current Opinions in Chemical Biology (63).

1.2.2.1 Ceramic scaffolds

Ceramics are non-metallic, in-organic, materials with mechanical properties strongly related to their porous crystalline-like structure (64). Hydroxyapatite (HA), β-tricalcium phosphate (β-TCP), and Bioglass® are generally considered bone bioactive ceramics (Figure 1.4) (65). These are materials which bond to surrounding osseous tissue and enhance bone tissue formation. Bioglass®, originally developed by Larry Hench and colleagues in 1969, has enjoyed considerable clinical success over the years for applications such as bone grafting, spinal fusion, revision arthroplasty, and middle ear repair (marketed under the name Novabone) (66;67). Bioglasses are interesting as they possess the ability to chemically bond with the host bone and form a bone-like hydroxyl-carbonate apatite surface layer in-vivo. Numerous studies have shown that bioglass® stimulates the growth and osteogenic differentiation of primary human osteoblasts and enhances bone formation in-vitro (68;69). Currently, a new generation of bioactive glasses are being fabricated thanks to advances in sol-gel processing techniques(70). These sol-gel derived bioactive scaffolds bear a unique interconnected mesoporous structure with hierarchical pore morphologies similar to trabecular bone (71). Further these bioactive sol-gel glasses have a number of advantages over traditional melt-derived glasses as tissue engineering scaffolds, including higher rates of hydroxyl-carbonate apatite (HCA) layer formation, faster bonding with bone, improved purity and
homogeneity, and enhanced degradability and in vivo resorption (57). Research is now focused on understanding the underlying reaction mechanisms between bioactive glasses and surrounding bone-derived cell types. This knowledge could lead to development of a new generation of bioactive glass implants for guided skeletal tissue regeneration.

![Image of ceramic based materials. (A) Image of a melt-derived bioactive glass powder (compliments of Yann Fredholm). (B) Image of a 3D porous hydroxyapatite scaffold fabricated for mandibular reconstruction (work of Professor Russ Jamison).]

**1.2.2.2 Polymer scaffolds**

Polymers such as polylactic acid (PLA), polyglycolic acid (PGA), copolymers of PLA and PGA (PLGA), polyanhydrides, polyorthoesters, polycaprolactones, polycarbonates, and polyfumerates have many properties that are well-suited for the purposes of tissue engineering (55). They are non-immunogenic, non-toxic, and bioresorbable, breaking down through hydrolysis into metabolic products (although the build-up of acidic degradation products is recognised to be cytotoxic (73)). They are also convenient to process into solid phase scaffolds for implantation or as solutions for injection and polymerisation at the site of damage. In-situ polymerizable compositions, including photocrosslinkable polyanhydrides and chemical crosslinkable polyfumerates (74;75), can be easily administered in the form of arthroscopically injectable gels and liquids, and have been shown to possess adequate initial mechanical properties, controllable degradation kinetics, and the ability to simultaneously incorporate cells and growth factors to promote wound healing and support bony in-growth in-vivo (76;77).

Scaffolds fabricated from unmodified synthetic polymers tend to have limited bioactivity and provide minimal biological cues to guide tissue regeneration. This can be overcome by the reintroduction of these cues in various forms – from functionalisation which alters surface charge density, to matching the underlying tissue architecture and the incorporation of peptide sequences that convey highly specific biofunctionality (Figure 1.5). For example, surface functionalisation, such as hydrolysis to expose carboxylic groups and aminolysis to expose primary and secondary amine groups, can be used to increase surface hydrophilicity, and has been found to encourage stronger interactions with serum and ECM proteins through electrostatic effects and hydrogen bonding (78). The presence of this adsorbed protein layer transforms the implant surface into a
biological landscape that supports cellular interaction and is important to numerous cell-fate processes (79).

**Figure 1.5** Enhancing material biofunctionality. Control over cellular interaction for bone and cartilage repair can be achieved through scaffold material design. A number of different examples are presented here, ordered by increasing biofunctional specificity. Unfunctionalised: Unmodified polymer surfaces nonspecifically absorb proteins through weak interactions between the protein-water and water-surface interfaces. Functionalised with charged groups: Chemical modification of the polymer surface with different charged end-groups (e.g. -OH-, -S-, -COO-, -NH3+) will increase electrostatic interactions and may lead to stronger protein absorption and structural rearrangements – which may expose hidden binding sites for cell attachment. Peptide functionalised: The incorporation of peptide motifs (RGD) can be used to increase the binding of specific cell receptors, directing cell behaviour. Peptide-polymer hybrid systems: By including peptides, such as protease sensitive degradation sites, within the polymer backbone, the scaffolds can be further enhanced to permit cell-mediated migration and degradation. rDNA protein systems: Synthetic artificial proteins can be designed to structurally and functionally resemble specific biological ECM constituents using recombinant DNA technology.

In addition to foams and fibres, synthetic polymer based scaffolds have also been formed into hydrogels. Due to biological and structural similarities to ECM, hydrogels can be applied as space filling agents, as delivery vehicles for bioactive molecules, and as 3-dimensional structures that organize cells and present stimuli to direct the formation of a desired tissue (80). However, due to the relatively weak mechanical properties of hydrogels, they have been limited to non-load bearing orthopaedic applications, such as chondrocyte encapsulation (81) and delivery vehicles of BMPs (82). Plant-derived alginate hydrogel scaffolds (83;84) and hydrophilic polymers such as poly(ethylene oxide), poly(ethylene glycol), poly(vinyl alcohol), and their derivatives have also
attracted interest for cartilage regeneration (85-88). Their high water content creates a cartilage-like, porous, water saturated, viscoelastic material which supports the chondrogenic phenotype, and enhances the synthesis of neocartilaginous extracellular matrix constituents. Additionally, incorporation of terminal acrylate groups within synthetic polymers permits in-situ photoinitiated polymerisation for non-invasive implantation and the safe encapsulation of cells and exogenous growth factors into the hydrogel.

Strategies, such as use of densely cross-linked dendrimer hydrogels (89) may also help to maintain scaffold mechanical properties through-out degradation. Their multivalent highly-branched structures provide the capacity to achieve high mechanical strength and high water content, advantageous for cartilage repair. Specifically, in one study, a hybrid block-copolymer hydrogel consisted of a tetrafunctional poly(ethylene glycol) (PEG) core bound using anhydride coupling to biodegradable poly (glycerol succinic acid) wedges (90). Terminally methacrylated groups allowed for in-situ photopolymerisation of the dendrimer network. This hydrogel system not only demonstrated promising results of chondrogenesis in-vitro, but also has the potential for facile tailoring of physical, biochemical, and mechanical properties by tuning the dendrimer concentration and subsequent cross-linking density.

1.2.2.3 ECM protein scaffolds

Natural polymers are used as tissue engineered scaffolds because they are generally biocompatible, have potential bioactive behaviour, are chemically versatile, and have controlled degradation rates. Another major advantage is they are biodegradable and are degraded by endogenous enzymes (91). These natural polymers are derived from animal or plant sources, including collagen (92-95), fibrinogen (96;97), chitosan (98;99), starch (100;101), and hyaluronic acid (102;103). Of these materials, collagen-based scaffolds have received a bulk of the attention for musculoskeletal tissue engineered constructs due to collagen’s highly organised structure, abundance in the body, ability to support a majority of tissue types, and the fact it can be osteoconductive, osteoinductive, and osteogenic (104;105). However, some drawbacks of these natural polymers are they have relatively weak mechanical properties, especially in compression, and can cause an immune response in patients due to their plant and animal derivation.

To address these challenges tissue engineers have created synthetic proteins from the combination of structurally and biologically important motifs identified from the major constituents of the ECM (106;107). Advances in gene and protein engineering have led to the design and creation of artificial peptide sequences taken from ECM proteins, such as elastin (108;109), collagen (110), fibrin (111), and fibronectin (112), commonly found in bone, cartilage, and wound healing matrices. With this emerging class of materials, it has become possible to precisely control the size, composition, sequence, topology, and stereochemistry of macromolecular architectures to produce
well-defined structural elements found in nature, such as β-sheets and α-helices. Once important structural and biofunctional motifs have been identified, scaffolds which target specific biofunctionality can be produced such as cell binding sites and enzymatic degradation domains (113;114) (Figure 1.5). For example, enhanced chondrocyte migration was conferred onto a PGA fibre scaffold by an artificial protein composed of multiple repeats of the collagen II D4 domain (115). Protein segments can be coupled to synthetic polymers to form hydrogel networks with enhanced mechanical stability, through various chemical (i.e. radical polymerisation, reaction of functional groups, addition reactions) or physical (i.e. hydrogen bonding, electrostatic interactions, metal complexation) cross-linking methods (116).

1.2.2.4 Hybrid materials

To date no biomaterial has been able to demonstrate properties sufficient for all musculoskeletal applications, given the very unique biochemical and biomechanical properties associated with each application. Therefore, recent research has concentrated on the fabrication of material hybrids. The properties of these composite materials can be widely varied based on the proposed application, and it is this versatility that makes them an attractive strategy for new musculoskeletal tissue engineering treatments. Sherwood et al., have developed a PLGA/PLA/TCP heterogeneous composite consisting of different porosities and pore structures for uses as an osteochondral substitute (117). Chondrocytes were seen to preferentially attach to highly porous regions of the construct, and biochemical and histological analysis revealed hyaline-like cartilage formation after 6 weeks in culture. The tensile properties were also similar to that cancellous human bone, suggesting that these scaffolds have desirable mechanical properties for in vivo applications. Another group has developed a multiphase composite for repair of osteochondral defects composed of poly(D,L)lactide-co-glycolide combined with PGA fibres, 45S5 Bioglass® and medical grade calcium sulphate (118). These additives were used to vary stiffness and chemical properties of the construct when implanted into goats. Histological sections of the defect sites showed a high percentage of hyaline cartilage and good bony restoration, with new tissue integrating well with the native cartilage.

Scaffolds can also be rationally designed with specific biofunctionality by combining synthetic polymers with short peptide sequences (Figure 1.5). The peptide sequence most actively investigated is the RGD-binding domain recognised by trans-membrane integrin receptors present on the cell surface (119). These short sequences are found within ECM proteins, such as collagen and fibronectin, and are known to promote osteoblast (120;121) and chondrocyte cell adhesion (122) and expression of ECM specific proteins (123). Recently, an RGD sequence found in bone sialoprotein (BSP), has been used to specifically promote osteoblast migration and adhesion (124;125). Receptor binding to RGD appears to be strongly influenced by the guanidinium side group.
of the arginine residue. A recent study demonstrated that it is also possible to synthetically approximate the binding affinity of the RGD peptide by including agmatine, the decarboxylation product of arginine, into synthetic polymer hydrogels, resulting in substantially increased cell adhesion (126).

Hybrid multifunctional networks can be formed from bioactive precursors containing chemically reactive functional groups (such as amines, thiols, carboxyls) or end-functionalised hydrophilic polymers (such as PEG) that act as physical or chemical crosslinkers. Additional structural control can be achieved by using monoacrylated derivatives for photocrosslinking or specific amino acid sequences to induce covalent or electrostatic protein-protein interactions. From their modular design, researchers have been able to create multifunctional networks that possess high levels of structural organisation and an array of biochemical and biophysical properties. Some of the most intelligent hybrid systems are focused on mimicking the ECM’s susceptibility to cell-triggered proteolysis, enabling cell invasion and subsequent remodelling of the matrix constituents. The most versatile and well-characterised of these bioresponsive systems are based on poly(ethylene glycol) (PEG)-peptide hydrogels (127-130). The peptides in these systems are designed as substrates sensitive to proteases, such as plasmin and matrix metalloproteinases (MMPs), expressed at the surfaces of migrating cells (Figure 1.5) (111;131;132). MMPs are a family of ECM degrading enzymes, with MMP-2, MMP-9, and MMP-13 implicated as the key players involved in ECM turnover during bone development and remodelling (133;134).

In one approach, vinyl sulfone-functionalised 4-armed telechelic PEG macromers were reacted with thiol bearing MMP- and integrin (RGD)-binding peptides via highly selective Michael-type conjugate additions (128;130;135). Polymerisation was induced in-situ using a di-cysteine MMP-sensitive peptide cross-linker. This is an effective system for influencing new tissue ingrowth and scaffold mechanics by way of controlled proteolytic degradation. Other methods of gelation and incorporation of bioactive molecules, such as enzymatic cross-linking (136) and photopolymerisation of acrylate-terminated precursors (111;137), have also been used to form PEG-based hydrogels. As an additional feature, exogenous growth factors, such as recombinant human bone morphogenic protein-2 (rhBMP-2) (130;135) and angiogenesis-inducing signal vascular endothelial growth factor (VEGF), can also be incorporated into the hydrogel matrix via physical entrapment (128). By exploiting their MMP-mediated degradation activity, these networks better mimic the natural bone remodelling process by the release of osteogenic factors following bioactive matrix degradation. For bone repair, these synthetic materials demonstrated integrin- and MMP-dependent cell infiltration in temporal and spatial synchrony with endogenous bone regeneration. Metalloproteinase sensitive materials have also been successfully developed for
cartilage repair and chondrocyte culture, showing a higher expression of matrix proteins as compared to gels insensitive to proteolysis (138).

Looking to the future of biomaterials, complex approaches utilising materials and fabrication techniques can be used to impose new levels of control over scaffold nanostructure and mechanics. Novel approaches not covered in this review include electrospinning (139;140), peptide self-assembly (141;142), and biomemetic mineralization(143;144). Further, emerging high-throughput, analytical technologies, such as polymer (145) and protein (146) microarrays, can be combined with recent advances in genetic engineering, to identify and create novel biomaterials that meet specific mechanical requirements and illicit particular cell-specific interactions. Whether scaffolds are used chiefly as support structures which adsorb ECM proteins and encourage tissue in growth, or as synthetic extracellular matrices, providing the cues needed for tissue regeneration, will depend on the requirements of the tissue type and application. The continued development of biospecific materials is essential to each of these approaches.

1.2.3 Cell Sources for Musculoskeletal Tissue Regeneration

For the purpose of regenerating orthopaedic tissues, there are many options of cell sources (Figure 1.6). Today, the use of autologous cells is the obvious first choice as it can reduce problems due to immune rejection, inflammatory response, and pathogen transfer. However, due to recent advances in stem cell research, the strict use of autologous cells is likely to change.

1.2.3.1 Differentiated Cells

Autologous cell transplantation is a widely accepted technique in the repair of skeletal tissues, particularly for treatment of cartilage defects. Since 1995, autologous cartilage transplantation (ACT) has provided a major advancement in the pursuit of joint surface engineering (147). ACT therapy, Carticel®, was the first biological cell therapy to be licensed by the Food and Drug Administration in the US, and has recently attained a milestone of treating 10,000 patients (www.carticel.com). In this process, articular chondrocytes are obtained arthroscopically from a minor weight bearing area of the joint, culturally expanded in a laboratory, and finally transplanted back into the patient’s knee and covered with a periosteal flap. However, these procedures are not without their challenges. Problems of reproducibility and consistency of results are faced due to variability in surgical procedure, cell culture expansion and manipulation using animal products, and loss of phenotype. The expansion of chondrocytes, for example, is often hampered by problems of cell senescence and de-differentiation, which can cause the formation of dysfunctional cartilage when transplanted (148-150). Furthermore, harvesting these cells results in an additional surgical site and increases the potential for another defect. Alternatively, stem cells, such as adult
stem cells (ASCs) and embryonic stem cells (ESCs), have become of recent interest in musculoskeletal tissue repair due to their capacity to differentiate into multiple cell lineages.

1.2.3.2 Stem Cells

Stem cells are undifferentiated cells that are capable, to some degree, of undergoing both self-renewal and differentiation into more functionally specialised mature cells (151). When a stem cell divides the daughter cells can, depending on the environmental stimuli, either remain a stem cell or become a more specialised cell called a progenitor cell, for example a blood, bone or brain cell (152). However, stem cells have varying degrees of differentiation potential, or plasticity. Embryonic stem cells are considered to be pluripotent, which means they can differentiate into almost all cell types arising from the three germ layers of a developing human (153). Adult stem cells are thought to be multipotent, which means they are only capable of giving rise to cells related to the germ layer in which they originated. Although, the limit of this “multipotency” is of recent debate due to research suggesting that “transdifferentiation” of adult stem cells occurs (154;155). Transdifferentiation is the ability of a cell of one tissue, organ or system to differentiate into a cell type characteristic of another tissue, organ, or system; e.g., for blood stem cells to become liver hepatocytes.

![Figure 1.6](image-url)

**Figure 1.6** Microscopy images of human embryonic stem cells (A), mouse embryonic stem cells (B), human mesenchymal stem cells (C), and human periosteal cells in monolayer culture. Image A shows a colony of human ES cells growing on a feeder layer of mouse embryonic fibroblasts. Image B shows colonies of undifferentiated mouse ES cells. Image C shows a dense colony of human MSCs and image D shows a heterogeneous population of human periosteal cells growing in monolayer (scale bar = 200 µm).
Chapter 1: Introduction

1.2.3.2 Adult Stem Cells

Adult stem cells (ASCs) are undifferentiated cells found among differentiated cells of a specific tissue. ASCs are more accurately called somatic stem cells as they can also come from foetuses, umbilical cords, and infants. Adult stem cells are believed to exist in small numbers in most organs throughout the body (including the bone marrow, liver, gut, blood and brain) and are called upon during biological repair mechanisms to replace damaged cells (156). The bone marrow has been found to contain undifferentiated ASCs that have the ability to give rise to progeny of different tissue types. Two of most researched subpopulations of these bone marrow stem cells are Mesenchymal Stem Cells (MSCs) and Haematopoietic Stem Cells (HSCs). MSCs capacity to differentiate into cells of the mesenchyme makes them an attractive cell source for musculoskeletal tissue engineering.

Mesenchymal stem cells

MSCs are non-haematopoietic, stromal cells that, under the appropriate stimuli, can differentiate into bone, cartilage, fat, tendon, and muscle cells (157). Originally isolated from bone marrow and stroma of the spleen and thymus, MSCs have more recently been isolated from various other sites, including periosteum, synovium, synovial fluid, vasculature, muscle, and tendon (158-160). MSCs are believed to be called upon during biological repair mechanisms to replace damaged cells, however, it is not yet clear to what extent they are responsible for this. Skeletal progenitor cell-based therapies offer many advantages over fully differentiated cell sources, including; reliable isolation, ease of expansion, maintenance of phenotype, relative lack of senescence, and evidenced low immunogenicity (161). In-vitro MSCs are characterised by their capacity for continuous self-renewal and their functional ability to form distinct fibroblast-like cell colonies (termed colony forming unit-fibroblastic) with the ability to differentiate into multiple mesenchymal lineages (Figure 1.6) (162;163). Utilising these advantages, musculoskeletal tissue engineering strategies can facilitate tissue regeneration by loading MSCs into prefabricated scaffolds tailored to fit into defect sites.

MSCs seeded constructs have enjoyed successes in several animal models. MSCs isolated from adipose tissue were seeded onto collagen-ceramic composites and implanted in large defects in rats (164). Healing was seen to occur after only 8 weeks. Similar results are seen in larger animal models, where MSCs loaded onto porous scaffolds were found to accelerate osteogenesis in large bone defects in both sheep and canine (165;166). MSCs have also been shown to elicit a healing effect in osteochondral defects. Subchondral implantation of porous hydroxyapatite-synthetic polymer composites loaded with MSCs and bone morphogenic protein-2 have been shown to repair full-thickness cartilage defects within 6 weeks in rabbits (167). More recently, these promising results from early animal models have championed for the use of MSCs in small-scale clinical
trials. Quatro et al. have shown complete restoration of limb function after 6-12 months when porous ceramic scaffolds loaded with MSCs were implanted into large bone defects in humans (168). Although these studies are promising, the development of large-scale MSC-based clinical therapies has been limited. This is due to insufficient knowledge of long-term stability of the regenerated tissue and MSC’s tendency to differentiate towards unwanted lineages.

**Haematopoietic stem cells**

In fetal and adult mammals, haematopoietic stem cells (HSCs) predominately reside in the fetal liver (FL) and bone marrow (BM) and can be identified by the presence of surface glycoprotein CD34. However, HSCs do not originate in FL or BM, but rather migrate from other tissues to these sites during embryonic development. Due to their relatively short life-span, HSCs continuously differentiate into multiple lineages of different blood cell types, and replicate themselves through self-renewal. The marrow contains specialised environments that regulate the balance of HSC self-renewal and differentiation and comprise what has been termed the stem cell niche. The concept of stem cell niches was first proposed by Schofield in the late 1970’s (169). However, it has been only recently that the HSC niches in adult BM were directly observed and partially dissected (170). From this study, two types of HSC niches have been identified in BM; the endosteal niche located on the surface of trabecular bone, vascular niche at the BM sinusoids, which are blood vessels with a fenestrated endothelium located in the center of BM. Osteoblasts, which are found lining the endosteal surface, were the earliest identified components of this stem cell niche. Osteoblasts seem to regulate HSCs though a complex interplay between cell-surface molecules, signalling molecules, cell-to-cell contact, and interaction with ECM components, that promote the proliferation of hematopoietic cells in culture and support the in vitro maintenance of HSCs (171;172). Direct evidence supporting osteoblasts as an essential component of the HSC niche comes from recent studies in which osteoblast numbers were experimentally increased or decreased, which resulted in the HSC number changing in a parallel manner. These interactions have been shown to be partly regulated by osteoblast cell-adhesion molecules N-cadherin and β1-integrin (173;174) and signalling ligands angiopoietin-1 (Ang-1), Notch ligand, Jagged1, Wnt proteins, and others (175-177).

Although it is known that mesenchymal stem/progenitor cells and their descendents play a role in hematopoeisis, much less is understood about stem/progenitor cell regulation by haematopoietic cells. Recently, in limiting dilution experiments of rat marrow stromal cultures, Jane Aubin found that osteoprogenitor cell populations are, in-part, regulated by non-osteogenic lineages in the bone marrow through both direct cell contact and secreted factors (178). Thus it is likely, that osteoprogenitor cell differentiation in the bone marrow stroma may be under the influence of a
complex hierarchy of cells and factors, not unlike the hierarchy of cells and factors controlling the differentiation and propagation of hematopoietic origins.

Interestingly, several reports have suggested that bone marrow and bone tissues share a common progenitor cell that can give rise to both osteoblasts and hematopoietic cells and is present in the non-adherent fraction of bone marrow in humans (179). Bone marrow, bone, and periosteum are anatomically contiguous tissues that show parallel age related changes and several genetic features, suggesting a close developmental cellular relationship (180-182). Thus a reasonable hypothesis is that hematopoietic cells present in the periosteum and surrounding vasculature harbour a stem cell with osteogenic potential or play an accessory role in the differentiation and self-renewal of periosteal progenitor cells through direct or indirect pathways.

**Periosteal Cells**

Periosteal cells can offer many advantages as a cell source for musculoskeletal tissue engineering. First, periosteal cells have demonstrated the potential for osteogenic and chondrogenic differentiation in numerous studies in-vivo and in-vitro (29;183;184;184-193). Second, periosteal cells have an extensive self-renewal capacity; with the ability to undergo significant self-replication during subculture whilst maintaining a stable phenotype (Figure 1.6). Further, this phenotypic stability can be maintained after enzymatic liberation and cryopreservation (184). On the basis of these studies, periosteal cells have been named multipotent mesodermal cells, osteoprogenitor cells, precursor cells, mesenchymal stem cells, and mesenchymal precursor cells. Compared to bone marrow aspirates which only contain $10^3$ progenitor cells/cm$^3$, periosteal progenitor cell isolation is a safer, more efficient, and less painful clinical procedure (194). In addition, the potential for continuous expansion and osteochondral differentiation of periosteal cells has been demonstrated in elderly individuals (187), whereas, the number of progenitors in bone marrow is seen to decline with age (195). Despite the promise of periosteal cells as a regenerative cell source, attempts to functionally control and characterise periosteal cell populations have resulted in varying levels of success. The discrepancies seen between studies can be attributed to numerous variables, although the two major players seem to be differences in species and particular culture conditions. I will now highlight some of these discrepancies.

Arnold Caplan’s group was the first to study periosteal cells in-vivo as a potential bone tissue engineering cell source, using chick periosteal cells and human rib periosteal cells in a series of nude mouse experiments (159;184;185). In the human periosteal cell study they found that cells isolated from donors younger than 19 years, consistently formed bone and/or cartilage. In the case of donors older than 22 years, cultured cells formed neither bone nor cartilage in-vivo (184) These early studies are supported by more recent studies, in which the chondrogenic potential of
periosteal cells is dependent on donor age and cell passage in culture (196;197). In the O’Driscoll rabbit periosteum studies this decline in chondrogenic potential is attributed to a decrease in the size of the precursor pool in the cambium layer, which is seen to thin during aging (198;199).

Conversely, the periosteum is known to actively participate in fracture healing and bone remodelling in-vivo, by generating hyaline cartilage, which then undergoes endochondral and intramembranous ossification (see section 4.1.4). These processes are seen to occur over the lifetime of the individual, regardless of age. This observation is supported by Koshihara et al (187), who concluded that the mineralization potential of periosteal cells is not age dependent, and Sang et al (200), where human periosteal cells are shown to maintain their progenitor phenotype after extensive passaging, regardless of donor age.

Between these two extremes, a study performed by De Bari et al demonstrated that this relationship is more complex (201). He found early passage periosteal cells isolated from young (<30) human explants had the ability to undergo spontaneous chondrogenesis (without the use of chondrogenic growth factors, or other culture techniques). This potential is rapidly lost after cell passaging and is not observed in donor older than 30 years. However, this group also reported that independent of age and passage number, periosteal differentiation toward the chondrogenic phenotype could be elicited reproducibly and consistently when a micro-mass pellet culture system was combined with TGF-β1 treatments. This suggests that periosteal cells isolated from elderly individuals and extensively passaged still possess the competence to respond to at least 1 chondrogenic signal.

These studies reveal that a direct connection between age and osteogenic/chondrogenic potential becomes more complicated and less certain as more studies are published on periosteum biology and its varying functionalities. Similar discrepancies are found regarding literature on the use of growth factors to enhance periosteal chondrogenesis and osteogenesis in-vitro. The large differences in the results can be attributed to the fact that the influence of growth factors is dependent on cell species, cell passage number, dose amount, dose regime, antagonistic/synergistic effects with other biomolecules and factors, and the culture methods used.

In highlighting these discrepancies found in literature, I am attempting to show that the research and understanding regarding the periosteum is still in its infancy. The true versatility of periosteal cells and their therapeutic potentials have yet be elucidated, let alone harnessed. Although investigations into the bipotentiality of animal and human derived periosteal cells cultured in-vitro serve as excellent models, there is still a need to establish a more accurate in-vitro model to control and predict the in-vivo behaviour of periostea for wide-spread clinical use. To develop such a model, extensive characterisation studies are required to elucidate the heterogeneous phenotypes and properties of human periosteal cells in-vitro.
1.2.3.2 Embryonic Stem Cells

While adult stem cells have been used successfully for over 30 years (bone marrow transplantation), embryonic stem cells have only recently been isolated from humans in 1998 (202). Pluripotent stem cells can be derived from the inner cell mass of blastocysts of preimplantation embryos (ES cells) or the primordial germ cells of embryos (EG cells) (202;203). Primordial germ cells can be first identified as a small cluster (~8 cells) within the proximal part of the epiblast in embryos that are 7 days post coitum. Human ES and EG cells have unique features including unlimited growth capacity, expression of specific markers, normal karyotypes, and an ability to differentiate into cells of all three embryonic germ layers (204-206). Differentiation generally leads to the formation of teratomas in-vivo and embryoid bodies (EBs) in-vitro, which contain derivatives of all three germ lines (Figure 1.6).

Human embryonic stem cells at the most immature stage are characterised by expression of the globo-series glycolipids SSEA-3, SSEA-4 (207;208), the keratan sulphate-related antigens TRA-1-60 and TRA-1-81 (209;210), and by a lack of expression of the lacto-series oligosaccharide SSEA-1 (211;212). Historically, SSEA-1 is a known marker of murine embryonal carcinoma, embryonic stem and germ cells (213). Recently, however, there is evidence to suggest that the SSEA-1 antigen is a differentiation marker in human embryonic stem cell cultures, positively staining for early intermediate stem cells (214). One group used the SSEA-1 marker for positive and negative selection of neural stem and precursor cell subpopulations in a hESC differentiation paradigm (215). Within this study they also consistently found SSEA-1+ cell subsets to co-express the pentaspan membrane glycoprotein CD133, a known somatic stem cell marker (216-218). Concurrently, in SSEA-1+ human ES cell cultures differentiated cells present at the periphery of embryoid bodies stained strongly positive for SSEA-1, whilst undifferentiated cells at the centre were unstained.

As compared to ASCs, ES and EG cells possess greater pluripotency and ability for self-renewal, which makes them a more versatile therapeutic option (219). Clinical applications of ES and EG cells remain restricted due to the potential of uncontrolled differentiation and proliferation and the ability to form tumours (220;221). The orthopaedic potential of ES and EG cells therefore relies on the directed differentiation of ESCs into skeletal cells, e.g. chondrocytes, osteoblasts, and tenocytes. To prevent the spontaneous differentiation of ESCs in vitro, precise culture conditions must be maintained with feeder cell layers and specific growth factors, such as leukaemia inhibitory factor in mouse ES cultures. The signalling pathways underlying self-renewal and pluripotency of ESCs in culture have only recently begun to be unravelled. The ability to guide ESCs towards a specific cell type without the use of animal products will require a further leap of understanding.
1.2.3.3 Stem cell enrichment methods

Despite the recent experimental and clinical interests in stem and progenitor cells, defining the function and phenotype of these cell types remains a difficult task. These difficulties stem from the low frequency of stem/progenitor cells in tissues (~0.001-0.01% in bone marrow aspirates) and their cellular heterogeneity; in terms of morphology, differentiation potential and lineage commitment (163;222;223). To add to this variability, there seems to be a number of compounding peri-cellular factors that influence stem/progenitor cell numbers in culture and regulate the proliferation and further differentiation of their progeny (224-226). As such, there are still few standardized methods for isolating and expanded pure stem cell populations. This lack of knowledge presents a major obstacle to the widespread clinical exploitation of stem cells’ therapeutic potential. Moreover, enrichment steps will be necessary when creating novel stem cell and gene therapies, which rely upon the selection and characterisation of rare stem cell populations identified in numerous adult tissues (227-232). To resolve these issues, numerous groups are focused on the development of simple protocols to identify and enrich stem/progenitor cell populations based on numerous intra-cellular and extra-cellular features, including morphology, protein expression cytoplasmic granularity, and cell-cycle.

To date, a variety of isolation strategies have been employed for this task, including fluorescent activated cell sorting (FACS), limiting dilution, density centrifugation, magnetic sorting, and adherence to glass and plastic surfaces (233;234). Most popular amongst these techniques is FACS, which is a specific type of flow cytometry that allows each cell to be characterised and sorted according to a range of distinct biochemical and biophysical characteristics (Figure 1.7A). Exploiting this strategy, rare cell types such as stem cells, antigen-specific B and T cells and circulating tumour cells have been identified and isolated (235-239). These successes are, in part, due to developments in fluorophore and antibody production and availability, which have led many researchers to focus their purification efforts on immunophenotypic assays utilizing cocktails of monoclonal antibodies (240-247). Of these antibodies, Stro-1 has been extensively utilised for enrichment efforts and is found to be expressed on a small population of clonogenic multipotent stem cells (248;249). In this way, rare stem/progenitor cell populations are targeted based on the expression of intra-/extra- cellular molecules.

Although these popular techniques have enjoyed widespread use over recent years, new analytical technologies offer the promise of improved analytical performance, enhanced functionality and robust versatility in a variety of cell-based assays (250-255). Amongst these technologies, droplet microfluidic systems offer the possibility of rapid, specific and detailed analysis of cell populations. In these systems, picolitre-sized droplets generated from immiscible phases can be
produced at high frequencies (in excess of 1 KHz) and can act as discrete and isolated reaction compartments to study a number of biological and chemical processes (Figure 1.7B) (256).

**Figure 1.7** Schematic representations of rare cell identification and isolation techniques; flow activated cell sorting (A) and droplet microfluidics (B). Both methods provide a means for sorting heterogeneous mixtures of cells based on specific light scattering and fluorescent characteristics. (A) In FACS, temporary voltages are applied to droplets carrying targeted cell phenotypes, after which the charged droplets then fall through an electrostatic deflection system that diverts the droplets into containers based upon their charge. (B) Schematic of a microfluidics device which when combined with a fluorescent detection system provides a novel analytical platform to isolate and study single cells in aqueous droplets. (C) Microscopy image of droplets flowing though microchannels (50 µm wide, 50 µm deep, and 4 cm long) containing entrapped mammalian cells.

In this context, droplet microfluidic systems have been used as an analytical tool to examine enzyme kinetics(257), libraries(258), and assays(259), protein crystallisation(260;261) and biological assays(262;263). More recently, droplet microfluidic systems have been successfully demonstrated to effect single cell encapsulation and manipulation (255;262;264). Accordingly, such droplet systems have the potential to define a new high-throughput screening platform that can sample, encapsulate, manipulate, process and detect single cells and more importantly the presence of rare stem cell phenotypes. For cell biologists and tissue engineers alike, the compartmentalization provided by the droplets is particularly valuable for precisely analyzing multiple cellular characteristics simultaneously. An initial demonstration of this biological potential was recently provided by Huebner et al (262) and Koster et al (265), where droplet-based microfluidic systems were able to quantify protein expression and cell viability and metabolism.
Further diagnostic studies are now needed to determine if microfluidics-based approaches can accurately identify and sort rare stem cell phenotypes without causing viability loss and changes to cell behaviour; both of which have plagued flow cytometry-based enrichment methods.

1.2.4 Control of cell differentiation

Cells interact with their local environment through a number of sensors, known as receptors, which mediate specific intracellular responses, including cell differentiation. These signalling pathways are regulated by numerous autocrine and paracrine molecules, including ions, growth factors, and proteins, and biomechanical environments.

Of the regulatory molecules, growth factors have received a great amount of attention. Growth factors are peptides that regulate cell growth, function, motility, and differentiation. Bone and cartilage growth factors influence the synthesis of new bone and cartilage by acting on local or circulating populations of differentiated and/or progenitor cells. Bone morphogenic proteins (BMPs) have been shown to play a critical role in modulating mesenchymal differentiation, inducing the complete sequence of endochondral bone formation (266). Similarly, the transforming growth factor-beta (TGF-β) and fibroblast growth factor (FGF) superfamilies, have been implicated in the regulation of several biological processes, including cell differentiation, cell growth, and embryonic pattern formation (267;268). There are vast amounts of cytokines, which could be talked about in the context of skeletal tissue development and maintenance; however this is beyond the scope of my project, which chooses to concentrate more on the role of mechanical stimulus.

The conversion of external physical forces into biological signals and physiological responses, is known as mechanotransduction, and is contained within the broad field of mechanobiology (269). The influence of mechanical forces on bone adaptation has been of interest since 1870, when Wolff first discovered that bone responds to external mechanical stimuli to either reduce or increase its mass as required (25). More recently, physical forces have been found to play a role in regulating not only functional adaptation, but also normal skeletal development and fracture healing on the organ, tissue, cellular, and molecular levels (270-273).

At the organ level, mechanical signals can be characterised in terms of loading history, which includes force, displacement, and deformation characterisation. At the tissue level, varying quantities of stress and strain have been related to differentiation (274-277). Stress and strain are tensor quantities defined by six components and a reference coordinate system. Pauwels et al, concluded that the important information for tissue mechanical signalling was contained in stress and strain invariants, which are scalar quantities (275). These stress and strain invariants result in distortional and hydrostatic stress or strain. In elastic, isotropic materials hydrostatic stress causes a
change in material volume, or volumetric strain, but no distortion. Conversely, distortional stresses cause material deformation, or distortional strain, with no change in volume. Building on these concepts, Carter et al and Giory et al, developed finite element models to relate mechanical loads to skeletal tissue differentiation (Figure 1.8) (278-280).

In this model, they formulated that compressive hydrostatic stress regimes guide cartilaginous matrix formation, whereas tensile strain regimes guide connective tissue formation. Fibrocartilage is formed in regions of high tensile strain and hydrostatic compressive stresses. Bone formation occurs in regions exposed to neither significant compressive hydrostatic stress, nor significant tensile strain. However, direct bone formation also requires vascularisation, so in the case of low oxygen tension, preosseous tissue can be directed to down a chondrocytic pathway (279). This basic model has been applied to studies looking at fracture healing (270;272), bone implant interface (280), and fibrous tissue formation in tendons (278).

Investigations into the mechanical environments of healing fractures and distraction osteogenesis, using finite element analysis, have revealed that the periosteal surfaces of bones experience a wide range of loading histories, which elicit different tissue differentiation patterns (281). Results revealed that periosteal tissue outside the fracture gap is subjected to relatively low hydrostatic and tensile strains, which promotes intramembranous bone formation. At the site of fracture, periosteum forms an osseous bridge across the gap, where it effectively shields the underlying softer, cartilaginous tissues of the callus from high stresses. This reduction of stresses, facilitates endochondral ossification, which proceeds from the outer ossified callus, abut the periosteum, toward the centre-line. If the fracture site is not well-immobilised high distortional strains can lead to high compressive stresses and tensile strains, which can lead to fibrous tissue formation and prevent effective tissue organization and vascularisation. Results of the distraction osteogenesis
studies reveal that the mechanical states and tissue differentiation patterns are mirror images to those found in initial fracture healing (269).

On the cellular level, changes in cell morphology (282,283), cell pressure (284-286), electric potentials (287) and oxygen tension (288-290) have been related to changes in ECM and enzyme production. The understanding of how gross mechanical forces translate into a cellular response requires an appreciation of the underlying mechanisms at the molecular level. The molecular level is the most complicated and least understood of the organisation levels. Mechanical stimulus at this level is seen to effect cytoskeleton arrangement, integrin binding, growth factors, and stretch activated ion channels, which all lead to changes in intracellular signalling (291-294). However, the exact mechanisms by which mechanical forces are transmitted across the cell membrane and translated into a response remain unknown.
Chapter 2

Scope of Thesis

The ultimate objective of this research is to develop novel therapies for the repair and regeneration of musculoskeletal tissues. Important to the success of regenerative strategies, is the ability to develop and combine biomimetic materials with reparative cell types to create novel constructs can interface with native tissues structurally, mechanically, and biofunctionally. However, prior to the clinical marriage of cellular and material systems, extensive explorative research is required to characterise, understand, and control the biological behaviour of cell-based tissue engineering constructs. Therefore, this thesis aims to develop and characterise material- and cell- based therapeutic models \textit{in-vitro} using polymeric biomaterials, differentiated and undifferentiated skeletal cell types, and physico-chemical cell signalling cues.

The purpose of \textit{Chapter 1} was to provide a background review of the field of musculoskeletal tissue engineering, including recent developments and challenges in biomaterial and stem cell research. \textit{Chapters 3-8} discuss the main experimental results of this thesis, and also include application specific literature reviews, experimental methodologies, and suggestions for future work. Final conclusions and general overview of future developments are presented in \textit{Chapter 9}. The following sections will highlight the particular scope and aspects of each results chapter.

2.1 Biomaterials

The advent of injectable polymer technologies has increased the prospect of developing novel, minimally invasive arthroscopic techniques to treat a wide variety of ailments. In \textit{Chapter 3}, a novel polyurethane-based injectable, in-situ curable, polymer platform synthesized by PolyNovo Biomaterials Pty. Ltd. (Clayton South, Australia), is evaluated to determine its potential uses as a tissue engineered implant for osteochondral repair. Films of the polymers were prepared by reacting two pentaerythritol-based prepolymers, and characterised for mechanical and surface properties, and cytocompatibility using primary human osteoblasts.

2.2 Cells

Periosteum and periosteum-derived cells are known to play an intimate role in the formation of bone and cartilage tissue during bone tissue development and repair and have demonstrated multilineage potential \textit{in-vitro}. As such, the periosteum represents an interesting new candidate for osteochondral tissue engineering strategies (30;31). To date, however, only a few groups have sought to exploit this potential from periosteum tissue derived from human donors (160;295;296).
Consequently, there are no standardised methods for the culture of adult human periosteum \textit{in-vitro} and little information is available on the behaviour and characteristics of periosteal cells. The full realisation of the periosteum’s regenerative capacity is contingent upon the development of model systems to study, explore, and characterise human periosteum and periosteal cells \textit{ex-vivo}. The knowledge gained from these systems will translate into more effective clinical therapies with reproducible outcomes.

As a necessary first step in this research, \textit{Chapter 4} is dedicated to the design and optimisation of simple and reproducible methods to harvest and culture human periosteal tissue without compromising the viability and/or therapeutic potential of the cells. Accordingly, this experimental process is broken down into two main parts, aimed at designing culture systems for human periosteum tissue explants and isolated periosteal cells respectively.

Following on from these results, \textit{Chapter 5} is focused on elucidating the heterogeneous cellular characteristics and behaviours of isolated human periosteal cells \textit{in-vitro}. Adherent periosteal cells isolated from multiple human donors are extensively expanded in monolayer and examined for changes in growth potential, morphology, and cell-cycle characteristics. Additionally, flow cytometry and real-time RT-PCR are used to analyse immunophenotype and gene expression profiles.

Building on the previous chapters’ characterisation studies, \textit{Chapters 6 and 7} are focused on the development of enrichment strategies to selectively identify, isolate, and further characterise therapeutic precursor cell populations present in human periosteal tissue. In \textit{Chapter 6}, a simple enrichment strategy to isolate periosteum cambium layer cells is developed using a series of four sequential digestions. The efficacy of this enrichment strategy is confirmed by examining the growth rates, morphology, colony forming ability, and phenotype of the different cell isolates in culture. Further, the ability of this system to preferentially isolate cell populations according to distinct periosteal cell layer is also investigated.

In exploring alternative enrichment strategies, \textit{Chapter 7} concentrates on the isolation and characterisation of rare progenitor cell phenotypes from heterogeneous periosteal cell populations using droplet-based microfluidics. In these systems, developed by Edel et al at Imperial College, picolitre-sized droplets generated from immiscible phases can be produced at high frequencies (in excess of 1 KHz) and can act as discrete and isolated reaction compartments to study a number of biological and chemical processes (256). The biological capacity of this platform technology to encapsulate, detect, and quantify rare periosteal cell phenotypes is investigated. Further the accuracy and reproducibility of this microfluidics systems is tested and compared to results
obtained using conventional flow cytometry. The advantages of this novel platform system over currently available biological sorting methods are also discussed.

2.3 Physicochemical signalling cues

In diarthroidal (synovial) joints, the development, maintenance, and destruction of bone, cartilage, and periosteum are regulated, in part, by mechanical forces. Physical cues influence the biology and function of cells through a number of mechanotransduction pathways (282,294). In previous studies, mechanical forces have shown the ability to induce lineage specific cell differentiation and extracellular matrix production in numerous skeletal cell types (297-299). In Chapter 8 the influence of mechanical strain on periosteal cell differentiation is investigated *in-vitro*. In collaboration with Queen Mary University London (QMUL), a 3D periosteal cell-agarose culture model is developed and tested for its ability to support cell viability, chondrogenesis, and mechanotransduction. The chondroinductive potential of mechanical stimulation is examined by applying dynamic compressive strains to periosteal cell constructs using a mechanical straining apparatus previously designed at QMUL. The temporal expression profile of early chondrogenesis is assessed by real time RT-PCR. To elucidate the relationship between physical and chemical factors, cell-seeded constructs are supplemented with TGF-β and subjected to loading.
Chapter 3

Synthesis of two-component Injectable Polyurethanes for Bone Tissue Engineering

3.1 Introduction

In recent years, skeletal reconstruction has become an increasingly common and important procedure for the orthopaedic community. Problems associated with joint disease and trauma may not always be severe, but their management can be complex. Treatments often involve choices between non-operative management and varying degrees of surgical intervention (300). Surgical treatments are extremely labour intensive, costing the patient both time and money, and often require bone grafting to restore normal function and physical integrity.

The recent development of injectable, biodegradable, and in-situ crosslinkable biomaterials seek to alleviate many of the challenges associated with current surgical techniques and prefabricated tissue engineered implants. Injectable systems have the advantage of arthroscopic use in which a gel can be introduced into a defect with complex geometries to provide a strong bond with surrounding tissues. Ideally, upon injection the material should (i) polymerise without detrimental effects to the surrounding tissue, (ii) have a cytocompatible environment, (iii) provide mechanical and physical integrity for selected application, (iv) guide cell attachment and growth, and (v) allow for diffusion of nutrients to the cells (301;302). For this purpose, synthetic polymer-based systems offer some distinct advantages over metal, ceramic, and natural biomaterials. Degradable polymer implants can provide initial structural integrity for damaged bone and subsequently degrade within a timeframe that allows for natural bone tissue in-growth and remodelling. The mechanical properties, degradation times, and chemical functionalities of synthetic polymers can be easily tailored to support the tissue type, providing issues such as cytocompatibility, sterilisation, and ease of handling are addressed (303). Thus, a key aim when designing injectable in-situ forming polymer systems is to select not only chemically and structurally versatile polymers but also ones that are cytocompatible.

To date, very few synthetic polymer-based injectable systems have been developed that possess suitable properties for orthopaedic applications (e.g. high strength, and controlled degradation rates) (304;305). The work of Mikos et al (8) and Anseth et al (74;304) has led to the development of two such in-situ polymerisable systems, based on the chemistries of poly(propylene fumerate)
(PPF) and polyanhydrides. These polymer systems have shown the ability to control both polymerisation and degradation rates, form highly cross-linked (strong) structures upon polymerisation, minimise exothermic temperatures during formation, and polymerise into 3-dimensional complex structures (8;304;306-308). Additionally, these systems were shown to be biocompatible in-vivo and encourage cell attachment, proliferation, and differentiated osteoblastic function in-vitro (8;74;307;309).

Polyurethanes can offer many advantages in the design of injectable and biodegradable polymer compositions. Polyurethanes have an established record of biocompatibility (310;311), the ability to be functionalised to improve cell growth and proliferation (311-315), and controllable degradation kinetics (316). Additionally, the mechanical properties of polyurethanes can be tailored for uses as hard (317-319) and soft tissue (320-323) biomaterials. Currently, novel non-toxic, biodegradable lysine-di-isocynate (LDI)-based polyurethanes are being developed for uses in tissue engineering. Zhang et al (324), have developed a new generation of peptide (LDI) based polyurethane foams that possess the versatility of polyurethanes, but lack the toxicology of other urethane products. These peptide based polymer systems can incorporate active moieties, which promote cell adhesion, viability, and proliferation with no adverse effects on the surrounding environment (311;325). This polymer system, however, has been synthesised as spongy foam, and therefore does not have the advantage of being arthroscopically delivered and cured in-situ.

This present study seeks to evaluate a novel two-component injectable and in-situ curable polymer system utilising LDI-based polyurethanes, developed by PolyNovo Biomaterials Pty Ltd. (Clayton South, Australia), for initial orthopaedic applications. Here we have investigated the initial surface and mechanical properties of the synthesised polymers, and analysed the cytocompatibility and cell-surface interactions with primary human osteoblasts. Preliminary results advocate for future investigation into the use of this injectable polymer platform as a bone cement, as the polymers have suitable mechanical properties and provide an adequate environment for osteoblast viability and proliferation.

### 3.2 Materials and Methods

Pentaerythritol, Glycolic acid (70% w/v in water) and Stannous Octoate were purchased from Aldrich (Sydney, Australia) and used as received. Ethyl 2,6-diisocyanato hexanoate was purchased from Kyowa Hakko Kogyo Co Ltd, Japan and β-tricalcium phosphate from Plasma Biotal Limited, UK.
Chapter 3: Synthesis of two-component Injectable Polyurethanes for Bone Tissue Engineering

3.2.1 **Prepolymer synthesis**

3.2.1.1 **Prepolymer A**

Pre-dried pentaerythritol (4.818 g) (Aldrich, Sydney, Australia) was weighed into a dry three-neck flask equipped with a magnetic stirrer, nitrogen inlet and drying tube. Ethyl 2,6-diisocyanato hexanoate (ELDI) (30.04 g) was then added to the flask followed by catalyst stannous 2-ethyl hexanoate (0.1 wt%) under nitrogen. The reaction mixture was stirred and heated to 50°C for 72h under nitrogen atmosphere. In polymers 76-6, 76-6a (Table 3.1), the prepolymer A was prepared by replacing PE with a polyol prepared according to the method described below.

3.2.1.2 **Prepolymer B**

Pre-dried pentaerythritol (27.23 g) was weighed in a dry three-neck flask equipped with a magnetic stirrer, nitrogen inlet and air condenser attached to the flask through a distillation head. Glycolic acid (136.21 g, 70% w/v) was then added to the flask. The flask was heated in an oil bath to 80°C for about 3 hours until all pentaerythritol dissolved. The temperature of the flask was then increased to 160°C and heating continued. Nitrogen was bubbled through the reaction mixture for fast removal of water formed during the reaction. The reaction was stopped after 24h and the reaction flask removed from the oil bath after carefully turning off nitrogen flow. The polyol was then transferred to a single neck RB flask and degassed at 120°C under vacuum (0.1torr) using Kuglerohor. Polyols PE-LLA and PE-DLLA were prepared using the same procedure, except L-lactic acid and dl-lactic acid respectively were used in place of glycolic acid.

3.2.1.3 **Prepolymer molecular properties**

The viscosity of prepolymer A was measured using a Bohlin Rheometer (CSR10) at 23°C. Hydroxyl number of the polyols was determined by p-toluene isocyanate method in accordance with ASTM method E1899-02.

The molecular weights of the prepolymers were determined using gel permeation chromatography (GPC). Analysis was performed on a Waters Associates Liquid Chromatography system (Waters 717, Rydalmere, Australia) equipped with a differential refractometer and four µ-Styragel columns (105, 104, 103 and 100Å). The mobile phase was tetrahydrofuran (THF) at a flow rate of 1 mL/min. Prepolymer was dissolved in THF by warming the solution at 50°C for 1h and filtered through 0.45 micron filter before analysis. The system was calibrated with narrow disperse polystyrene standards and molecular weights are reported as polystyrene equivalents.

3.2.2 **Sample preparation for mechanical testing**

In a typical procedure, prepolymer A (1.0 g, 0.96 mmol) was mixed with prepolymer B (0.493 g, 0.96 mmol) along with stannous 2-ethyl hexanoate catalyst (0.1 wt%) using a spatula for few
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minutes and degassed at ambient temperature under a vacuum of 0.1 torr 5 min. The degassed viscous polymer mixture (0.33 g) was then injected into cylindrical cavities [6 mm (D) x 12 mm (L)] of a Teflon® mould and cured at 37°C overnight. The polymer sample with β-tricalcium phosphate granules (10 wt%) was prepared by high speed mixing using Silversion Mixer (Chesham Bucks, UK) and cured similarly.

The batch size of the reaction mixture was about 1.5 g with a gelling time of 15 minutes under these conditions. During the 15 minute ‘working time’ the mixture was injected into the moulds using a 14 gauge syringe needle. The temperature of the reaction mixture was monitored by inserting a thermocouple. The maximum temperature reached during the reaction was approximately 40°C.

### 3.2.3 Preparation of polymer films

Four different types of in-situ cured polymers were prepared from different prepolymer mixtures (see Table 3.1), degassed, and dispensed through a syringe onto glass coverslips (~100µm in thickness). The cover slip was placed in an oven at 37°C overnight with nitrogen circulation to cure the polymer film. The completion of curing was monitored by observing decrease in NCO absorption peak intensity using IR spectroscopy. The films were sterilized by gamma radiation (25 kGy dose).

<table>
<thead>
<tr>
<th>Sample Code</th>
<th>Prepolymer A</th>
<th>Prepolymer B*</th>
<th>Prepolymer MW (Mn/PD)</th>
<th>Additives</th>
</tr>
</thead>
<tbody>
<tr>
<td>76-2</td>
<td>PE-ELDI</td>
<td>PE-GA</td>
<td>2250/3.21</td>
<td>-</td>
</tr>
<tr>
<td>76-2 TCP</td>
<td>PE-ELDI</td>
<td>PE-GA</td>
<td>2250/3.21</td>
<td>10 wt% β-TCP</td>
</tr>
<tr>
<td>76-6</td>
<td>PE-LLA-ELDI</td>
<td>PE-GA</td>
<td>2091/1.83</td>
<td>-</td>
</tr>
<tr>
<td>76-6a</td>
<td>PE-DLLA-ELDI</td>
<td>PE-GA</td>
<td>1816/1.66</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 3.1 In-situ cured polymer films. Where PE: Pentaerythritol; ELDI: ethyl lysine diisocyanate; GA: glycolic acid; LLA: L-lactic acid; DLLA: DL-lactic acid, β-TCP: β-tricalcium phosphate. *Prepolymer B molecular weight = 513 based on hydroxyl number.

### 3.2.4 Mechanics

The mechanical properties of Novosorb™ polymers 76-2 and 76-2 TCP were determined using an Instron Universal Testing System (model 5568, Instron corporation, Grove City, PA, USA) equipped with 5 KN load cell and carried out with cross head speed of 1 mm/min (ASTM F451). Cylindrical test specimens were prepared according to ASTM F451-95 and equilibrated for 24h at ambient conditions before testing. Tests were performed at ambient temperature (23°C) in 45-50 %
humidity to calculate the maximum compression stress, the elastic modulus, yield stress, and strain at break. The results are presented as an average of five replicates.

A standard screw pull-out test was also carried out according to ASTM F543-02. The test specimen was prepared by curing the liquid polymer sample 76-2 with a screw (3.5 mm diameter) so that 17.5 mm of the screw was embedded in the polymer. The sample was mounted in a custom jig to ensure that the screw was pulled only along its long axis. The screw was pulled using an Instron Universal Testing System under displacement control, until failure, at a rate of 5 mm/min. Load and displacement data were obtained and the pull out strength (PS) was determined as the maximum force on the load-displacement curve.

3.2.5 Wettability

The wetting behaviour of ‘wet’ Novosorb™ films was quantified by dynamic contact angle analysis (DCA). To determine the wettability of each hydrated polymer film, captive bubble contact angle techniques were performed as described elsewhere (326) using a Drop Shape Analyser (DSA 10 MK2, Krüss GmbH, Hamburg, Germany). Films cured to glass microscope slides were placed horizontally into the measurement cell and filled with Delbucco’s-modified phosphate buffered solution (D-PBS). Dynamic measurements were made as air-bubble volume was increased and decreased at a constant rate (40 μl/min), using a custom microsyringe pump unit. The captive bubble measurements at the three-phase (air/liquid/solid) interface were recorded by VCA Optima software (AST Productions, MA, USA) and displayed as a function of time, drop radius, drop volume, and drop angle, which was then converted to advancing (θadv) and receding (θrec) angles (Figure 3.1).

![Figure 3.1](image)

**Figure 3.1** Schematic representation of the Drop Shape Analyser apparatus used to determine dynamic contact angles of Novosorb™ samples and appropriate controls. This is software controlled closed-loop system, where advancing (θa) and receding (θr) angles are determined by placing a sessile air drop onto our surface and mathematically assessing the three-phase contact angle with visual software.
Contact angle hysteresis, the difference between advancing and receding angles, was also recorded to highlight physicochemical heterogeneities present on the surfaces. For controls, thermanox coverslips and poly-d,l-lactide (PDLLA) films were used. Briefly, PDLLA (Purac Biochem Goerinchem, The Netherlands) was dissolved in chloroform to produce a polymer weight to solvent volume ratio of 4% (w/v) and the mixture stirred for 2-3 h to obtain a homogeneous polymer solution. The PDLLA solution was spread over glass cover discs and dried at room temperature. Films were sterilised by UV irradiation on each side for 1 h prior to testing for contact angle. Each sample was characterised by mean values derived from 18 measurement profiles: 6 readings per sample surface (n=3). To statistically assess differences in wetting behaviour between Novosorb™ films and controls a Mann-Whitney U test was used ($p < 0.05$).

3.2.6 Energy dispersive X-ray analysis

Chemical analysis of samples 76-2 TCP and 76-2 was performed with a LEO 1525 scanning electron microscope equipped with an energy dispersive X-ray (EDX) detector (Carl Zeiss SMT ltd, Hertfordshire, UK). To quantify the chemical content of small areas (~ 60 μm$^2$) on the sample surface, 3 distinctly different topographical areas were chosen from 3 different sites per sample and examined at 10kV using INCA Energy 3000 software (Oxford Instruments, High Wycombe, UK). Results were corrected for the carbon coating used and are presented as weight percentage and Ca/P ratio averages ± standard deviation (n=3).

3.2.7 Cell culture

The primary human osteoblasts used in this study were isolated from the trabecular bone of femoral heads taken from patients undergoing total hip arthroplasty (age: 75 and 82) at The Chelsea and Westminster Hospital with full ethical consent (London, UK). Cells were grown in monolayer until passage 2 in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS, 2 mM L-glutamine, 50 μg/ml penicillin, and 50 μg/ml streptomycin (all Invitrogen, Paisley, UK). The cells were maintained at 37°C and 5% CO2 and media was changed every two to three days. After three weeks of culture, cells were detached, counted, and seeded on the Novosorb™ films (1.3 cm$^2$) at a density of 10,000 cells/cm$^2$. Media was changed every 2-3 days during the assay period. Prior to the experimental use of these cells, the phenotype of the cells was confirmed using osteoblast markers Alkaline Phosphatase and Collagen Type I. The results of the immunophenotyping examination are presented in Appendix A.

3.2.8 Cell viability

Cell viability on the films was demonstrated using the Live/Dead viability/cytotoxicity assay (Molecular Probes, The Netherlands). The experiment was run in triplicate, with each of the 4 types of polyurethane-based films and thermanox discs as controls, at 1, 4, and 7 days in culture. At each
timepoint assayed samples were incubated with Live/Dead solution (2µM Calcein-AM and 4µM ethidium homodimer) at room temperature in the dark for 20 minutes. Microscopy was carried out using an Olympus BX-51 reflected fluorescence microscope (Olympus UK LTD., London). Six random fields were imaged at 10 times magnification with an Olympus DP070 digital camera and analysed using Olympus DP Controller software (Olympus Imaging, London, UK).

### 3.2.9 Cell metabolic activity

The effect of the polyurethane-based films on cell metabolic activity was investigated using Cell Titer 96® Nonradioactive Cell Proliferation Assay (MTS Assay, (Promega, UK) after 1, 4, and 7 days. Metabolic activity is assessed calorimetrically, using the tetrazolium compound MTS [3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt], which is bioreduced by metabolically active cells into a colored formazan product that is soluble in tissue culture medium. Metabolic activity was determined by optical density and analysis of data was performed using a one-way ANOVA, with a Tukey post-hoc test to establish significance ($p < 0.05$).

### 3.3 Results

#### 3.3.1 Prepolymer chemistry and properties

**3.3.1.1 Prepolymer A**

The reaction described in section 2.1 yields a viscous prepolymer consisting of a mixture of star and hyperbranched structures (Figure 3.2). The number average molecular weight and polydispersity of the prepolymer were 1348 and 1.73, respectively based on GPC analysis. The prepolymer instantaneous viscosity was 8.7x104 cSt.

![Figure 3.2 Schematic of prepolymer A synthesis - pentaerythritol end capped with ethyl lysine diisocyanate.](image)
3.3.1.2 **Prepolymer B**

In all cases Prepolymer B was a four arm polylol prepared from PE and glycolic acid (Figure 3.3) using condensation polymerisation as described in section 2.1. The molecular weight and polydispersity of the polylol was determined to be 513.3 and 1.08 respectively, based on hydroxyl number determination.

![Figure 3.3 Schematic of prepolymer B synthesis: condensation reaction.](image)

The cured polymers are a cross-linked network formed by the reaction of terminal isocyanate and hydroxyl groups present respectively in prepolymer A and B. The main functional groups present in the polymer network are ester and urethane/urea, all susceptible to hydrolytic, enzymatic and other types of degradation pathways present in biological systems.

3.3.2 **Mechanics**

The mechanical properties of samples 76-2 and 76-2 TCP were studied in compression and represented as stress, strain, and elastic modulus. In-situ crosslinked polymer networks 76-2 TCP and 76-2 exhibit excellent mechanical strength, with ultimate compressive strengths (UCS) of 139 ± 11 MPa and 136 ± 14 MPa and elastic modulus (E) of 2.3 ± 0.03 GPa and 2.0 ± 0.01 GPa, respectively. By comparison, these samples had a UCS significantly greater than, and an E within the range of, cancellous bone (Table 3.2). To further highlight the mechanical strength of the polymers, the UCS were compared to those of European and FDA approved acrylic bone cements (73-117 MPa) and injectable, photocrosslinkable polyanhydrides (30-50 MPa), recently developed for orthopaedic applications (304;327). In measuring strain resistance, sample 76-2 and 76-2 TCP were found to have ultimate compressive strains of >40%, with recoverable elastic strains of ~5% (Figure 3.4).

When compressed, the polymers demonstrated viscoelastic-like properties, evidenced by an initial linear elastic region and a plastic deformation region seen prior to failure (Figure 3.3). A similar type of viscoelastic behaviour is also demonstrated in natural bone (328). Stress-strain plots (Figure
3) revealed that the incorporation of 10 wt% β-TCP particles (5 µm) to sample 76-2 caused an increase (not significant) in E, yield strength, and UCS.

Table 3.2 Initial mechanical properties in longitudinal compression of samples 76-2 and 76-2 TCP compared with properties of natural bone, widely used calcium phosphate based bone cements, and injectable and photocrosslinkable polyanhydrides. Values for mechanical properties were obtained from information contained in these papers: a(328;329), b(327), c(307). Some values were not reported in literature (---).

<table>
<thead>
<tr>
<th>Material</th>
<th>Ultimate Stress (MPa)</th>
<th>Stress @ Yield (MPa)</th>
<th>Modulus (GPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>76-2</td>
<td>136 (14)</td>
<td>84 (15)</td>
<td>2.0 (0.3)</td>
</tr>
<tr>
<td>76-2 TCP</td>
<td>139 (11)</td>
<td>76 (13)</td>
<td>2.30 (0.01)</td>
</tr>
<tr>
<td>Cortical Bone</td>
<td>180-220</td>
<td>180 (13)</td>
<td>18.6 (3.5)</td>
</tr>
<tr>
<td>Cancellous Bone</td>
<td>5-10</td>
<td>---</td>
<td>6.1 (5.4)</td>
</tr>
<tr>
<td>Acrylic bone cements</td>
<td>73-117</td>
<td>---</td>
<td>0.05-0.1</td>
</tr>
<tr>
<td>Polyanhydrides</td>
<td>30-50</td>
<td>---</td>
<td>---</td>
</tr>
</tbody>
</table>

Sample 76-2 was also tested for its pull out strength. The pull out force of sample 76-2, 5954 ± 101 N, was found to be significantly higher compared to the pull out force of screws in healthy bone and curable PMMA cements, demonstrated to be 1540 ± 361N (330) and 2301 ± 283 N (331) respectively.

Figure 3.4 Stress-strain curves of cylindrical specimens 76-2 and 76-2 TCP loaded in longitudinal compression and measured using an Instron Universal Testing System.

3.3.3 Wettability

The wetting behaviour of each of the polymer films was assessed by dynamic contact angle analysis, previously evaluated as a sensitive method to detect interfacial changes at biomaterial/biosystem interfaces (332). The combination of captive bubble techniques with Drop
Shape Analysis image processing, allowed for the accurate and objective measurement of dynamic contact angles in a wet, biologically relevant environment. The contact angle measurements of the polyurethane films and control films, Figure 3.5, are presented as advancing angle, $\theta_{\text{adv}}$, and receding angle, $\theta_{\text{rec}}$.

In comparing receding contact angle results, we found all polyurethane films to be significantly less hydrophilic than thermanox ($p<0.05$). The films, however, were found to be significantly more hydrophilic than control film PDLLA ($p<0.05$). Cross comparing contact angles of the polyurethane films, suggested that sample 76-2 TCP is the most hydrophilic, followed by samples 76-2, 76-6a, and 76-6, although these differences were found not to be significant.

Additionally, all polyurethane films were found to have similar hysteresis values (~21°), with sample 76-2 TCP having a slightly higher value (21.85°). Whereas, thermanox and PDLLA, expressed lower contact angle hysteresis, with values of 11.6° and 16.7° respectively. This signified a more physically or chemically heterogeneous surface on the polyurethane films compared to controls.

![Figure 3.5](image_url)

**Figure 3.5** The advancing ($\theta_{\text{adv}}$) and receding ($\theta_{\text{rec}}$) dynamic contact angles of captive bubbles on Novosorb™ films and controls in dulbecco’s modified phosphate buffered solution (D-PBS). The controls are poly (DL-lactic acid) (PDLLA) films and thermanox. The results are displayed as means ± SD and samples are arranged in terms of decreasing hydrophilicity, seen as an increase in the receding angle. Significance was assigned using a Mann-Whitney test ($p<0.05$). Thermanox was found to have a $\theta_{\text{rec}}$ significantly less than all other samples (*), Novosorb™ films were found to have a $\theta_{\text{rec}}$ significantly less than PDLLA (a,b,c,d) and comparable to one another.
3.3.4 Energy dispersive X-ray analysis

SEM/EDX analysis of unseeded samples 76-2 and 76-2 TCP was employed to examine surface topography and determine chemical composition of film surfaces. This enabled correlations between chemical content and topographical heterogeneity on the sample surfaces. These two samples were compared because they have identical polymer chemistries and fabrication techniques, however sample 76-2 TCP was supplemented with β-TCP particles during film fabrication. EDX results revealed that calcium and phosphorus were solely concentrated at topographically heterogeneous areas on the film surface of sample 76-2 TCP (Figure 3.6). These areas of heterogeneity (sample areas 1 and 3 in Figure 3.6A) appeared as topographically raised areas, with inter-dispersed pore-like structures. In contrast, no calcium and phosphorus was detected on the adjacent flat and nonporous area on the same sample surface (sample area 2 in Figure 3.6A). Additionally, in sample 76-2, no calcium and phosphorus was detected on all surface areas scanned (sample areas 1, 2, 3 in Figure 3.6B). The Ca/P ratios of the heterogeneous areas on sample 76-2 TCP were averaged to be 1.61 ± 0.27 (n=9).

<table>
<thead>
<tr>
<th>Sample Area</th>
<th>Ca (wt %)</th>
<th>P (wt %)</th>
<th>Ca/P Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>A)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>13.0</td>
<td>9.0</td>
<td>1.4</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>0</td>
<td>--</td>
</tr>
<tr>
<td>3</td>
<td>9.3</td>
<td>5.7</td>
<td>1.6</td>
</tr>
<tr>
<td>B)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
<td>--</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>0</td>
<td>--</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>0</td>
<td>--</td>
</tr>
</tbody>
</table>

Figure 3.6 SEM/EDX surface analysis of unseeded films 76-2 TCP (A) and 76-2 (B). Concentrations of calcium (Ca) and phosphorus (P), represented as weight percentages, are found strictly on the topographically heterogeneous areas of sample 76-2 TCP (sample area A1 and A3). Chemical spectral analysis of sample 76-2 surfaces reveals undetectable concentrations of calcium and phosphorus present (sample area B1,B2, B3).(Scale= 20 µm).
3.3.5 *Assessment of cell viability on Novosorb™ films*

Primary human osteoblasts were cultured on the polymer films for 1, 4, 7 days and evaluated for cytotoxicity using Live/Dead assay. By this qualitative method, live and dead cells were stained green and red, respectively. As indicated in Figure 3.7, a majority of the cells remained viable on the samples for periods of up to 7 days, seen as positive live to dead cell ratios (>95%). The live cells adhered well to the films surfaces, and exhibited normal, healthy osteoblastic spindle-like morphology for periods of up to 4 days. In comparing the samples, it is seen that sample 76-2 TCP maintained the best cell viability at longer time points. In samples 76-2, 76-6, and 76-6a, good cell viability is seen for periods of up to 4 days and is followed by a slight decrease in cell viability. Osteoblasts on thermanox controls were found to have similar spindle-shaped morphology as compared to the polyurethane films, however cells were found to be confluent after 4 days in culture (data not shown). The images shown in Figure 3.7 are representative of the results seen for all polyurethane films at 6 different locations on the surface (n=3).

**Figure 3.7** Live/Dead analysis of Novosorb™ films seeded with primary human osteoblasts for 1, 4, and 7 days. Images show combined images of live (green) and dead (red cells) cells on all samples. Non-cytotoxic surfaces are seen on all sample types for periods of up to 7 days, with images showing sustained cell viability and extremely positive live to dead cell ratios (>95%). (Scale bar = 200 µm).
3.3.6 Assessment of cell metabolic activity on Novosorb™ films

Changes in the metabolic activity of primary osteoblasts cultured on the films were evaluated by MTS proliferation assay at 1, 4, and 7 days. MTS measures metabolic function in cells via mitochondrial activity, where a higher absorbance signifies either an increase in cell proliferation or a higher rate of metabolism of the MTS. A reduction in cellular metabolic activity is an early indication of cellular damage, which could potentially lead to cell death. Results for all films, presented in Figure 3.8, show a general increase in cell metabolism for a period of 7 days. This same cell proliferation profile was obtained on the thermanox control. In determining significance, it was found that sample 76-2 exhibited significantly decreased metabolic activity for each time point as compared to the thermanox control (p<0.05), although an increase in metabolic activity was noticed for a period of 7 days. Samples 76-6 and 76-6a were found to have comparable increases in cell proliferation to cells on thermanox for a period of 4 days. Sample 76-2 (TCP) was comparable (no significant difference) to cells on thermanox over the entire period of 7 days. No metabolic activity was observed from any of the unseeded samples, signifying that the samples did not react with the MTS reagents (data not shown).

Figure 3.8 MTS assay for primary human osteoblasts cultured on Novosorb™ films and thermanox controls for 1, 4, and 7 days. Significance is assessed compared to thermanox control for each time point assayed (* = p< 0.05).
3.4 Discussion

The preliminary evaluation of toxicity performed in this study indicated that the films provided generally cytocompatible surface characteristics that allowed for initial cell attachment, sustained viability, and increased cell proliferation profiles. Moreover, these results confirmed polymer synthesis and curing processes to be adequate for initial implantation, since cell viability and increased proliferation was noted for a 7 day period.

Results of Live/Dead and MTS suggested that in-situ sample 76-2 TCP provided an optimal environment for osteoblast attachment, sustained viability, and proliferation as compared to the rest of the films. A possible explanation for this result was the incorporation of β-TCP granules during polymer film synthesis. β-TCP is a resorbable bioceramic material and can be used in the form of blocks, granules, or cements in scaffolds for bone regeneration. Previous literature suggests that TCP may enhance osteoblast function and overall bone formation (333;334). Although the exact mechanisms whereby this happens presently cannot be elucidated, evidence suggests that TCP fillers increase the initial anchoring and spreading of serum proteins on polymer surfaces (335). Therefore, one can assume that sample 76-2 TCP should have a more osteoconductive environment compared to a similar sample film without β-TCP (i.e. sample 76-2). This was observed in this study, as sample 76-2 TCP demonstrated enhanced viability and proliferation of osteoblasts over a 7 day period compared to sample 76-2. Additionally, β-TCP is also believed to act as a buffering agent, neutralising any acidic by-products and maintaining pH (308), which could be another explanation as to why better viability and proliferation were found on this film.

Initial mechanical support and bonding of an implant to the surrounding tissue, is of particular importance to biomaterials for orthopaedic applications. It is integral that these mechanical properties are analogous to the properties of the tissue being repaired. While many properties are important when characterising materials for orthopaedics, compressive properties are most consistently recorded in literature, and therefore provide for a better comparison between materials. After cured, samples 76-2 and 76-2 TCP attained compressive strengths of 136 and 139 MPa respectively. Although insignificant, the slightly enhanced mechanical properties found in sample 76-2 TCP (Table 3.2 and Figure 3.4) are most likely due to the incorporation of β-TCP particles; a typical reinforcement effect noted previously (302). Both samples, however, had compressive strengths well above those for cancellous bone (5-10 MPa) and are nearly within the range of cortical bone (180-220 MPa) in compression (328). These compressive strengths are almost twice the critical minimum (70 MPa) permitted under standard ISO 5833, regulating injectable bone cements (336). Similarly, both polyurethane samples exhibited elastic modulus (~2 GPa) in accordance with permitted minimums under the same standard (>1.8 GPa). Additionally, the polymers are found to exhibit mechanical properties better than those of a novel calcium phosphate
based bone cements and widely used acrylic cements, both of which have gained FDA and European approval for uses in distal radius and tibial plateau fractures, craniofacial applications, and screw fixation (327;337;338).

Further comparisons of mechanical properties of the polyurethane polymers to natural bone, revealed that polymers 76-2 and 76-2 TCP were designed to withstand the peak compressive strains in limb bones incurred during rigorous functional loading. In bone, principal compressive strains have been found to reach peak magnitudes of 2000 µε (339), well within the elastically recoverable strain range of polymers 76-2 and 76-2 TCP (<50,000µε). Undoubtedly, the result of plastic deformation to a weight bearing implant will lead to loss of form and function, and will eventually fail, resulting in both pain and an additional surgical procedure for the patient. Moreover, the ratios of ultimate compressive strength to elastic modulus, are comparable in both bone and our polymer constructs (~1/100) (327). This analysis showed that the polyurethane constructs, although not mechanically identical to cortical bone, possess a similar balance between compressive elasticity and strength found in natural bone.

Mechanical screw pull-out tests, showed sample 76-2 to have a fixation strength greater than that of bone and PMMA bone cements, found to be 1540 ± 361 N and 2301 ± 283 N respectively (330;331). This strong initial bonding strength possessed by polymer 76-2 may act to reduce interfacial failure and bond loosening by strengthening the cement-implant and cement-bone interface (340). The strength of this interface with the surrounding host bone tissue is of paramount importance to the clinical success of injectable polymer systems in orthopaedic applications. The strong initial bonding of this injectable polymer gel to the host bone will allow for quick restoration of voids; leading to immediate fracture stability, an earlier return to full-weight bearing, and faster restoration of tissue function.

The wetting behaviour was assessed by dynamic contact angle analysis, previously evaluated as a sensitive method to detect interfacial changes at biomaterial/biosystem interfaces (332) Dynamic contact angle analysis is considered a valuable technique for characterising the wetting behaviour of rough biomaterial surfaces, because the three-phase contact angle is sensitive to the outermost 3-20 Å of a surface and its chemistry (341). This same area is also important to many cell-surface interactions including adhesion, motility, and signalling. It is known that surface wettability plays a vital role in the cell-biomaterial interface, modulating both protein adsorption and cell adhesion (342), where cell adhesion and motility increases on more hydrophilic surfaces (343). In this study an increase in the value of the receding contact angle provided a reliable indication of a less hydrophilic surface (344). All polyurethane films were found to have receding angles significantly more hydrophilic than PDLLA; a biodegradable polymer approved for in vivo orthopaedic applications by the Food and Drug Administration of the USA (61;345). This surface
hydrophilicity, correlated with the favourable cell viability and proliferation results seen for all samples.

To better understand the wetting characteristics of these films, hysteresis values obtained from DCA, were used to assess surface roughness. The heterogeneity of the surface can be observed indirectly when measuring contact angles. If a surface is not smooth, large deviations in attainable contact angle measurements will be seen as the three-phase contact line is moved, leading to large hysteresis values (346). These irregular contact angles arise from a phenomenon known as pinning (stick/slip). It was previously shown, that if size of the heterogeneity (347) or roughness feature (348) was larger than 0.1µm, the three phase contact line had a tendency to be stopped by a pinning mechanism on local surface imperfections and could not move without introducing additional external force (i.e. rapid influx of air into the captive bubble). This creates a series of metastable states, with contact angle hysteresis used as indicator as to the extent of these surface heterogeneities. All polyurethane films were seen to be rough, heterogeneous surfaces, evidenced by significantly larger values of contact angle hysteresis (~21°) compared to smooth, homogeneous thermox surface (11.6°). Sample 76-2 TCP was found to have a slightly higher hysteresis value as compared to the other films. This was most likely due to the fact that sample 76-2 TCP, was composed of two regions, having different wetting characteristics: the guest material (β-TCP 5µm particles) and the primary host material (LDI-based polyurethane). The different chemistries present on this surface likely influence the wetting behaviour by causing both morphological and chemical changes in the system (341). Although not statistically significant, this suggested that sample 76-2 TCP had a rougher surface with more heterogeneity, in support of SEM micrographs of the surfaces of samples 76-2 and 76-2 TCP (Figure 3.5), which showed a more topographically heterogeneous surface on sample 76-2 TCP. Although surface roughness was not quantitatively assessed in this study, one can assume that the surface roughness of sample 76-2 TCP has created a more hydrophilic environment, shown as a reduction in contact angle. This observation is in agreement with previous investigations, which concluded that roughness makes hydrophilic surfaces (contact angle < 90°) more hydrophilic and hydrophobic surfaces (contact angles > 90°) more hydrophobic (349).

EDX analysis confirmed the presence of calcium and phosphorous on the surface of sample 76-2 TCP whilst these elements were undetectable on sample 76-2. Furthermore, results revealed calcium and phosphorus to be solely concentrated at topographically heterogeneous areas on the film surface of sample 76-2 TCP (Figure 3.5B). The fact that no concentrations of calcium or phosphorous were found on sample 76-2, suggested that β-TCP had a role in creating topographical heterogeneity. This demonstrates a possible correlation between the β-TCP filler and increased chemical and physical heterogeneity. These areas of chemical heterogeneity were found to consist
of Ca/P ratios (1.61 ± 0.27) similar to native bone (1.71) and natural stoichiometric HA (~1.67), known to have osteoinductive and osteoconductive capacities (350;351). More studies, however, are needed to examine the extent of the relationship between physiochemical heterogeneity and osteoblast behaviour in sample 76-2 TCP.

3.5 Conclusions

This study presents a novel two component polyurethane platform that can be administered arthroscopically, polymerise at biological temperatures in-situ, and provide appropriate bonding strength and mechanical support, with mechanical properties superior to widely used bone cements. These novel polymers provided a favourable in-vitro environment for initial cell adhesion, maintained cell viability, and normal rates of proliferation of human osteoblasts for periods of 7 days. Physicochemically, this biocompatibility can in-part, be attributed to the hydrophilic surface characteristics of these polymers, and, in the case of sample 76-2 TCP, the combined presence of heterogeneous topographies and chemical moieties on the surface. The addition of β-TCP filler during film fabrication enhanced cell viability, increased surface heterogeneity and mechanical properties in compression, and significantly increased cell proliferation.
Chapter 4

Development of an *in-vitro* Human Periosteum Tissue Culture System

In the previous chapter, I discussed the development of a novel synthetic polymer platform which can be used to create scaffolds that support cells and incorporate cues which guide tissue repair. In the next 4 chapters, I will shift my focus to the cellular aspects of the tissue engineering paradigm. In particular, my aim is to isolate, culture, and extensively characterise adult human periosteal cells for the development of new and interesting stem/progenitor-cell therapies. The necessary first step on this path to wide-spread clinical use is the design and optimisation of methods to harvest and culture human periosteal tissue without compromising the viability and therapeutic potential of the cells. Therefore, this chapter is dedicated to the intensely iterative process of developing a simple and reproducible human periosteum tissue culture system. Accordingly, this chapter is broken down into 2 main sections, or mini-chapters, for the purposes of designing culture systems for periosteum tissue explants and isolated periosteal cells respectively.

4.1 Harvest and Culture of Human Periosteal Explants: An Organ Culture Model

4.1.1 Introduction

The rapidly growing interest in cell and tissue transplantation for cartilage repair stems from the well-known fact that damaged articular cartilage is incapable of healing itself. Full-thickness osteochondral defects that penetrate the subchondral bone, if left untreated, can ossify or fill with fibrocartilage and cause clinical symptoms such as pain and joint dysfunction that can lead to the development of osteoarthritis (352;353). In-vivo, cartilage regeneration has been shown to in experimental animals (354-357) and human patients (17;358) by whole tissue transplantation using periosteum. The regenerative potential is associated with the presence of undifferentiated mesenchymal stem cells resident to the cambium layer of the periosteum (31;159;160;359). However, the in-vivo results are not always predictable or reproducible and do not result in the regeneration of fully functional hyaline-like cartilage (352).

Therefore, the development of periosteum-based osteochondral composites in-vitro would serve as a promising alternative approach for cartilage repair and a novel means to study chondrogenesis.
Advantages of exploiting periosteum over cell cultures for skeletal tissue engineering include the fact that it is a source of intrinsic chondrogenic growth factors and can serve as three-dimensional template for directional evolution of neotissue (360). Towards this end, periosteal explant culture systems have been developed for cartilage tissue engineering platforms and chondrogenesis models (190;361;362). However, these culture models have utilised explants harvested from animals and may not accurately reflect the characteristics and behaviour of human tissue. Therefore, we aimed to develop a periosteal organ culture model using human donor derived tissue to engineer osseous and cartilaginous tissues in-vitro. This preliminary study will validate this model by assessing the viability and chondrogenic potential of human periosteal explants in culture.

**4.1.2 Materials and Methods**

**4.1.2.1 Harvest of periosteal explants**

Periosteal explants, measuring 1.0 x 1.5 cm\(^2\), were harvested by subperiosteal elevation from patients undergoing elective knee surgery at The Chelsea and Westminster Hospital. In each case, explants were harvested from the medial proximal tibia of patients by the same skilled surgeon. Explants were cleaned of fat and blood tissue in theatre and immediately transported to laboratory in Earl’s balanced salt solution (EBSS; Gibco, Paisely, UK).

**4.1.2.2 Histological evaluation of periosteal explants**

To identify the quality and variability of the harvested tissue, histological examination of periosteal explants was performed. Tissue samples were fixed in 10% v/v neutral buffered formalin (Agar Scientific, Stanstead, UK) for 12 hours at room temperature, dehydrated through an increasing alcohol series and cleared with xylene (Sigma-Aldrich, Poole, UK) before being embedded in paraffin wax. Each sample was cut perpendicular to the bone-interfacing surface, into four 5μm thick histological tissue sections at equal intervals from the proximal to distal end, and stained with haematoxylin and eosin (both Sigma-Aldrich, Poole, UK). Photomicrographs (x100) were taken of each section, to identify the presence distinct cellular layers and determine any donor-donor variability present.

**4.1.2.3 Preparation of explant-agarose constructs**

To study the viability and therapeutic potential of human periosteal explants a modified agarose culture system was designed based on previously developed 3-dimensional culture systems (184;190;361;362) Harvested explants were rinsed three times in PBS and cut into ~3 mm\(^2\) pieces under aseptic conditions. To create the agarose-explant constructs a specially designed Perspex® mould consisting of 6 x 5 x 4 mm\(^3\) wells was used. Each well of the mould was pre-coated with approximately 60 μl of molten low gelling temperature agarose (type VII; Sigma) and allowed to gel for 5 minutes at RT. Agarose suspensions were prepared in Earl’s Balanced Salt Solution
(EBSS; Gibco Ltd., Paisley, UK) at concentrations varying from 2-5% (w/v) depending on the study performed. After this time an explant was placed into each well and another 60µl of molten agarose was used to cover the explant and complete the mould. The full constructs were then allowed to gel in the mould at 4°C for 10 minutes. Agarose/explants constructs were cultured for periods of up to 28 days in a 24 well-plate, one construct per well, in one millilitre of medium.

### 4.1.2.4 Explant viability

Cell viability within periosteal explants and agarose-explant contracts was examined, first, to determine the negative effects of explant isolation and transport, and second, to establish the feasibility of this agarose-explant culture system. The LDH cytotoxicity test is based on lactate dehydrogenase (LDH) leakage upon cell membrane injury. LDH release into the culture medium indicates compromised membrane integrity of dead or dying cells (363-365). Cell death was quantitated by measuring the release of cytoplasmic enzyme lactate dehydrogenase (LDH) using the CytoTox 96® Non-Radioactive Cytotoxicity Assay kit (Promega, Madison, USA) that is based on a coupled enzymatic reaction that results in the conversion of a tetrazolium salt into a red formazan product. The increase in the amount of formazan produced in the culture supernatant directly correlates with the increase in the number of lysed cells. Formazan was quantified spectrophotometrically by measuring its absorbance at 490 nm. Explant cytotoxicity was measured directly after explant transport from theatre (t=0) and after 1 and 4 days in static culture in 2%, 3%, 4%, 5% (w/v) agarose gels and in explants not in gel constructs (no gel). Cell death was expressed as a ratio (%) of released LDH/total LDH (content in medium+ cells) (n=3 per time point). To measure the amount of LDH in media, aliquots were taken of culture media from each experimental well. This data was then combined with the measures of LDH released from the accompanying cell lysate obtained by homogenising (hand-held homogeniser, Fisher, UK) the explant in 1% Triton-X100 lysis solution. Due to the interference of phenol red (λ max ≈ 550 nm) with the coloration of the LDH assay (λ max ≈ 490 nm), phenol red-free DMEM was used in all cultures assayed for LDH release. To assess statistical differences in cell death within data from individual donors the Mann-Whitney U test was employed (p<0.05).

### 4.1.2.5 Chondrogenesis

To assess the chondrogenic potential of periosteal explants, 3% (w/v) explant/agarose constructs were cultured for 2 and 4 weeks in DMEM containing 10% FBS, 1 mM proline, 50 µg/ml ascorbic acid, penicillin/streptomycin (50 U/ml/50 µg/ml) and either 10 ng/ml of TGF-β1 and FGF-2 or no growth factors (361). The cultures were maintained in 5% CO2 in air at 37°C with the culture medium replaced every other day. This experiment was performed on explants harvested from 2 different donors, with 3 explant/agarose constructs per experimental group per time pt (n=3). At 14 and 28 days the constructs were rinsed with phosphate buffered saline (PBS), and cleaned of any
residual agarose gel remaining on the explant. Each explant was then fixed in 10% neutral buffered formalin for 12 h before paraffin embedding.

### 4.1.2.6 Histochemical and immunohistochemical analysis.

Safranin-O and hematoxylin/eosin staining of the explants was performed at each time point. Sections of 5 µm were cut perpendicular to the cambium surface at regular intervals from medial to distal end. Immunohistochemical analysis was performed using monoclonal antibodies obtained from Chemicon (MAB8887, Millipore, UK) to detect the presence of collagen Type-II. Prior to staining, an enzymatic retrieval of antigens was completed using 1% (w/v) pepsin and rinsed 2x in PBS. Primary staining was performed at a 1:200 dilution for 1 hr at RT. For secondary staining, horseradish peroxidase was used at the same concentration. In all cases, positive staining was assessed visually and images were taken using an Olympus BX51 reflected microscope fitted with a Olympus DP070 digital camera (Olympus Imaging, London, UK). Sections of human nasal cartilage were used as positive controls.

### 4.1.3 Results and Discussion

#### 4.1.3.1 Explant histology

To preserve viability and reduce inter-donor variation, explant harvest procedures were carefully performed as described previously (366) using sub-periosteal elevation by an experienced orthopaedic consultant. Additionally, explants were only harvested from the medial proximal tibia of visually healthy periosteum in non-osteoporotic patients. This anatomical location was selected as the optimal donor site due to its easy accessibility during both arthroscopic and open-joint surgery and enhanced therapeutic potential (359;367). This procedure is advantageous to other autologous tissue harvest procedures, such as bone marrow aspirates, which often involve painful surgeries and face heightened risks of donor-site morbidity. The isolation procedure detailed here results in a small wound-site, which is easily repaired by normal wound-healing processes and does not affect the integrity or functionality of the underlying bone (observations by orthopaedic consultant Mr. Williams). Haematoxylin and eosin staining of the periosteum tissue explants reveals the presence of two distinct tissue layers in all donors (Figure 4.1). The cambial layer is visible as a cell rich outer layer adjacent to the bone, with the fibrous layer, consisting of widely dispersed cells on the outside of the cambium. Cambium layer thicknesses varied between 84-111 µm, with the mean for all donors found to be 95.5 ± 13.4 µm. Fibrous layer thicknesses demonstrated larger donor-to-donor variation, with mean values ranging from 1061.7-1525.5 µm. Further, a network of blood vessels was found in the fibrous layer of explants that were surrounded by a dense collection of cells. A full evaluation of the cellular and extracellular characteristics of
the individual periosteal tissue layers is performed in Chapter 6 and includes an analysis of age-related changes in tissue architecture.

4.1.3.2 Explant culture

For both short-term and long-term culture periosteal explants were further dissected into small tissue pieces, measuring 1.5 x 2 mm, as cartilage formation was found to be significantly better in smaller explants (368). The use of growth factors TGF-β1 and FGF-2 were used to promote chondrogenesis in long-term explant cultures. In-vivo, TGF-β has a broad range of cellular activities including the control of the proliferation and expression of differentiated phenotype of several cell types specific to bone, among them mesenchymal precursor cells, chondrocytes, osteoblasts, and osteoclasts (369;370). Due to this TGF-β plays a central role in regulating the complex cascade of cellular events involved in soft-tissue and the formation of cartilage and bone, as occurs during fracture healing (371;372). In-vitro, studies have suggested that both TGF-β1, 2, and 3 may regulate osteogenesis and chondrogenesis by affecting replication, gene expression, and structural protein synthesis in nearly all cell types associated with bone formation (373-375). Importantly, TGF-β1 has been shown to not only induce the differentiation of periosteal mesenchymal stem cells into osteoblasts and chondrocytes but also stimulate these cells to proliferate and synthesize the ECM proteins characteristic of bone and cartilage tissue (31;190;362;376;377). FGF-2 is another important factor found within the cartilage matrix and has been shown to be a potent mitogen for chondrocytes and primitive marrow stromal cells (378-381). In combination with TGF-β1, FGF-2 was recently shown to support efficient expansion of articular chondrocytes while maintaining their capacity to differentiate (382). Recently, FGF-2, during the early stages of periosteum explant culture in the presence of TGF-β1, significantly enhanced periosteum cellularity and chondrogenesis by increasing the number of committed cells (361). In this way, the addition of growth factors into in-vitro cultures is considered as an adjuvant in bone and cartilage tissue engineering.
4.1.3.3 Explant viability

Extra-cellular LDH concentrations were measured as a means to quantify the cytotoxic effects of periosteal explant isolation, transport, and culture on cells in the tissue. The mechanical processes of explant isolation and transport resulted in the release of large amounts of LDH from membrane damaged, dead or dying cells. Across the 4 explants (4 donors) examined we see initial percentage of total cell death range from 11-43% (Figure 4.2). Upon culture, the cell death initial found in the explants was found to stay consistent or rise over a period of 4 days. Although, in explants harvested from a 28 yr old female we found a consistent increase in cell death over the 4 day culture period in each of the experimental conditions (Figure 4.2C). We hypothesized that nutrient and metabolite diffusion limitations would lead to increased cell death in agarose gels in a concentration dependent manner. However, we were unable to establish any significant differences between the different agarose gel concentrations analysed and found that the added 3-dimensional support of agarose gels neither improved nor worsened the cell viability of the explants. Equally, we also did not find differences in LDH release between explants cultured in the presence or absence of agarose over the culture period (Figure 4.2A and B). The inability to extract statistically
significant differences between the experimental conditions is due to the large deviations in LDH release measured. This variability is demonstrated between both donors and dissected tissue pieces derived from one explant. The high levels of cell death and variability found in periosteal explants shown here highlights the potential limitations in the use of a periosteal explant culture model and warrants further analysis.

Figure 4.2 LDH released from periosteal explants after harvest and transport \((t=0)\) and following 1 and 4 days in culture in the presence \((2\%, 3\%, 4\%, \text{or } 5\% \text{ w/v})\) or absence \((\text{no gel})\) of agarose. Cell death is expressed as a ratio \((\%)\) of released LDH/total LDH \((\text{content in medium+ cells})\) with an \(n=3\) per time point. Results collected from 4 donors of different ages \((16-73\text{ yrs.})\) and sexes are shown to highlight donor-donor variations.

### 4.1.3.4 Chondrogenesis

In the normal, homeostatic state, defined here as time 0, the cambium layer contains a heterogeneous population of spindle-shaped cells, 5-6 cell layers in thickness \((\sim 80-100\ \mu\text{m})\). Whereas, the fibrous layer is much less cellular and is composed of mainly fibroblastic-like cells. In the presence of growth factors TGF-β1 and FGF-2, it was observed that the cellularity of both layers increased substantially over the 14 and 28 day period (Figure 4.3B-D). In Figure 4.3, only day 28 images are shown as they are representative of results found in explants examined at 14 days. Although it was not quantified, the increase in the cellularity within the cambium layer was more pronounced than in the fibrous layer in the donors tested. This stimulation of cell
proliferation in each layer is reflective of the early stages of chondrogenesis noted in previous periosteal explant studies (190;361;362).

However, unlike previous studies there appeared to be no concomitant increase in the weight or thickness of the periosteum explants studied here. Equally, we did not observe the sequential progression of cell morphologies related to developing neochondrocytes during periosteal chondrogenesis (383). This lack of morphologically distinct hypertrophic and mature chondrocytes in our explants is in agreement with the absence of positive safranin-O and collagen type II staining noted in each section of each explant examined (Figure 4.3F-H & J-L). Although, the explants displayed an abundance of collageneous matrix, as evidenced by fast green staining, there was no synthesis of cartilage-specific proteoglycans or collagen type II. The specificity of the safranin-O and collagen type II staining was confirmed by the use of human nasal cartilage as a positive and negative control (Figure 4.3E & I). Therefore, the combination of agarose with growth factors TGF-β1 and FGF-2 did not favour the differentiation of chondrogenic cells or formation of cartilage-like matrix within all periosteal explants across all donors. This negative result is made all the more unique given the use of proven differentiation factors TGF-β1 and FGF-2 and demonstrated chondrogenic potential of the periosteum ex-vivo. Within similar culture conditions, the appearance of neo-cartilaginous tissue was noted after 2 weeks in rabbit periosteal explants (361). This may reflect species related differences in periosteal cell characteristics and functionality noted previously in periosteal cultures in-vitro (384).
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Figure 4.3 Histochemical and immunohistochemical images of haematoxylin and eosin (A-D), safranin-O (E-H), and collagen type II (I-L) staining of human periosteal explants and nasal cartilage. Periosteal explants embedded in 3% (w/v) agarose were cultured for 28 days in chondrogenic media supplemented with TGF-β1 and FGF-2. Representative images of stained explant sections from 3 donors have been selected to show the cellular and extracellular features of cultured explants. Safranin-O stains proteoglycans red, fast-green stains fibrous tissue green, and positive collagen type II staining is seen as dark brown. Human nasal cartilage was used as a control to show positive staining of cartilaginous matrix and negative staining of the perichondrium. Scale bars = 200 µm.
4.1.4 Conclusion

The organ culture model examined here demonstrated compromised cell viability and an inability to induce chondrogenesis in human periosteal explants in-vitro. The interpretation of these findings were influenced and further complicated by the inherent inter- and intra- donor variation that is apparent in these explants. To circumvent the issues surrounding this explant culture system we must consider the use of isolated and ex-vivo expanded periosteal cells in the treatment of a wide range of skeletal abnormalities. However, for such a cell-based system to enter wide-spread clinical use it will be necessary to identify conditions of culture that support the rapid expansion of periosteal cells with therapeutic potential. From this prospective, further studies will now focus on the development of an isolated human periosteal cell culture model. The layout of the next experimental mini-section in this chapter will follow the iterative process of design optimisation outlined in Figure 4.4.

![Figure 4.4](image.png)  
Figure 4.4 Schematic outline of iterative experimental process towards the development of an optimal isolated human periosteal cell culture model.
4.2 Isolation and Culture of Human Periosteal Cells: A Cell Culture Model

4.2.1 Introduction

Although in-vitro multilineage differentiation of periosteal cells has provided proof of principle for the creation of an autologous tissue engineered implant (159;160;190), we still lack a well-defined protocol for the isolation and expansion of these cells to make this technique available for clinical purposes. For tissue engineering applications, therapeutic cell types need to isolated, purified, and expanded to large numbers whilst controlling cell fate. For example, on the basis of using a scaffold containing 8 million cells/mL (385), 20 mL of tissue engineered implant used for spinal fusion surgery would require about 160 million cells. Since the cell expansion of primary human cells is a labor intensive and expensive process, the use of an optimal culture system is desirable. Surgical skill (386), explant size (368), donor site (359) and donor age (387) have been found to significantly influence the quality of functionality of periosteal grafts previously. Additionally, micro-environmental factors such as media composition, cell density, growth factors, and culture time are all known to influence the propagation of colony forming mesenchymal progenitor and stem cell types in-vitro (225;246;388). Therefore, it was necessary to determine the optimal cell isolation and monolayer culture conditions for the expansion of viable periosteal cell populations of interest. For this purpose, we examined different enzymatic isolation methods and the effects of cell culture media and cell seeding density on cell phenotype, initial cell adherence, short-term growth characteristics, and colony forming ability.

4.2.2 Materials and Methods

4.2.2.1 Enzymatic isolation of human periosteal cells (experiment I)

To establish the optimal cell isolation protocol in terms of cell yield, viability, and time, a series of 13 periosteal explants were harvested from patients aged between 25-84 yrs and subjected to a variety of different isolation methods based on enzymatic digestion protocols developed previously. In each case, the explant was transported back to laboratory in Earl’s balanced salt solution at 37°C in a mini-portable incubator and was processed within 1.5 hrs of harvest. After being cleaned of fat and blood tissue, the explants were rinsed 3x in PBS and finely minced into 2 x 2 mm pieces.
Isolation Method 1

This isolation protocol is based on methods developed by Nakahara et al for isolating rabbit periosteal cells (186). Finely minced explants were transferred into a 15 ml conical tube and rinsed three more times with Tyrode's salt solution (T 2145: Sigma, UK). A 0.25% (w/v) Collagenase solution (CLS Type II, Worthington Biochemical, Reading, UK) was added to the tube and the periosteum were incubated for ten minutes in a 37 °C water bath. After the incubation the periosteum had settled to the tip of the conical tube so that the collagenase solution could be removed and discarded, followed by two rinses with Tyrode’s salt solution solution. Fresh 0.25% collagenase solution was then added and the periosteum were incubated for two hours at 37 °C with vortexing for ten seconds every 30 minutes. After this incubation trypsin was added to a final concentration of 0.2% (w/v) (TRL-3: Worthington Biochemical, Reading, UK) and the periosteum were further incubated for 30 minutes at 37 °C. After the incubation the collagenase-trypsin solution was inactivated by adding an equal volume of fetal bovine serum. The digestate was passed successively through 100-, 70-, and 50-µm cell filters (Falcon; Fisher Scientific, Loughborough, UK). The cells were centrifuged and resuspended in complete medium (DMEM containing 10% foetal bovine serum, 1% L-glutamine, 1% antibiotic/antimicrobial).

Isolation Method 2

This isolation protocol is based on manufacturer’s instructions and modified methods developed to enzymatically isolate periosteal cells (201;389). Finely minced explants were transferred into a 15 ml conical tube and digested by 0.25% collagenase D solution (Roche Applied Science, West Sussex, UK) in Earl’s Balanced Salt Solution for 3 hrs at 37 °C at 5% CO₂. The resulting digestate was passed successively through 100- and 70-µm cell filters (Falcon; Fisher Scientific, Loughborough, UK), centrifuged, and the pellet was washed two times in complete medium.

Isolation Method 3

This protocol is identical to method 2, except that a 0.6% collagenase solution was used to enzymatically digest the periosteal explants for periods of 2-3 hrs. The digestate was processed as above.

Isolation Method 4

This digestion method was developed to optimise and improve upon the previous enzymatic protocols examined here. Finely minced explants were placed in a enzymatic solution consisting of 3 mg/ml collagenase D (Roche Applied Science, West Sussex, UK) and 3 mg/ml collagenase type II (Sigma, Poole, UK) and 5mM CaCl₂ in 5 ml complete media (DMEM containing 10% foetal bovine serum, 1% L-glutamine, 1% antibiotic/antimicrobial). The tissue was placed in an incubator at 37 °C and 5% CO₂ and allowed to incubate for periods between 4.5-8.0 hrs under
gentle agitation. Following incubation, the cell digestate was passed through a 70 µm cell filter, collected by centrifugation, washed twice and resuspended in complete media.

4.2.2.2 Cell counting

After each isolation method, the cells were resuspended in 5 mls of complete medium (DMEM containing 10% foetal bovine serum, 1% L-glutamine, 1% antibiotic/antimicrobial) and live/dead cells were counted using a haemocytometer and trypan blue dye. These numbers were then used to determine total cell yield, cell release rate (cells/hr), and percentage cell death. Mononuclear, red blood cells were not counted.

4.2.2.3 Effects of culture medium on cell expansion (experiment IIa)

Two different growth media solutions were selected from literature for their well-defined ability to propagate isolated periosteal cell cultures whilst eliciting minimal influence over the cells’ differentiation profile. The growth media solutions used in this study are shown in Table 4.1.

<table>
<thead>
<tr>
<th>Media Name</th>
<th>Media Contents</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>DMEM, 10% FBS, 1 mM proline, 50 µg/ml ascorbic acid, and penicillin/streptomycin (100 U/ml/100 µg/ml)</td>
<td>(361)</td>
</tr>
<tr>
<td>2</td>
<td>DMEM, 10% FBS, 1% L-glutamine, penicillin/streptomycin (100 U/ml/ 100 µg/ml)</td>
<td>(190;201)</td>
</tr>
</tbody>
</table>

To study the effects of different culture environments on cell expansion, periosteal cells were isolated from a tissue explant using enzymatic isolation method 4, described above, and cell migration methods described previously (296;390). Briefly, a periosteal explant (750 g) was harvested from the proximal tibia of a 37 yr old male undergoing ACL revision surgery at The Chelsea & Westminster Hospital. The explant was cut in two equal pieces by weight and the cells were isolated by enzymatic digestion and explant migration methods. The 9.3 x 10^5 enzymatically liberated cells were separated into separate T75 tissue culture flasks containing 15 mls of either growth medium 1 or 2 (Table 1). The remaining explant was minced into 1mm^2 pieces and were equally distributed into two T75 tissue culture flasks containing 15 mls of either growth medium 1 or 2 (Table 1). All flasks were then cultured at 37°C and 5% CO² where the medium was changed every 2-3 days to remove non-adherent cell subsets. To assess the initial adherence, viability, and proliferation of the cells in each condition, light microscopy images were taken 4 days post-tissue harvest (termed passage 0 cells) and 7 days after the first passage (termed passage 1 cells).

4.2.2.4 Effects of culture medium on population phenotypes (experiment IIb)

To assess the effect of the effect of the growth medium on the propagation of specific cell populations, flow cytometry was performed on enzymatically liberated passage 2 cells using...
fluorescently-conjugated monoclonal antibodies directed against Stro-1 (progenitor cells), ALP (osteoblasts), CD34 (haematopoietic cells), and CD45 (leukocytes). This cocktail of antibodies examined here identify all populations of progenitor, osteogenic, and haematopoietic cell types present in the periosteum and represent the largest populations of cells isolated from human tissues. This is based on results in this thesis. A full analysis and description of each mAb, including the preparation and labelling of the cells for flow cytometry will be discussed in full detail in Chapter 5. Cell fluorescence was evaluated by flow cytometry using a FACSCalibur instrument (BD Biosciences, Oxford, UK) and data analysed using FlowJo software (Tree Star Inc, Ashland, USA). At least 30,000 cells were collected per sample and percentage positive expression for each mAb was defined as the level fluorescence >99% of the isotype-matched control antibodies.

4.2.2.5 Effects of cell seeding density (experiment III)

To allow for a more standardised and reproducible periosteal cell culture system, it was important to establish the effects of plating density on total cell yields and proliferation rates. Passage 2 isolated periosteal cells from a 31 yr old male and a 39 yr old male were cultured at 10 cells/cm\(^2\), 50 cells/cm\(^2\), 500 cells/cm\(^2\), and 5000 cells/cm\(^2\) in 78 cm\(^2\) petri dishes for a period of 12 days in growth media 2. Every 2 days, cells from 3 plates from each culture density were harvested and counted on a haemocytometer. This data was then used to calculate average cell yields, fold increase, population doublings, and cell doubling times of each culture.

4.2.2.6 Colony forming ability (experiment IV)

To study the effects of plating density and culture time on cell growth and colony formation, passage 1 periosteal cells were plated at 500, 10\(^3\), 10\(^4\), 10\(^5\), 10\(^6\) cells per 78 cm\(^2\) petri-dish (Falcon, Fisher Scientific, UK) in 3 dishes per cell concentration in growth media 2 (n=3). After 7 and 12 days, the dishes were stained with 0.5% (w/v) crystal violet in methanol for 5 minutes, rinsed twice in distilled water and the number of colonies per dish counted by 2 independent observers. Based on previously described criteria (391) colonies < 1 mm in diameter (~50 cells) and faintly stained colonies were discounted. Mean values for each ech density for each time point were derived from three Petri dishes.
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4.2.3 Results and Discussion

4.2.3.1 Cell isolation (experiment I)

Since human periosteal tissue is a dense connective tissue composed mainly of collagen and reticular fibers, cell isolation required the use of bacterial collagenases to digest the extra-cellular tissue. The collagenase most commonly used for tissue dissociation is a crude preparation containing clostridiopeptidase A in addition to a number of other proteases and peptidases. Crude collagenase is well suited for tissue dissociation since it contains the enzyme required to attack native collagen and reticular fibers in addition to the enzymes which hydrolyze the other proteins, polysaccharides and lipids in the extracellular matrix of connective and epithelial tissues. Bacterial collagenase is a protease with specificity for the X-Gly bond in the sequence Pro-X-Gly-Pro, where X is most frequently a neutral amino acid. Such sequences are often found in collagen, but only rarely in other proteins. While many proteases can hydrolyze single-stranded, denatured collagen polypeptides, clostridiopeptidase A is unique amongst collagenases in its ability to attack and degrade the triple-helical native collagen fibrils commonly found in connective tissue.

To evaluate the efficacy of each isolation method a minimum of 2 explants (2 donors) were used per method. Further, to reduce the potential for experimental variation explants, measuring ~ 1.0 x 1.5 cm, were harvested from the same donor site by the same skilled surgeon and were processed immediately. Data from the 4 different isolation methods examined in this study is displayed in Table 4.2 and critically analysed in Figure 4.5.

The protease solution in method 1 utilised a combination of collagenase and trypsin and resulted in a digestate containing low cell yields and large populations of dead cells (>25%). In isolation method 2, we switched to a collagenase with greater enzymatic activity and suspended the use of potentially harmful trypsin in order to improve cell yields and lower cell death. Therefore, Collagenase CLS 2 (Worthington Biochemical), with enzymatic activity of 278 U/mg Mandl, was replaced with Collagenase D (Roche Applied Science), with an enzymatic activity of 432 U/mg Mandl. For comparison purposes, collagenase activities reported here were converted into Mandl units (1 µmol leucine liberated from collagen in 5 hrs at 37 °C). Isolation method 2 resulted in enhanced viability, however, did not significantly increase the numbers of cells isolated. To improve upon this result, we increased the concentration of this collagenase D solution from 0.25% to 0.60% (w/v), in hopes of increasing cell yields. As hypothesized, isolation method 3 resulted in more than doubling cell yields. However, this increased collagenase concentration caused a significant amount of cell death (>80%) during the isolation process regardless of incubation time. For isolation method 4, we reduced the concentration of collagenase D to 0.3% and added 0.30 % (w/v) of the less proteolytic, collagenase Type I (Gibco, Invitrogen, UK), to this solution.
### Table 4.2 Human periosteal explant digestion methods and results

<table>
<thead>
<tr>
<th>Digestion method</th>
<th>Donors</th>
<th>Wet weight mg</th>
<th>Digestion time hrs</th>
<th>Isolation Yield total cells</th>
<th>Isolation rate cells/hr</th>
<th>Dead cells (% of total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>68 (f)</td>
<td>338</td>
<td>3.5</td>
<td>161,000</td>
<td>46,000</td>
<td>46,000 (28.6%)</td>
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<tr>
<td></td>
<td>58 (f)</td>
<td>331</td>
<td>3.5</td>
<td>120,000</td>
<td>34,285</td>
<td>30,000 (25.0%)</td>
</tr>
<tr>
<td>2</td>
<td>30 (m)</td>
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<td>283,000</td>
<td>94,333</td>
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<tr>
<td></td>
<td>84 (f)</td>
<td>513</td>
<td>3.0</td>
<td>210,000</td>
<td>70,000</td>
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</tr>
<tr>
<td>3</td>
<td>56 (m)</td>
<td>480</td>
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<td>720,000</td>
<td>240,000</td>
<td>620,000 (86.1%)</td>
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<tr>
<td></td>
<td>64 (f)</td>
<td>440</td>
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<td>256,000</td>
<td>430,000 (84.0%)</td>
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<tr>
<td>4</td>
<td>43 (f)</td>
<td>615</td>
<td>4.5</td>
<td>630,000</td>
<td>140,000</td>
<td>15,500 (11.1%)</td>
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<tr>
<td></td>
<td>31 (m)</td>
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<tr>
<td></td>
<td>74 (f)</td>
<td>421</td>
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<td>710,000</td>
<td>157,778</td>
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<tr>
<td></td>
<td>62 (m)</td>
<td>628</td>
<td>4.5</td>
<td>666,667</td>
<td>148,148</td>
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</tr>
<tr>
<td></td>
<td>72 (f)</td>
<td>357</td>
<td>5.0</td>
<td>810,000</td>
<td>162,000</td>
<td>142,000 (17.5%)</td>
</tr>
<tr>
<td></td>
<td>25 (f)</td>
<td>431</td>
<td>8.0</td>
<td>1,450,000</td>
<td>181,250</td>
<td>980,000 (67.5%)</td>
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<tr>
<td></td>
<td>39 (m)</td>
<td>660</td>
<td>7.5</td>
<td>1,230,000</td>
<td>164,000</td>
<td>733,200 (59.6%)</td>
</tr>
</tbody>
</table>
In addition we added 5 mM CaCl$_2$ (molecular biology grade, Sigma, Poole, UK) to EBSS, as > 5mM of Ca$^{2+}$ ions are necessary for adequate collagenase activation. EBSS alone only contains 2.4 mM CaCl$_2$ and thus requires supplementation. The resulting collagenase cocktail (pH= 7.29), utilised in method 4, allowed for increased cell yields while limiting cell death in incubations of 4.5 hrs (circled in Fig 1). After 4.5 hrs, cell death and total cell yields are found to increase proportionately with time in solution. Long incubation times of 7.5-8.0 hrs are found to result in the complete disaggregation of periosteal explants and led to higher cell yields and an equally high level of cell death. Taken together, this data showed that isolation method 4 combined with incubation times of 4.5 hrs provided the optimal environment for the release of large populations of viable cells from human periosteal explants (Figure 4.5). The donor cells isolated using this optimised method can now be placed into monolayer culture and expanded for future characterisation studies.

**Figure 4.5** Plot of periosteal cell isolation results from 13 human donors. To determine the efficacy of each of the 4 enzymatic digestion methods, the data is categorised according to percentage viability and total cell yield. Isolation method 4 at 4.5 hrs incubation represents the optimal conditions for isolating the largest numbers of viable cells (circled data).

### 4.2.3.2 Effects of culture media on periosteal cells (experiment II)

Visual assessment of isolated periosteal cells from both enzymatic digestion and explant migration cultures reveal a faster rate of proliferation and enhanced cell viability in early passage cells cultured in growth media 2 (Figure 4.6). Representative images of cells cultured in growth media 1 show a large proportion of the cells to be round, floating “dead” cells and small, non-proliferating, attached cells (Figure 4.6A, B, C, D). Due to this unsuitable environment, cultures propagated in growth media 1 never reached confluence and did not survive past passage 2. This is compared to cultures propagated in growth media 2, which reached confluence between 7-12 days, and were
cultured for more than 20 passages without arrested growth (see Chapter 5 for results). To further highlight the superiority of growth media 2 over growth media 1, the unattached cells found in the growth media 1 culture supernatant were collected and recultured in growth media 2. After 24 hours in growth media 2, the cells attached and spread on the culture plate and subsequently reached confluence after 7 days in culture.

To further investigate the impact of culture conditions on periosteal cell cultures, we compared the presence of numerous cell subpopulations in cells grown in both growth media. The cocktail of cell specific mAbs used in this flow cytometry study was selected to identify cell types of interest; namely progenitor cells (Stro-1+/ALP-), haematopoietic stem cells (CD34+), preosteoblasts (Stro-1+/ALP+), and osteoblasts (ALP+/Stro-1-). As shown in Table 4.3, periosteal cells cultured in growth medium 1 contain dramatically smaller populations (≥ 50% less in each case) of the cell types investigated as compared to cells grown in medium 2. The capacity to propagate each cell subpopulation during subculture is integral to our ability to fully and extensively characterise isolated periosteal cells in future studies. Therefore, it is observed that growth medium 2 provided optimal culture conditions to allow for the expansion of heterogeneous cell types in monolayer. A complete analysis of these specific cell types and the long-term effects of subcultivation on their growth and differentiation is demonstrated in Chapter 5.
Table 4.3 Summary of flow cytometry data showing the effect of the different media conditions on the presence of distinct cell populations within passage 2 human periosteal cells isolated from a 38 yr old male. Data represents the number of cells staining positive for 5 different cell type/lineage specific mAb(s) (top line) as a percentage of the total cell population examined. To establish positive events from background staining, individually matched isotype controls were used for each mAb. More than 30,000 total events were recorded to determine the percentages in the table.

4.2.3.3 Effects of plating density on expansion of periosteal cells (experiment III)

To determine the short-term proliferation rates and examine the impact of the microcellular environment on the growth capabilities of heterogeneous periosteal cells, cultures were plated at four different densities. Passage 2 periosteal cells isolated from two different donors were plated at densities ranging from low (10 cells/cm²) to high (5000 cells/cm²) and cultured for 12 days under identical conditions. Calculations of total cell yields and fold expansion rates (Figure 4.7) shows that periosteal cell growth rates and behaviour are extremely sensitive to plating density in cultures derived from both donors. Cells (31 yr old male) plated at densities of 5000 cells/cm² and 10 cells/cm² both expanded approximately 5-fold in 12 days. However, cells plated at 5000 cells/cm² yielded 630,000 cells per 25 cm² culture plate, whereas cells plated at 10 cells/cm² yielded only 1300 cells. Expansion rates of cells from the other donor (39 yr old male) displayed similar results. Densities of 5000 cells/cm² showed a 7-fold increase in cell number over 12 days, yielding over 900,000 cells. This fold increase was slightly higher than the remaining densities, 500 cells/cm², 50 cells/cm², and 10 cells/cm², which saw 6-, 2-, and 4-fold increases respectively.
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Figure 4.7 This figure shows the relationship between initial plating density and expansion of periosteal cells. Passage 2 periosteal cells from 2 donors were plated in 25 cm² culture flasks at 10, 50, 500, 5000 cells/cm². The cells were harvested and counted every 2 days for 12 days. Total cell yield (A and C) and fold increase (B and D) per flask are shown for each donor. Data are expressed as mean ± standard deviation (n=3).

In both donors, the peak doubling rate per day for the three lowest plating densities (10, 50, 1000 cells/cm²) was between day 2 and day 4 (Figure 4.8). Cell doublings per day (CD) was calculated using equation 4.1, where $N_f$ = final cells counted and $N_i$ = initial cells seeded.

$$CD = \frac{24hrs}{48hrs} \times \frac{\ln(2)}{\ln\left(\frac{N_f}{N_i}\right)}$$

The fastest doubling rate was noted in cultures seeded at 10 cells/cm² at day 2, with an average doubling time of 24 hrs for both patients. This initial burst of cell proliferation found in the 3 lowest seeding densities was subsequently followed by periods of arrested growth, seen as periods of 0 population doublings and decreased cell numbers. Comparatively, cultures seeded at high density (5000 cells/cm²) expanded at a consistent rate until confluence, which occurred between 10 and 12 days in both donors. Although their peak doubling rates were found to be low, high density (5000 cells/cm²) cultures maintained the most consistent expansion and doubling rates during the 12 day growth period. Due to the increased cell yields and consistent and reproducible growth over a single passage, the plating density of 5000 cells/cm² was selected as the optimal density for our periosteal cell culture model.
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4.2.3.4 Colony forming efficiency (experiment IV)

Colony-forming-units fibroblasts (CFUs) were initially referred to in seminal work by Friedenstein and colleagues, who demonstrated the *in-vitro* growth of adherent colonies of cells morphologically resembling fibroblasts derived from explants of bone marrow (222;392;393). These colonies are formed by the rapid proliferation of stem-cell like precursors from non-haematopoietic tissues and as such these cells are often referred to as mesenchymal stem cells or mesenchymal progenitor cells. Since the colony forming efficiency of cultures is highly dependent on the microenvironment (225;388), a range of seeding densities (500 to $10^6$ cells per 78 cm$^2$ Petri-dish) was preliminarily examined to establish the optimal cell density for cell growth and distinct colony formation in our periosteal cell culture system. It is well known that adult stem cells are highly sensitive to plating density, and early progenitors are rapidly lost if the cultures are grown to confluency (224). The results demonstrate that periosteal cells seeded at 10,000 cells/Petri dish and cultured for 12 days produce the greatest number of distinct colonies (>1 mm) and did not suffer from colony growth inhibition as the higher seeding densities did (Figure 4.9). Indeed, the optimal conditions, established here, allows for the expansion of periosteal progenitor cell populations, which is important for the development of cell-based therapies. More comprehensive functional colony forming efficiency studies are demonstrated in Chapter 5 across multiple passages using cells isolated from multiple patients.

![Figure 4.8](image-url) Figure shows the effect of plating density on periosteal cell doublings per day. Passage 2 periosteal cells from two donors were plated in 25 cm$^2$ culture flasks at densities of 10, 50, 500, 5000 cells/cm$^2$. Every other day for 12 days, cells were harvested and counted to calculate cell doublings.
Figure 4.9 Crystal violet staining (0.5% w/v) of passage 1 periosteal cell colonies seeded at varying densities and cultured for periods of 7 or 12 days. To highlight the effects of seeding density and time in culture on colony formation, representative images of the stained dishes are displayed and the number of colonies quantified for each respective condition (n=3).
4.2.4 Conclusion

To consider human periosteal cells as a viable cell source for cell and tissue therapies they need to possess the ability to be expanded in culture relatively quickly under conditions in which they retain their potential to proliferate and differentiate into multiple cell lineages. However, as seen in MSC cultures, as the cells are expanded they lose their proliferative and multilineage differentiation capacity; both of which are sensitive to cellular microenvironment, particularly plating density (224;225;228). It is apparent that a large number of variables and parameters must be considered in expanding adult stem cells for clinical purposes. In human periosteum, which is composed of two-distinct anatomical layers, cellular and extracellular heterogeneities are intrinsic to the tissue. These in-vivo heterogeneities are further complicated in-vitro by age-, donor site-, species-, explant size-, and isolation method- specific variations in cell characteristics and differentiation potential in-vitro (359;368;384;386;387;394). Therefore, the development and optimisation of cell isolation and culture methods, presented here, was fundamental to the production of a simple, reproducible and robust human periosteal culture model to study and influence cell behaviour and function.
Chapter 5

In-vitro Expansion and Characterisation of Human Periosteal Cells

5.1 Introduction

The periosteum is a specialised connective tissue forming the outer lining of long bones and is intimately involved in driving the cell differentiation processes of bone development and repair (47;395;396). The periosteum itself is comprised of two discrete layers; an outer fibrous layer and an inner juxta-osseous cambial layer (29). The outer fibrous layer appears to be composed of fibroblastic cells immersed in a matrix of collagen and elastin fibres, along with a distinct nerve and microvascular network (39;397). The inner cambium layer is highly cellular and contains a variety of different cell types including fibroblasts, osteoblasts, and osteochondral precursor cells (398). Mesenchymal precursor cells in the periosteum differentiate into neochondrocytes to produce cartilage tissue during embryogenesis and contribute to bone apposition during intramembraneous ossification by differentiating into osteoblasts (399).

A number of studies have aimed to utilize this potential, using either periosteal explants (191;361;387) or isolated cells (384;390;400) to generate bone (30;401) or cartilage (31;361;362;402;403). Periosteal cell phenotype and properties are influenced by numerous factors, including sex steroids (404;405), endogeneous growth factors (406;407), and mechanical loading environment in vivo (408;409) and isolation (386), culture methods (30;190;201;361;376), and the donor site (359) in vitro. While the osteo- and chondrogenic capacity of periosteal cell cultures has been investigated using functional assays in growth-factor supplemented media, little quantitative work has been performed on the initial phenotype of isolated periosteal cells and their behaviour during prolonged culture. Examination of these cells as reported here has numerous benefits. New information regarding putative undifferentiated mesenchymal precursor cells in the periosteum could aid in understanding bone formation and fracture healing in vivo. Additionally, for successful cell-based tissue engineering and regenerative medicine strategies, changes in growth potential and protein and gene expression during expansion need to well-studied and controlled. Thus, the aims of this study were to isolate and expand periosteal cells derived from adult human donors using a simple, well-defined culture technique that uses enzymatically liberated cells, and to characterise changes in growth kinetics, morphology and phenotype in the heterogeneous cell populations during subculture.
Here we obtained periosteal tissue from a number of patients undergoing scheduled knee surgery. Cells were then enzymatically liberated and expanded in monolayer to analyze proliferation, cell division, and morphology. To evaluate the phenotypic changes that occur in culture, we performed dual-colour flow cytometry and real-time reverse transcriptase polymerase chain reaction (RT-PCR) to determine the expression patterns of target proteins and genes using a panel of monoclonal antibodies and genetic probes (Table 5.1); indicative of haematopoietic cell types, osteoblasts, chondrocytes and mesenchymal stem cells. The presence of additional cell types, such as primitive stem cells, smooth muscle cells, and adipocytes was also investigated. Following the seminal work of Friedenstein and colleagues (222;391;392), the presence of highly proliferative, progenitor cell phenotypes, were further examined by staining and counting colony forming units (CFUs). The osteogenic potential of the Stro-1\textsuperscript{+} subpopulation was confirmed by selectively depleting the cell population of Stro-1\textsuperscript{+} cells, and comparing the capacity of the two populations to form bone.

5.2 Materials and Methods

5.2.1 Harvest of periosteal tissue and isolation of cells

This study was approved by the NHS ethical committee (project RREC2700). Periosteal tissue specimens were obtained from patients providing informed consent (25-74 years old) undergoing elective knee surgery at The Chelsea and Westminster Hospital, London. Explants were harvested from the proximal tibia using a periosteal elevator, and transported in Earl’s Balanced Salt Solution at 37°C. Explants were weighed, finely minced, and enzymatically digested with 3 mg/ml Collagenase D (Roche Applied Scienc, West Sussex, UK) and 3 mg/ml collagenase type II (Sigma-Aldrich, Poole, UK) and 5 mM CaCl\textsubscript{2} in growth medium (high glucose Dulbecco’s Modified Eagle Medium containing 10% v/v foetal bovine serum, 1% v/v L-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin [all from Invitrogen, Paisley, UK]). After incubation at 37 °C for 4.5 hours, the cell suspension was washed, passed through a 70µm cell strainer (Falcon, Becton Dickinson, Oxford UK), and counted. A proportion of the freshly isolated cells were analysed using flow cytometry, the rest being placed into culture and allowed to attach to tissue culture plastic for a period of four days. The media was changed after 2 days to remove non-adherent cells and the attached cells were further cultured as passage 0 until confluence. All characterisation experiments were carried out using isolated periosteal cells serially expanded from passage 0 through to at least passage 15.

5.2.2 Cell culture

To investigate the growth characteristics of periosteal cultures, cells isolated from 8 donors aged 25-74 yrs were separately expanded in monolayer in growth medium. Upon confluence, adherent periosteal cells were harvested with 0.25% trypsin and 1mM EDTA for 5 minutes at 37°C, and
replated at the optimised density of 5000 cells/cm² in a T75 flask containing 15 mls of growth medium; this process was repeated every 7-14 days for at least 15 passages. Viable cell number was determined using trypan blue dye exclusion and a haemocytometer. Based on the cell counts cumulative cell number, population doublings, and fold increase were calculated. Cultures were terminated after seeded cells failed to double for three consecutive passages.

5.2.3 Cell cycle analysis

To further characterise the growth characteristics and study the effects of passaging on proliferation rates in human periosteal cell cultures, cell cycle analysis was performed to determine the percentage of quiescent (G₀) and proliferating (G₁, S, G₂/M) cells. Periosteal cells at passages 1, 7, and 15 from a young (25 yrs female) and old (62 yrs female) donor were plated at 5000 cells/cm² in T-25 tissue culture flasks (n=2) (Corning, Fisher Scientific, UK) and cultured for a period of 12 days in standard growth medium. Every 2 days, cells were collected by trypsinisation, counted using a haemocytometer, and fixed in methanol. The fixation method employed here was adapted from procedures optimised by Jacobberger et al for intracellular antigen staining (410;411). This method allows for the long-term preservation of cell morphology without covalently modifying cellular DNA, RNA, or proteins. Additionally, the use of methanol results in significantly less cell debris and aggregates. Briefly, 90% (v/v) methanol, pre-chilled to -20 °C, was added drop-wise to the cell pellet during gentle agitation on a vortex. The cells were stored at -20°C until cells from all time points and donors were collected. For immunofluorescent staining, cells in fixative were washed 2x-times in flow buffer containing 2 mg/ml bovine serum albumin (Sigma, Poole, UK) and 0.1% Sodium Azide (Sigma, Poole, UK) in PBS. Cells were thoroughly resuspended in 100 µl flow buffer at densities between 1.0 - 4.0 x 10⁶ cells/ml. Cells were then incubated with FITC-conjugated monoclonal antibody against proliferation marker Ki-67 (clone B56; BD Pharmingen, UK) at a concentration of 20µl/10⁶ cells for 30 min at RT. Isotype controls were stained in parallel. After rinsing, cells were counterstained in 100 µl of 0.1% (v/v) Triton X-100 in PBS containing 20 µg/ml PI (propidium iodide, Invitrogen, UK), and 2 mg/ml RNase A (Sigma, Poole, UK) for 20 minutes at 37 °C. DNA content and expression of Ki-67 were measured on a FACS Calibur instrument (BD Biosciences, Oxford, UK), where at least 20,000 events were collected per sample. The resulting data was analysed using a combination of FlowJo (Tree Star Inc, Ashland, USA) and WinMDI software (The Scripps Institute, La Jolla, CA, USA). DNA content was assigned to G₀/G₁, S, or G₂/M phases based on the method of Ormerod (412).

5.2.4 Evaluation of cell morphology

Changes in cell morphology were progressively examined in passage 0-20 human periosteal cultures derived from 6 donors aged 31-64 years. At each respective passage, photomicrographs were taken randomly of cell areas that were roughly 60-70% confluent under a phase contrast
microscope at 10x magnification. At this level of confluence, periosteal cells were found to be in
the log-phase of growth. An important experimental condition, considering that confluence level is
known to influence the morphology and frequency of cell populations in heterogeneous cultures
(226). Post-digital image processing and measurement were performed using ImageJ software
(NIH, Frederick, USA). From the projected cell area and perimeter measurements it was possible
to determine the cell shape factor coefficient (Sfc). As described by Allen et al (413), Sfc is a
measure of cell shape and is given by the equation:

$$Sfc = \frac{4\pi \times Area}{Perimeter^2}$$

(5.1)

By definition, an Sfc value of 1 corresponds to a perfect circle,
whereas a straight line has a value of 0. Thus, the more elongated the cell
shape is, the lower the Sfc value. The number (n) of cells measured per passage was determined in
each culture using the formula:

$$n = \left[ \frac{z_{\alpha/2} \times \sigma}{E} \right]^2$$

(5.2)

where,

$z_{\alpha/2}$ is the positive $z$ value that is at the vertical boundary for the area of $\alpha/2$ in the right tail of the
standard normal distribution.

$\sigma$ is the population standard deviation.

$n$ is the sample size.

This formula was used to determine the sample size necessary to establish, with a confidence of
95% ($z = 1.96$), mean cell areas within an error margin of $\pm 10\%$. Samples sizes ranging from 50 to
100 single cell measurements were used to statistically determine changes due to passaging and
also compare morphologies across donors. In all cases mitotic cells were ignored.

### 5.2.5 Phenotypic analysis

#### 5.2.5.1 Immunofluorescence

Early and late passage periosteeal cells from two donors were grown on four-well chamber slides
(Bectin Dickinson Falcon) at 5,000 cells/well for one week. Cultures were washed twice with
0.01M, pH 7.4 phosphate buffered saline (PBS), then fixed and permeabilized in cold acetone:
methanol (1:1 v/v) for 20 minutes at 4 °C. Cells were then washed with PBS and blocked with PBS containing 2% (v/v) BSA, 0.1% (v/v) normal mouse serum, and 0.1% (v/v) foetal bovine serum for 45 minutes at room temperature (RT). After blocking, the cells were stained for 30 minutes at 37 °C with the primary antibodies anti-α-smooth muscle actin (10 µg/ml) and phycoerythrin conjugated anti-SSEA-1 (5 µg/ml) (Both R&D systems). Cells stained for α-smooth muscle actin were further incubated for 30 minutes at 37 °C with a secondary goat F(ab’)2-anti-mouse IgG-FITC (5 µg/ml) (R&D systems). Both incubations were followed by thorough washing with PBS. The chambers were then detached from the culture slide, and coverslips were mounted with Vectashield containing DAPI (Vectashield). Images were taken using an Olympus BX-51 reflected fluorescence microscope (Olympus UK LTD., London) fitted with an Olympus DP070 digital camera and analysed using Olympus DP Controller software (Olympus Imaging, London, UK). As a positive control for SSEA-1 reactivity, passage 15 mouse embryonic stem cells (mES) were cultured for 4 days on gelatin (0.1% w/v) coated chamber slides in the presence of leukaemia inhibitory factor and stained in parallel. Secondary antibody controls, devoid of primary antibodies, were processed simultaneously using the same protocol and were found to be negative for staining. To determine the percentage of cells expressing α-smooth muscle actin, positively stained cells were counted per chamber well and divided by the total number of cells per well, which was calculated using a haemocytometer (n=4).

5.2.5.2 Oil-Red O staining

To demonstrate the presence of adipocytes (figure S2B), Oil-Red O staining was employed using early and late passage periosteal cells grown for ten days in complete medium. The cells were then fixed in 4% w/v paraformaldehyde for 20 min and stained with filtered Oil-Red O solution (6 parts 0.5% v/v Oil-Red O stock: 4 parts dH2O) for one hour at room temperature. Nuclei were counterstained with Harris Haematoxylin (Sigma; Poole, UK) for 1 min. and mounted with 10% v/v glycerol in PBS. A series of washes with PBS were performed after each step. As a positive control, human epithelial lung carcinoma (A549) cells were cultured for 4 days and stained as above.

5.2.5.3 Flow Cytometry

Antibodies were tested for their specificity, titrated, and used at concentrations varying from 1-2.5 µg/10⁶ cells. Details of all monoclonal antibodies (mAbs) are given in Table 5.1, but briefly, mAbs used were raised against Stro-1, human bone/liver/kidney alkaline phosphatase (ALP), CD34, CD31 and CD45. Approximately 1-3 x 10⁵ periosteal cells of passages 0-15 were incubated in 1 ml of blocking buffer (phosphate buffered saline pH 7.4 0.01M (PBS) containing 2% (w/v) bovine serum albumin (BSA), 0.1% (v/v) foetal bovine serum, and 0.1% (v/v) normal mouse serum) (all Sigma-Aldrich) for 30 minutes at room temperature to limit non-specific binding. For direct
staining, cells were then incubated with conjugated mAbs for 45 minutes at 4°C, washed in PBS containing 2% BSA (w/v) + 0.1% (w/v) Sodium Azide (flow buffer), centrifuged, and resuspended in 300 µl of flow buffer prior to analysis. For indirect staining using primary mAb Stro-1, the cells were first incubated with the primary mAb (45 min at 4°C), washed, and incubated with a fluorescein isothiocyanate (FITC)-conjugated fragment F(ab')2 goat anti-mouse secondary Ab (Jackson ImmunoResearch, Suffolk, UK) for 45 minutes at 4°C. Matched isotypes were used as negative controls and prepared at the same concentrations as their matched primary Ab. Cell fluorescence was evaluated by flow cytometry using a FACSCalibur instrument (BD Biosciences, Oxford, UK) and data analysed using FlowJo software (Tree Star Inc, Ashland, USA). At least 20,000 events were collected per sample and percentage positive expression for each mAb was defined as the level fluorescence >99% of the isotype-matched control antibodies.

5.2.6 Real-time Reverse Transcriptase Polymerase Chain Reaction

Total RNA was extracted from monolayered periosteal cells by the addition of 10µl β-mercaptoethanol in 1ml RLT-buffer (QIAGEN, West Sussex, UK). Total RNA was isolated using the RNeasy mini kit, treated with RNase-free DNase 1 (both QIAGEN), according to the manufacturers protocol and quantified using the ND-1000 UV-Vis Spectrophotometer (Nanodrop®, Wilmington, USA). 1000ng of total RNA was reverse transcribed (RT) into cDNA and a mastermix prepared for each reaction containing; 10µl Taqman universal mastermix (Applied Biosystems, CA, US), 7µl 0.1% v/v diethylpyrocarbonate (DEPC) water (Invitrogen Ltd, Paisley, UK), 2µl extracted cDNA and 1µl Taqman® probe (Applied Biosystems, CA, USA). TaqMan® Gene Expression Assays used to amplify genes are detailed in Table 5.1, but include Runx 2, Sox 9, bone-specific ALP and Col 1A. 18S was used as a control gene. Each reaction was carried out in triplicate. The PCR reaction was initiated by a 2 minute 50°C and 10 minute 95°C step to optimise thermal cycling conditions for the ABI Prism 7700 sequence detection system (Applied Biosystems, Ca, USA) used to detect relative quantification of gene expression. This was followed by PCR amplifications performed for 40 cycles in a Corbett Rotor-Gene 6000 (Corbett Life Science, Sydney, Australia) at 95°C for 15 seconds and 60°C for 1 minute. The target signal was plotted against the number of cycles and the threshold level was set at 0.05. Comparison of all data was taken at the intercept, where sample reactions crossed this phase of amplification. Our results were correlated using the comparative C_T method (414). Fold changes in gene expression are presented as mean ± SE change relative to passage 1 cells from 3 different patients.
Table 5.1 Antibodies and Taqman™ gene probes used in this study, with details of their sources and specificities.

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<thead>
<tr>
<th>Antibodies</th>
<th>Concentration [µg/10⁶ cells]</th>
<th>Catalogue number</th>
<th>Manufacturer</th>
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<tbody>
<tr>
<td>Stro-1</td>
<td>2.5</td>
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<td>R&amp;D Systems</td>
<td>Progenitor cells</td>
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<tr>
<td>Alkaline phosphatase (ALP)</td>
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</tr>
<tr>
<td>CD34</td>
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<td>Abd Serotec</td>
<td>Haematopoietic stem cells</td>
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<tr>
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<td>Primitive stem cells</td>
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<tr>
<td>α-smooth muscle actin</td>
<td>10.0</td>
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<table>
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<tr>
<th>Taqman™ gene probes (Applied Biosystems)</th>
<th>Gene name</th>
<th>Symbol</th>
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<th>Accession number</th>
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<tr>
<td>Alkaline phosphatase</td>
<td>ALP</td>
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<td>NM000478</td>
<td>Chr. 1 21769385 - 21772879</td>
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<tr>
<td>SRY (sex determining region-y)-box 9</td>
<td>Sox9</td>
<td>Hs00165814</td>
<td>NM000346</td>
<td>Chr. 17 67,630,455 - 67,634,156</td>
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<tr>
<td>Runt-related transcription factor 2</td>
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<td>Hs00298328</td>
<td>NM001015051</td>
<td>Chr. 6 45,622,542 - 45,626,797</td>
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<tr>
<td>Bone gamma-carboxyglutamate protein</td>
<td>Osteocalcin</td>
<td>Hs01587813</td>
<td>NM199173</td>
<td>Chr.1 154,478,629 - 154,479,006</td>
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<tr>
<td>Platelet/endothelial cell adhesion molecule</td>
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5.2.7 Colony forming unit assay

A range of seeding densities (500 to $10^6$ cells per 78 cm$^2$ Petri-dish) was examined previously to establish the optimal cell density for cell growth and distinct colony formation (Chapter 4). Based on these results, periosteal cells from 4 donors were seeded at 10,000 cells/dish and cultured in growth medium for 12 days. After 12 days, the cells were stained with 0.5% crystal violet in methanol for 5 minutes, rinsed twice in distilled water and the number of colonies per dish counted by 2 independent observers. Based on previously described criteria (391) colonies < 1 mm in diameter (~50 cells) and faintly stained colonies were discounted. Mean values for each passage for each patient were derived from three Petri dishes. Cells of passages 0-5, 7, 10 and 12 from four different patients were used.

5.2.8 Cell Sorting

Stro-1$^+$ cells were selected from passage 10 periosteal cells (32 yr old male) using a cell-sorting kit utilising Streptavidin coated magnetic beads (Biotin binder kit, Invitrogen, UK) according to manufacturer’s instructions. Briefly, $10^7$ periosteal cells were incubated with the primary mAb (anti-human Stro-1, R&D systems, UK) at a concentration on 2.5 µg/10$^6$ cells for 1 hr at 4°C. Following a wash in 2% (w/v) BSA buffer, cells were incubated with Biotin-SP-conjugated fragment F(ab’)$^2$ goat anti-mouse secondary antibody (Jackson ImmunoResearch, Suffolk, UK) at a concentration of 1.5 µg/10$^6$ cells for 45 min at 4°C. After washing, biotin-conjugated magnetic beads were added to the cell suspension and incubated for 20min. The bead-cell suspension was placed into a magnetic field and Stro-1$^+$ cells collected. The Stro-1$^+$ population was counted using a haemocytometer and the viability determined using the trypan blue dye exclusion assay. Both the viability and purity of the sorted population were determined to be >98%.

5.2.9 In-vitro mineralisation

To study the differential osteogenic potential of late passage periosteal cells, both unsorted (total population) and sorted (Stro-1$^-$) cell populations were seeded at 10,000 cells/cm$^2$ in 48-well plates (n=6) and induced to mineralise. Cells were maintained at 37 °C and 5% CO$_2$ in standard growth medium until confluence, when mineralization was induced by addition of osteogenic supplements, 10 mM β-glycerophosphate, 50 mg/L L-ascorbate, and 10 nM dexamethasone (all Sigma-Aldrich, Poole, UK). Alizarin Red was used to assess the formation of mineralised matrix after 28 days. Cell cultures were rinsed with PBS and fixed in 4% formaldehyde in PBS for 15 min at RT, rinsed with distilled water, then stained with 1 ml of 40 mM Alizarin Red Stain (pH 4.1) per well for 30 minutes at RT with gentle shaking. After aspiration of unincorporated dye, the wells were washed 5-times with dh$_2$O and finally rinsed in a water bath. Images of stained nodules were immediately taken using both phase-contrast and fluorescent microscopy at 10x magnification (DP-70 Olympus
Microscope, Watford, UK) and the number of fluorescent nodules per well counted. To quantify the amount of mineralised matrix per well we modified a dye extraction protocol developed previously (415). Briefly, 800 µl of 10% (v/v) acetic acid was added to each well, and the plate incubated at RT for 30 min on a plate shaker. The monolayer was scraped from the well using a pipette tip and transferred to a 1.5-ml microcentrifuge tube with a wide-mouth pipette. After vortexing for 30 seconds, the slurry was overlaid with 500 µl mineral oil (Sigma-Aldrich), heated to 85 °C for 10 min, and transferred to ice for 5 min. The slurry was then centrifuged at 20,000g for 15 min and 500 µl of the supernatant removed to a new 1.5-ml microcentrifuge tube. Then 200 µl of 10% (v/v) ammonium hydroxide was added to neutralise the acid. Aliquots of 150 µl of the supernatant were read in triplicate at 405 nm in a 96-well plate using opaque-walled, transparent-bottomed plates (Fisher Lifesciences, Loughborough, UK).

5.2.10 DNA assay

To normalise mineralization data to average cell number per well, total DNA was determined in sorted in unsorted periosteal cell populations with the use of Hoechst 33258 (Sigma, Poole, UK) dye. DNA was extracted from cell lysed by one freeze/thaw cycle (30min at -80°C and 20min at 37 °C). Hoechst 33258 was added to the lysate and allowed to react for 5 minutes in the dark. Fluorescence was measured using a fluorescent plate reader set to 360nm (Excitation wavelength) and 460nm (Emission wavelength). To quantitate the amount of DNA per well, Calf Thymus DNA was used (Sigma, Poole, UK) at concentrations of 0, 0.78, 1.56, 3.13, 6.25, 12.5, 25 and 50ug/mL in TNE buffer (pH7.4) to determine a standard curve.

5.2.11 Statistics

SigmaStat and SPSS statistical software packages were used to analyse the data. Unless otherwise stated all data is presented as mean ± SD. A two-way analysis of variance (ANOVA) was applied to flow cytometry, quantitative RT-PCR, and CFU data with a Tukey post-hoc test. To identify statistical differences in morphology and mineralization data, the non-parametric Mann-Whitney U test was performed. In all cases, p-values < 0.05 were deemed statistically significant. For morphology data the following symbols were used to display significant differences (p<0.05) with (*) passage 0, (♦) passage 1, (†) passage 2, (□) passage 5, ($) passage 10, (‡) passage 15, and (○) passage 20 cells.

Linear regression analysis was performed on cell morphology and proliferation data to find the Pearson’s product-moment correlation coefficient or, in the instances of unequal variances between variables, Spearman rank correlation coefficient. A one-way ANOVA was used to establish the statistical significance of the Pearson’s product-moment correlation between the two variables.
Whereas, a two-tailed student t-test was used to statistically compare Spearman's rank correlations. All correlations were deemed statistically significant at $p$-values < 0.05.

A Pottoff analysis was used to test the statistical homogeneity of growth rates (slopes of population doubling/culture time) between donor periosteal cultures. The Pothoff method is essentially an Analysis of Covariance (ANCOVA) that utilizes a multiple regression model of the form: $Y = a + b_1C + b_2G + b_3C*G$, where $Y$ is the criterion variable (population doublings), $C$ is the continuously distributed variable (culture time), $G$ is the grouping variable (donor) and $C*G$ is the interaction between $C$ and $G$. To test the null hypothesis that the slope for predicting growth rates is the same in each donor, we compared the full regression model (all variables) to the reduced model (no interaction term) to determine the partial $F$-statistics between donors.

$$F = \frac{SS_{\text{full}} - SS_{\text{reduced}}}{(f-r)MSE_{\text{full}}}$$  \hspace{1cm} (5.3)

In this model, $SS$ is the sum of squares for each model, $(f-r)$ is the degrees of freedom, and $MSE$ is the mean square that is equivalent to the pooled error variance. From the partial $F$-statistic the associated $p$-value was determined and deemed significant at $p<0.05$. 


Chapter 5: *In-vitro* Expansion and Characterisation of Human Periosteal Cells

5.3 Results

5.3.1 Cell culture

The in-vitro growth patterns of periosteal cells during culture expansion were analysed to assess their long-term self-renewal capacity. Periosteal cells were expanded ex vivo by successive cycles of trypsination, seeding at 5000 cells/cm², and culturing for periods of 7-14 days. Upon plating, periosteal cells experienced a lag phase of 1-4 days before entering the log phase of growth, where cells proliferated with an average population doubling time of 72.0 ± 9.4 hrs over 15 passages (Figure 5.1A & B and Figure 5.2A & B). This growth period was followed by a confluent growth-arrested phase. Under these conditions, there was an average 2.8-fold increase in cell number after each passage. However, across all donors early passage cultures demonstrated heightened growth rates compared to late passage cultures. Early passage cultures (passage 0-5) experienced a 3.7 ± 1.7-fold increase in cell number after each passage compared to cultures at passage 6-10 and 11-15, which experienced lower increases of 2.6 ± 0.8 - and 2.5 ± 1.0 -fold respectively.

The growth curves of periosteal cells from donors of various ages were linear for > 20 population doublings, with no progressive donor age-associated decline in their growth rates (Figure 5.1B). Statistical analysis of periosteal cell growth profiles, however, did reveal significant differences in cell proliferation rates between donors (Figure 5.1B & C). Regression lines fit to each culture expansion profile were used to identify Pearson’s product-moment correlation coefficients (R-values) and growth rates (slope ± standard error) for each donor-derived periosteal cell culture. This information was then used to conduct a Pothoff analysis (ANCOVA) of slope homogeneity between donors to identify differences in cell proliferation (Figure 5.1C). Cultures derived from a 59 yr old female demonstrated growth rates that were significantly slower than all other donors examined (p<0.05). Whereas, cultures derived from a 74 yr old female and a 52 yr old male demonstrated growth rates that were significantly faster than all other donors. The remaining donors had similar growth rates between 0.125 - 0.164 PD/day.
Figure 5.1 Growth characteristics of human periosteal cells from 8 donors of different ages. Growth kinetics are displayed as accumulated cell number (A) and total population doublings (B) calculated from passage 0. Cells were plated at 5,000 cells/cm² and expanded in monolayer with serial passages upon confluence. In all donors, except 59 yr old female, growth curves were linear for over 20 population doublings. To assess donor dependent differences in growth, regression lines were fit to growth data (B) to determine growth rates (slope), standard errors, and pearson’s product-moment coefficients (C). From this information an analysis of covariance was used to determine statistical differences in growth rates (slopes) between donors.
5.3.2 Cell cycle analysis

To more accurately determine the fraction of actively dividing cells during expansion, detailed cell cycle analysis of proliferating and quiescent cell populations was performed in periosteal cultures isolated from 2 donors using flow cytometric analysis combining FITC-labelled anti-Ki-67 antibodies and PI DNA staining. Ki-67 is a nuclear antigen present exclusively in proliferating cells, including cells in the G₁, S, and G₂ phases of the cell division cycle as well as mitosis (416). Quiescent or resting cells in the G₀ phase do not express the Ki-67 antigen and thus represents an excellent operational marker to determine the growth fraction of a given cell population. The multi-step analytical method used to analyse the DNA cell cycles and calculate the percentages of cells in G₀ and G₁ is described in full in Appendix B.

DNA cell cycle analysis revealed that a vast majority (>75%) of periosteal cells from both donors were in the G₀/G₁ phases of the cycle. In further separating proliferating and non-proliferating populations, we found 76.9 ± 12.0% of the cells in the quiescent, G₀, phase of the cell cycle at each passage (Figure 5.2E & F). The proportion of proliferating, Ki-67⁺, cells were seen to peak between 2-6 days after seeding in culture and decrease non-monotonically thereafter (Figure 5.2C & D). This expression profile correlated with the log and confluent phases of growth found in cultures derived from both donors and highlights the influence of confluence level on growth rates (Figure 5.2A & B). Additionally, this temporal pattern of Ki-67⁺ expression did not change drastically with increasing passage number passaging and was markedly similar between donors.
Figure 5.2 Growth pattern and cell cycle analysis of human periosteal cells in culture. To identify the growth fraction and study the effects of subculture, passage 1, 7, and 15 periosteal cells from two different donors were grown in monolayer for a period of 12 days and stained with propidium iodide and proliferation marker Ki-67. Cell counts were performed every other day to determine the changes in growth profiles over 12 days (A, B). Dual staining periosteal cells with PI and Ki-67 allowed for the examination of cell cycle distribution and the identification of proliferating (Ki-67+) (C, D) and quiescent (G0) (E, F) cell populations. Each data point on figures C, D, E, and F represents the percentage of total cells analysed using flow cytometry (> 20,000 events).

5.3.3 Cell morphology

Periosteal cells obtained from enzymatic dissociation and maintained in adherent culture consisted of a morphologically heterogeneous population. Two major subpopulations were observed (Figure 5.3A): 1) small cells with an elongated, spindle-shape with two processes that extend in opposite directions from the cell body, and 2) large cells with a flattened, polygonal shape and a plainly visible nucleus with or without short processes. Based on these morphological classifications,
periosteal cells in early passage (P0-P2) cultures were mainly (>70%) small, spindle shaped cells. During cultivation, however, a progressive change in cell morphology was observed, with cells becoming wider and flatter with more projected podia. At passage 15, small spindle-shaped cells represented <15% of periosteal cells in cultures across all donors.

Figure 5.3 Characterisation of the cell morphology of human periosteal cells during long-term subculture. (A) Representative images taken of periosteal cultures derived from a 52 year old male are used to highlight the heterogeneous cell morphologies present across 15 passages. (B) Distribution histograms of periosteal cell areas measured digitally between passage 0-15 in cultures derived from 6 different donors. Together these data demonstrate the shift in cell morphology during culture from small spindle-shaped cells to large flat cells.
Cell size measurements reveal a definitive positive correlation between cell area and time in culture (R-values ≥ 0.88, p < 0.05), where average cell areas are found to significantly increase during expansion in each donor examined (Figure 5.4). Early passage cultures derived from each donor contain a smaller, more homogenous, distribution of cell areas, as highlighted in cell area frequency histograms in Figure 5.3B and standard deviations in the bar graphs displayed in Figure 5.4. Consequently, the heterogeneity of cell areas increased with time in culture, with the largest distributions and standard deviations in cell area found at passages 10 and 15 (Figure 5.3B and Figure 5.4).

31 yrs - male

38 yrs - male

59 yrs - female

\[
\begin{align*}
\text{31 yrs - male} & : y = 51.2x + 2102.2, R = 0.97, p\text{-value} = 0.01 \\
\text{38 yrs - male} & : y = 58.8x + 999.1, R = 0.97, p\text{-value} = 0.01 \\
\text{59 yrs - female} & : y = 62.2x + 1952.8, R = 0.99, p\text{-value} = 0.01
\end{align*}
\]
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**Figure 5.4** Characterisation of cell areas in human periosteal cell cultures from 6 donors between passages 0-20. At each passage, areas of 50-100 cells were measured at 60-70% confluence using ImageJ digital software. For each donor, cell areas (mean ± SD) are displayed as both bar graphs and scatter plots versus passage and time in culture, respectively. Bar graphs demonstrate the significant differences in cell area between two passages. Symbols represent statistically significant differences in cell area (p < 0.05) with (*) passage 0, (♦) passage 1, (†) passage 2, (§) passage 5, ($) passage 10, (‡) passage 15, and (○) passage 20 cells. Scatter plots display results of linear regression analysis, which shows a significant positive correlation between cell area and time in culture (R-values ≥ 0.89 and p < 0.05 in all donors).
Equally, this pattern of increasing morphological heterogeneity can be seen in pooled cell area data, where the mean value of cell area for each donor and all donors combined is plotted versus passage number (Figure 5.5A). Here we found very small donor-donor variations in cell areas in early passage (P0, P1, P2) cultures, however, these inter-donor variations increased substantially at later passages. In further comparing changes in cell area to donor age, we found no significant positive or negative correlations at any of the passages examined (t-test, p > 0.05) (Figure 5.5B).

To study changes in cell shape we calculated the shape factor coefficient of cells, which is a measure of cell circularity. Linear regression analysis of each of the donor derived periosteal cultures, apart from those of the 31 yr old male, showed no significant time-dependent increase or decrease in cell circularity during culture (one-way ANOVA, p > 0.05) (Appendix C). Between donors correlation coefficients were found to vary from -0.33 to 0.98.

5.3.4 Antibody titration and specificity

Due to the heterogeneous nature of periosteal cells, it was necessary to titrate each monoclonal antibody and establish their exact binding specificities using a range of different cell types. Full results and figures of this optimisation study using flow cytometry are presented in Appendix D. Importantly, Stro-1, a progenitor cell marker, showed a high level of reactivity in MG63 cells (>98%) and low to undetectable levels of expression in Saos-2 cells and primary osteoblasts and fibroblasts (<2%). This profile of tissue specificity is similar to a previous study which extensively characterised the Stro-1 antibody in a panel of normal and transformed cell types. Although Stro-1 reactivity is not restricted to bone derived cells, the screening process shown here demonstrated that the Stro-1 antibody defines a population of developmentally immature cells of the osteoblast lineage.
5.3.5 Periosteal cell phenotype

Immunocytochemical staining of passage 0 periosteal cells revealed the presence of a very small population of smooth muscle cells (<1% of total cell population) and no positive staining for primitive stem cell marker SSEA-1 (Figure 5.6A & C). Additionally, Oil-Red O staining of passage 0 cells demonstrated the presence of adipocyte-like cells (<2% of total cell population) containing large deposits of lipids (Figure 5.6B). The lack of expression of lipid deposits in late passage periosteal cells demonstrates that these cell types are quickly lost in culture. For a full display of cytochemical staining micrographs of periosteal cells from multiple passages and donors see Appendix E, Figure E.1 and E.2.

![Figure 5.6](image)

**Figure 5.6** Expression of cell specific and developmental markers in passage 0 human periosteal cells cultured on 4-well chamber slides for 1 week in complete media. Using immunofluorescence and oil-red O staining, small populations were found to be positive for α-smooth muscle actin (A) and cytoplasmic lipid droplet accumulation (B) and completely negative for primitive stem cell marker SSEA-1 (C). On immunofluorescent images nuclei were stained with DAPI (blue) to show the presence of cells.

In passage 0 cells we detected the expression of Stro-1, CD34, CD45, CD31, and ALP in all donors (Figure 5.7). Cumulative bar graphs of flow cytometry results (Figure 5.7) derived from fluorescence dot-plots (Appendix E, Figure E.3) revealed that ALP⁺ and CD34⁺ cells represented the two largest populations within freshly isolated periosteal cells. Together they constituted 27.9 ± 4.5% and 26.6 ± 6.1% (mean ± SE) of the total cell population, respectively (Figure 5.7). Antibodies for CD31, an endothelial cell marker, and CD45, leukocyte common antigen, demonstrated low levels of binding (<3%) in passage 0 cells. Additionally, a distinct subpopulation of granulocytes was identified within the CD45⁺ population of passage 0 cells (Appendix E, Figure E.3B).

After placing the isolated periosteal cells into culture and allowing them to adhere and proliferate for periods of 7-12 days (passage one), we found a significant decrease in the expression of CD34⁺ cells to 5.3 ± 1.9% and a complete loss of CD31⁺ and CD45⁺ positive cell types. The largest cell population over 15 passages was Stro-1⁺/ALP⁺, which was significantly higher than all other cell types ($p<0.001$, 2-way ANOVA).
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Figure 5.7 Flow cytometry results detailing the phenotypic heterogeneity and donor-donor variations in antigen expression levels on freshly isolated, passage 0, human periosteal cells. Periosteal cells enzymatically isolated from 6 different donors were stained with cell specific mAbs against Stro-1, ALP, CD45, CD34, and CD31 and analysed using dual-color flow cytometry. Individual values are shown as percentage positive expression of total cells analysed (>20,000 events), with positivity defined as the level fluorescence >99% of the isotype-matched control.

The continuous passaging of human periosteal cells had little effect on the population of Stro-1+ cells, with no significant changes measured across the 15 passages (2-way ANOVA) (Figure 5.8). Stro-1+/ALP- cell populations comprised 5.4 ± 1.5% of the total cell population for all donors. Additionally, individual values only ranged from 1%-16% over the 15 passages, indicating that the exact proportion of Stro-1+/ALP- cells is donor independent for the patient samples studied.

In contrast to Stro-1+/ALP- expression, the profile of ALP+/Stro-1- populations peaked at passages 1 and 2 (2-4 weeks in culture) and declined subsequently, with the lowest frequency of ALP+ cells occurring at passage 15 (8.4 ± 2.6%). This profile of increased and then decreased ALP expression was seen in all donors (Figure 5.8). In addition, a small population of cells were also found to coexpress the ALP and Stro-1 (Stro-1+/ALP+). As with the (Stro-1+/ALP+) population, the double
positive cell population (Sto-1^+/ALP^+) was maintained throughout 15 passages in culture at consistent expression levels (5.7 ± 1.9%).

![Graph showing cell surface marker expression](image)

**Figure 5.8** Bar chart of flow cytometry data showing cell surface marker expression as a function of passage number. Mean change in expression of cell surface proteins on periosteal cells isolated from 6 patients. Values are shown as percentage positive expression of total cells analysed using flow cytometry (>20,000 events). Positivity for each antibody was defined as the level fluorescence >99% of the isotype-matched control antibodies. CD45+ and CD31+ expression are displayed as the same data set, as those antibodies were expressed by periosteal cells in almost identical numbers and temporal profiles.

### 5.3.6 Real-time Reverse Transcriptase Polymerase Chain Reaction

Real-time RT-PCR confirmed the increased expression of osteoblast markers ALP and collagen I (Col I) in human periosteal cells cultured for up to 15 passages (Figure 5.9). At passage 2 we observed a rapid, 5.0- and 7.2- fold, increase in ALP and Col I expression, respectively. After long-term expansion, however, these levels were found to decrease slightly to 1.7- and 2.1-fold. Expression levels of osteogenic transcription factor, Runx2, were found to transiently increase over 15 passages, although these changes represented nominal differences compared to passage 2 cells. In contrast, expression of chondrogenic transcription factor, Sox9, decreased steadily from passage 1 to passage 15. No mRNA expression of osteocalcin, collagen II or CD31 was detected (data not shown).
5.3.7 Colony forming unit assay

At passage one, periosteal cells from four donors formed the highest numbers of colonies as compared to subsequent passages (mean for four donors ± SD: 41.5 ± 7.7) (Figure 5.10). During the subculture of periosteal cells, we found a significant decrease in the colony forming ability of plated cells (all samples \( p < 0.001 \), two-way ANOVA of passage one vs all other passages). This was seen as a relative decline in the number of colonies counted (per 10,000 plated) at different passages during expansion for each of the four different donors (Figure 5.10). In comparing the colony forming ability of periosteal cells to the percentage of progenitor, Stro-1⁺, cell populations in culture we found no significant positive correlation (\( R = 0.26, p > 0.05 \)).

Figure 5.10 CFU assay of the number of colonies (mean ± SD) formed per 10,000 cells seeded into 78 cm² petri dish (n=3). Colony scoring was performed by 2 independent observers and was based on colony size being ≥ 1mm in diameter and not inhibited by contact with other colonies. Results are displayed to show the change in colony forming ability over passaging for four different patients.
5.3.8 Mineralisation

Both counting of nodules (Figure 5.11B) and measurement of the intensity of alizarin red dye extracted from stained cultures (Figure 5.11A) showed that significantly more mineralised tissue had been formed by cultures which retained Stro-1$^+$ cells compared with those that had been depleted of Stro-1$^+$ cells. Micrographs showed a greater number and size of positively stained nodules within total population cultures that contained Stro-1$^+$ cells (Figure 5.11C). Secondly, this result demonstrates that periosteal cells retain their functional ability to mineralise during long-term subculture.

![Graphs of alizarin red dye desorbed from wells, normalised to total DNA content (A) and the numbers of fluorescent nodules counted per well (B) (* = p ≤ 0.05). The lower micrographs show representative images of unsorted (C) and sorted (Stro-1 depleted) (D) cell populations grown for 28 days in the presence of osteogenic media and stained with alizarin red dye. Scale bar on images = 1.0 mm.](image)

Figure 5.11 Graphs of alizarin red dye desorbed from wells, normalised to total DNA content (A) and the numbers of fluorescent nodules counted per well (B) (* = p ≤ 0.05). The lower micrographs show representative images of unsorted (C) and sorted (Stro-1 depleted) (D) cell populations grown for 28 days in the presence of osteogenic media and stained with alizarin red dye. Scale bar on images = 1.0 mm.
5.4 Discussion

The periosteum serves as a reservoir of osteochondral precursor cells (417) and represents an interesting new candidate for tissue engineering and cell-based therapies. To fully exploit this therapeutic potential, new work must concentrate on exploring the link between in-vitro periosteal phenotype and properties, and in-vivo functionality. Perhaps most importantly, this knowledge could provide powerful new insight into musculoskeletal development, aging, and healing processes.

Using a simple and well-defined culture model, human periosteal cells were easily isolated from small tissue explants and expanded for 15-20 passages, equivalent to 20-30 population doublings, without growth arrest. Although significant variations in proliferative potential were noted between particular donors, similar growth kinetics were measured throughout expansion regardless of donor age. This robust proliferative capacity might be attributed to the length of telomeres in periosteal cell populations or telomerase-independent mechanisms (418), as telomerase activity was not detected in adult human periosteal cells previously (160). This level of ex-vivo amplification allowed us to identify heterogeneities present in human periosteal cell populations and study the effects of prolonged passaging on periosteal cell properties and phenotype.

Morphologically, two distinct cell subpopulations of small spindle-shaped cells and large flat cells were identified throughout expansion in varying proportions. In early passage cultures, spindle-shaped cells represented >70% of periosteal cells. Serially passages, however, resulted in the progressive decrease in the proportions of spindle-shaped cells in concert with the appearance of large flat cells. The downstream effects of this morphological transition were seen in the growth rates and colony forming efficiency of periosteal cells. This suggests that cell-cell interactions, and/or exposure to autocrine or paracrine factors secreted in the culture medium are responsible for this transition.

We found that the growth potential of periosteal cells was inversely related to cell size, across all donors (Figure 5.12). The progressive increase in average cell area found during expansion resulted in a concomitant decline in the cell yield per passage ($R = -0.81$, $p = 0.00$). Interestingly, a significant inverse correlation was also identified between the rate of increase in cell area and the rate of expansion for each donor ($R = -0.87$, $p = 0.03$) (Figure 5.13). Donors that maintained larger proportions of small cells (<3000 cm$^2$) during expansion exhibited the greatest rates of growth. In support of this, small spindle-shaped cells were previously classified as “immature”, rapidly-cycling, multipotent phenotypes in MSC cultures, while large flat cells were characterised as “mature”, slowly-cycling, cell types with diminished differentiation potential (225;226;228;419). In further agreement with Prockop et al (247), we also found large-flat cells to have an inhibitory
effect on the propagation of small, rapidly dividing periosteal cell populations in colony forming unit and expansion cultures. Interestingly, however, this does not seem to affect the long-term proliferative ability of adult periosteal cells.

Figure 5.12 Effects of periosteal cell area on growth rates. Mean periosteal cell area and fold increase in cell number were calculated in the same culture between passages 0-20 for each donor and plotted against each other. Each data point represents the mean values at one passage in one donor. In total 6 donors were examined between passages 0-20. Non-parametric correlation measurements revealed a significant inverse relationship between cell size and growth (R-value = -0.81, p = 0.00).

Cell cycle analysis of quiescent (G_0) and actively dividing (G_1, S, G_2/M) cells identified a small fraction (23 ± 10%) of proliferating cells that remained comparable in early, intermediate, and late passage cultures. This small, yet consistent fraction of mitotically active cells demonstrates that the average doubling time (72.0 ± 9.4 hrs) observed in monolayer expansion cultures is not representative of the actual cell cycle time of the proliferating population. Additionally, this result also highlights certain features unique to adult stem cell cultures. In many tissues, stem cells rest in a quiescent state for extended periods of time and are responsible for the self-renewal potential of the tissue microenvironment *in-vivo* (420). They re-enter the cell cycle only when stimulated by signals to promote repair or regeneration processes. In the bone marrow, this stem cell niche is regulated by accessory cell types, which function to maintain and regulate quiescent haematopoietic stem cells (421). Although this has not been clarified here, our data suggests that the ex vivo expansive potential of periosteal cultures resides on the self-renewal capacity of a subset of quiescent cells. Additionally, the small, yet consistent population of Stro-1^+ progenitor cell populations identified in periosteal cells throughout expansion, adds further support to this claim.

Phenotypically, initial periostem-derived cell isolates present a mixed cell population, but with subsequent passages we observed a reduction in the population of haematopoietic cell types from 31.8 ± 6.9% to 3.5 ± 1.6% of the total population, and the disappearance of adipose, endothelial,
leukocyte, and smooth muscle cells. The cell population contained both osteogenic and chondrogenic cells. In the absence of specialised cell media, the expression of sox9 decreased linearly with passage, leaving the cultured cells largely osteogenic; with cells expressing Stro-1^−/ALP^+ accounting for ~40% of the cell population and Stro-1^+/ALP^+ a further 10%. These population percentages, particularly the measures of Stro-1^+ cells, are consistent with early passage cells derived from known mesenchymal stem cell sources; such as the trabecular bone explants (242), bone marrow, synovium, adipose tissue, muscle, and most relevant, periosteum (422). Our research however, examines the cell phenotype changes over 15 passages, while the previous study examines cells from a single passage. Although, a definitive marker set for the identification and enrichment of a “pure” population of multipotent stem cells has remained a difficult and elusive task, recent developments in antibody technology, have led several laboratories to produce mAbs targeted for stromal stem cell populations. Of these, Stro-1 has been the most extensively characterised and found to be expressed on a small population of clonogenic multipotent stem cells (240;248;249;423),

![Figure 5.13](image)

**Figure 5.13** Relationship between rate of periosteal cell expansion and rate of increase in periosteal cell area. For each donor the respective rates were calculated from linear regression analysis of cell growth (population doublings/day) and cell area (µm²/day) profiles during subculture; where the slope of the regression line = rate of change. Correlation statistics for 6 donors, reveals a significant inverse relationship between the rate of increase in cell area and the rate of expansion in human periosteal cell cultures (R-value = -0.87, p = 0.03).

The overriding osteogenic phenotype of cultured periosteal cells was not surprising, given the periosteum’s anatomical position adjacent to bone and developmental origins. The presence of a consistent Stro-1^+ population was a more striking feature of the periosteal cultures studied. Two Stro-1^+ cell subpopulations were identified, Stro1^+/ALP^+ and Stro1^+/ALP^−. Based on the study of developmental phenotypes of stromal cell cultures, it has been suggested that Stro-1^+/ALP^− cells represent a less differentiated osteoblastic phenotype, Stro-1^+/ALP^+ cells represent an intermediate cell stage, and Stro-1^+/ALP^+ cells are the most mature (242;424). Other researchers have also
identified primitive precursor populations within periosteal cultures using primitive osteoblast and mesenchymal stem cell markers CD105, CD166, and HOP26 (295;425;426). In contrast to previous studies where Stro-1 expression was found to vary between 2-80% in 28 day bone marrow stromal cultures (427), the proportion of Stro-1+ cells in this study was donor independent (for 6 donors). Most importantly, we did not find that the proportion of Stro-1+ cells decreased with time in culture. The difference between our results and those of Simmons and Torok-Storb (248) who found the percentage of Stro-1+ cells to decrease to 15% of their maximum frequency after six weeks of culture, reflects the difference between cell types and culture conditions used. Removing Stro-1+ cells from the general cell population resulted in a decrease in overall culture osteogenicity, and further confirmed the importance of these progenitor cells in bone formation.

Historically, the colony forming unit (CFU) assay has been used to reflect the number of MSCs or precursor cells in a sample population. Each adherent colony is thought to be derived from the rapid proliferation of a single non-haematopoietic stem-cell, morphologically resembling a fibroblast, and as such are often referred to mesenchymal stem cells or mesenchymal progenitor cells (162;222;392). Interestingly, however, we found no correlation between CFU number and the presence of Stro-1+ progenitor populations (R=0.26, p>0.05). Recently Bruder et al and Banfi et al found that the multipotentiality of human bone marrow derived CFUs was progressively lost during subculture, with progenitor cell populations retaining only their osteogenic differentiation potential at late passages (26;428), consistent with the pattern of expression of osteogenic markers and mineralisation capacity noted here. This progressive shift from multilineage potential to osteoblastic phenotype has been shown to be associated with a progressive change in cell morphology, from small, rapidly dividing spindle-shaped cells to large, flat slowly proliferating cells (419). This is consistent with the significant changes in cell area and morphology shown here. The decrease in the colony forming efficiency of periosteal cells is therefore the likely result of this morphological transition.

These results emphasize an important feature of CFUs, namely, that changes in observed CFU progenitor quantity or colony forming ability may be dependent on environmental conditions such as the presence of growth factors and changes in cell population rather than an actual reduction of stem cell numbers (178;429). Due to rich heterogeneity of periosteal cells characterised here, it seems plausible that CFU efficiency is affected by combination of cell-cell interactions and secreted factors present in the medium. It is interesting to note that CFU efficiency is highest in the early passage cells, which contain large proportions of haematopoietic non-mesenchymal cell types. Hematopietic cells, commonly regarding as contaminants in mesenchymal stem cell cultures, may play a critical role in the regulation and development of progenitor cells and their descendents during expansion. In bone marrow stromal cultures containing both MSCs and HC populations, a
significant increase in the numbers of CFU-F and CFU-O colonies was found when non-haematopoietic (CD45-) cells were grown in the presence of haematopoietic (CD45+) cells (429). This suggests the existence of positive interactions between CD45+ and CD45- cells, which is likely mediated by their respective endogenous secretion of soluble factors, similar to what is observed between early haematopoietic cells and their progenitors (430). Haematopoietic cells secrete regulatory molecules that form the basis of intracellular cross-talk networks, which in turn regulate the survival, growth, and function of mesenchymal progenitor cells in an autocrine or paracrine manner (430;431).

In the absence of cell specific media supplements, the shift of periosteal cell populations to a more osteogenic phenotype was confirmed by quantitative measurements of RNA expression (real-time RT-PCR) which show steady or increased expression of bone related genes Runx2, ALP, Col I and decreased expression of chondrogenic gene Sox-9 after initial plating. Gene expression of collagen II, a marker of mature chondrocytes, was not found. This low initial chondrogenicity of isolated periosteal cells, confirms results in the literature (432), and indicates that the chondrogenic cells in non growth factor supplemented media are derived from precursor cells, rather than from mature cells in the periosteum. As this population remains stable over a number of passages, this helps explain previous findings that show that periosteal cells in chondrogenic media retain their cartilage-forming capacity over a number of generations, which would be required to support chondrocyte populations (201).

5.5 Conclusions

In conclusion, we have identified a number of cell types within enzymatically liberated human periosteal cells based on the expression of distinct morphological features and cytosolic and surface proteins. Periosteal cells expanded for over 20 population doublings in the absence of differentiation stimuli experienced time-dependent changes in cell area and morphology and exhibited stable growth kinetics, a large expansive potential, and a small, but consistent subset (23%) of proliferating cells. Further, we were able to identify cells at different stages of osteoblast differentiation and demonstrate phenotypic changes over passaging based upon the expression of the Stro-1 and/or ALP antigen and lineage specific genes. As shown here, a population of progenitor cells can be initially isolated in viable form from human periosteal explants and cultured for up to 10 passages without a loss in population numbers or ability to form mineralised tissue. The existence of a large subpopulation of quiescent (G0) cells in periosteal cell cultures seems to be important, since their number and properties should be enough to sustain a steady supply of cells that upon differentiation and commitment may serve as precursors for a number of non-haematopoietic tissues. Whether late passage periosteal cells are fully committed to the osteogenic
pathway remains to be elucidated in our culture model, however, there is evidence to suggest that these precursor cell populations retain the potential for multilineage differentiation (160).
Chapter 6

Sequential Digestion of Human Periosteum

6.1 Introduction

The periosteum is a specialized connective tissue that covers all surfaces of long bones except articulating joints and is known to play a major role in bone apposition during development and fracture healing (433;434). During healing, the periosteum provides cells, bioactive factors, and a blood supply that aids in the formation of the cartilage-like soft callus which is eventually replaced by calcified bone matrix (45;435;436). This role is substantiated in early distraction studies, which show that new bone formation is less likely to occur when the periosteum is severely damaged (437-439). In addition to osteogenic potential, the periosteum has also demonstrated significant chondrogenic potential in-vivo (31;185;402), as exploited for the repair and resurfacing of large bone and articular cartilage defects (354;358;440-442).

Anatomically, the periosteum is a bilayered membrane consisting of an outer fibrous layer and an inner cambial layer, which lies against the bone (396;443). The outer fibrous layer is composed mainly of fibroblast-like cells scarcely distributed in a dense matrix of collagen and elastin fibers and a network of microvasculature, nerves, and lymphatic vessels (39;397;444). The inner cambial layer, is a thin (3-5 cell layers) highly cellular layer containing a variety of cell types, including mesenchymal stem cells, progenitor cells, fibroblasts, and osteoblasts (29;445;446). The regenerative capacity of the periosteum appears to be well correlated to the presence of primitive precursor cells (i.e. multipotent stem cells and lineage restricted progenitor cells) resident to the cambium layer.

To harness the therapeutic potential of the periosteum, research is now focusing on elucidating the biological mechanisms regulating cambial cell osteogenesis and chondrogenesis. To this end, periosteal grafts, explants, and enzymatically liberated cell isolates have shown promising results in numerous animal models and in-vitro culture systems. Most interesting, isolated periosteal cells have compared favourably to bone marrow stromal cells in terms of growth kinetics and multilineage potential (160;422;447) and have been successfully applied to bone tissue engineering applications with various scaffold materials (448-452). As such, cultured periosteal cells may represent an attractive alternative to existing cell-based regenerative therapies. However, the isolated periosteal cells examined in these studies represent a mixed cell population of multiple
cell phenotypes liberated from both the fibrous and cambial layers. The inability to clearly identify and isolate specific cell types with reparative potential could lead to reduced functional control and reproducibility, thus posing a significant hurdle towards the development of periosteal cell-based tissue engineered therapies. Previously, we observed that enrichment techniques based on selective adherence to tissue culture plastic is an inadequate method to isolate homogenous periosteal cell populations and target specific cell phenotypes (Chapter 5). Therefore more efficient enrichment strategies are needed to purify the reparative cell types from heterogeneous periosteal cell isolates. To date, a number of enrichment strategies exist for purifying heterogeneous populations, including fluorescent activated cell sorting (FACS), magnetic cell sorting, limiting dilution, and density centrifugation. Such strategies, however, are time-consuming and rely on the purchase of expensive equipment and/or antibodies and regents. A fast, simple and reproducible enrichment method would considerably benefit on-going periosteal cell research and aid in the development of clinical applications.

The objective of this present study is to develop and characterise a sequential digestion method to selectively isolate human cell types present in the cambium layer of periosteal explants. Using this technique, we aim to identify the optimal digestion time necessary for the enrichment of highly-proliferative progenitor cells types. Based on the thickness and cellularity of the cambium and fibrous layer, we hypothesized that periosteal cells isolated from each of the digestion steps would contain tissue layer specific differences in population phenotypes and behaviour, with early digestions isolating a unique population of cambial progenitor cells. In this context, this sequential isolation approach would not only allow for the enrichment of therapeutic cell types but would also enable us to target, separate, and extensively characterise distinct human periosteal cell populations from each distinct tissue layer. This level of cellular information would be useful for dissecting the heterogeneities present in human periosteal cell cultures and designing improved periosteal cell-based therapies. Therefore, isolated and monolayer-expanded cells from each of sequential digestions, from one donor, were examined and compared for their proliferative capacity, morphology, colony forming ability and surface protein expression using flow cytometry.

6.2 Materials and Methods

6.2.1 Assessment of thickness and cellularity of human periosteum

To develop an accurate and reproducible isolation method, the thickness and cellularity of the individual periosteal layers was measured and compared across a number of donor explants. A total of 7 periosteal explants were harvested from the medial proximal tibia of female patients aged between 16-91 undergoing knee surgery at The Chelsea and Westminster Hospital. The explants all measured approximately 1.0 x 1.5 cm and were elevated by subperiosteal dissection with a sharp
periosteal elevator by the same skilled surgeon. The explant was then cut into two pieces; a 5 x 5 mm piece for histology, with the rest being used for a preliminary digestion study. For histology, tissue samples were fixed in 10% v/v neutral buffered formalin (Agar Scientific, UK) for 12 hours at room temperature, dehydrated through an increasing alcohol series and xylene (Sigma-Aldrich, Poole, UK) before being embedded in paraffin wax. Each sample was cut perpendicular to the bone, into four 5μm thick histological tissue sections at equal intervals from the proximal to distal end, and stained with haematoxylin and eosin (both Sigma-Aldrich, UK). Three photomicrographs (x40, x100, x200 magnifications) were taken of each section, for a total of 36 photomicrographs per donor. All histological sections were cut and stained by one person. The total thickness of the periosteum and the thickness of the individual fibrous and cambium layers were measured in micrometers from photomicrographs taken at 40x magnification. The cell density of the respective layers was determined using a modification of a previously described technique (359). For the cambium cell density, a photomicrograph measuring, 436 x 328 µm² (area), was taken twice, at random, over the cambium layer of each section. To determine cell density (cells/µm²), all nuclei within the imaging area were counted and the result was divided by the sampling area (thickness of the cambium x width of the photomicrograph). For the fibrous layer cell density, the same procedure was employed in photomicrographs measuring 880 x 663 µm².

6.2.2 Cell isolation and culture

6.2.2.1 Sequential enzymatic digestion

A periosteal explant was harvested from the medial proximal tibia of a 71 yr old female during total knee replacement surgery at the Chelsea and Westminster Hospital. The explant, measuring approximately 1.0 x 1.5 cm and weighing 0.293 g, was elevated by subperiosteal dissection with a sharp periosteal elevator. The explant was transported to the laboratory in Earls balanced salt solution at 37°C in a mini-portable incubator. After being cleaned of fat and blood tissue, the explant was subjected to a sequence of digestions in an enzymatic solution consisting of 3 mg/ml collagenase D (Roche Diagnostics) and 3 mg/ml collagenase type II (Sigma) in 5 ml complete media (DMEM containing 10% v/v foetal bovine serum, 1% v/v L-glutamine, 1% v/v antibiotic/antimicrobial). To optimally design a sequential digestion technique, we conducted a pilot study to examine the average time needed to enzymatically digest a periosteal explant, using our standard collagenase solution. Based on this pilot study and the aims of this study, a sequence of four serial digestion steps were performed at 37°C in 5% CO₂ for 30, 60, 90, and 90 minutes for a cumulative digestion time of 4.5 hours (Figure 6.1). The digestions from steps 1-3 were kept at 37°C in 5% CO₂ until the last digestion (step 4) was complete, so as to negate the potential for time-dependent differences of collagenase exposure on cellular behaviour. After 4.5 hours, 5 ml of FBS was added to each digestate, passed through a 70μm filter, and centrifuged. The cell pellets
from the individual digestions were then rinsed in PBS, counted, and the dissociation rates calculated. Based on the dissociation rates, cells from isolation 1 and 2 were pooled together and in this study will be termed “initial digestion cells”. Isolated cells from digestion step 4 were also kept, and will be termed “final digestion cells”.

Figure 6.1 Schematic diagram of sequential enzymatic digestion method and results. A periosteal explant was treated for 4.5 hours in 0.6% (w/v) collagenase solution. Collagenase solution was replaced at 30, 60, 90, and 90 minutes and cell release rate was measured by counting cells after each of the digestion steps. The majority of the cambium layer cells were liberated during the first 1.5 hrs of digestion (Step 1+2). The remaining cambium and fibrous layer cells were isolated during the final 3 hrs of digestion (Step 3+4) with digestion step 4 consisting of mostly of fibrous layer cells. This was confirmed by histology and by determining the numbers of cells released per digestion step and converting this into a cell release rate (cells released/hr).

6.2.2.2 Full enzymatic digestion

For comparison purposes, an equally sized periosteal explant (0.255 g) was harvested as above from the medial proximal tibia of a 74 yr old female undergoing total knee replacement surgery. In this instance, however, the explant was subjected to a full 4.5 hour digestion in the same enzymatic
solution and environment as above, after which the liberated cells were strained, washed, and counted as above. These cells will be termed “full digestion cells”.

6.2.3 Cell proliferation- Standard density

To investigate the proliferation characteristics of the different cell isolates, the harvested cells were separately plated at the standard density of 5000 cells/cm² in complete media into a 24 well-plate. For continuous subculture and expansion, adherent cells were harvested with 0.25% (v/v) trypsin and 1mM EDTA for 5 minutes at 37°C, and replated at 5000 cells/cm² in a T75 flask containing 15 ml of complete media; this process was repeated every 7-14 days (~90% confluence) for 5 passages. Cells were fed every other day. Viable cell number was determined using trypan blue and a haemocytometer. Based on the cell counts, cumulative cell number, population doublings, and fold increase were calculated.

6.2.4 Cell proliferation- Low density

To study the effects of cell density on cell proliferation, the cell isolates were also plated separately into T75 culture flasks at 50 cells/cm² and cultured for periods of 7 and 14 days (n=3 per time point). At both time points, cells were harvested and counted as above and the growth curves were determined for each digestion using the average number of cells per flask.

6.2.5 Cell morphology

The differences in initial cell morphology between the cell isolates was examined in passage 0 standard density cultures at day 11 under a phase contrast microscope. Photographs were taken of at least 8 random sites on each culture plate. The cell areas and circularity of individual cells were measured quantitatively, as performed previously (Chapter 5), using imaging software (NIH Image J). In all cases mitotic cells were ignored.

To further determine differences in cell size and granularity, passage 0, 1, and 5 cells from each of the isolates were detached with 0.25% trypsin/EDTA, resuspended in PBS containing 2% (w/v) bovine serum albumin (BSA) and assayed using flow cytometry (FACSCalibur; BD Biosciences, Oxford, UK). The forward- and side-scattering (FSC and SSC) properties were measured on a linear scale of 1024 channels. The forward threshold was set to 55 to eliminate small particulate debris. Data from all the digestions was collected under identical parameters and analysed by collecting 20,000 events.

6.2.6 Colony forming unit assay

Based on previous optimization studies (Chapter 4), freshly isolated cells from each digestion step were plated at 10,000 cells per petri dish (78 cm²) in complete medium. After 12 days in culture,
the dishes were stained with 0.5% (w/v) crystal violet in methanol for 5 minutes, rinsed twice in distilled water and the number of colonies per dish counted by 2 independent observers. Based on previously described criteria (391) colonies < 1 mm in diameter (~50 cells) and faintly stained colonies were discounted. Mean values for each cell digestion were derived from three petri dishes (n=3). To estimate colony proliferation rate, colony area was examined using low magnification (1.5x) microscopy. Average colony area for each digestion, was determined by imaging 5 randomly selected colonies per Petri dish (n=15), and subsequently measuring total area using Motic imaging Software (Motic, Ipswich, UK).

### 6.2.7 Immunophenotype

The following antibodies were tested for their specificity, titrated, and used at concentrations varying from 1-2.5 µg/10⁶ cells: mAbs against mesenchymal progenitor cell surface antigen Stro-1 (R&D Systems, Abingdon UK), phycoerythrin (PE) conjugated CD34 hematopoietic precursor cell marker and CD31 endothelial cell marker (Abd Serotec, Oxford UK), allophycocyanin (APC) conjugated anti-human bone/liver/kidney alkaline phosphatase (ALP) and leukocyte adhesion molecule CD45, and fluorescein isothiocyanate (FITC)-conjugated α-smooth muscle actin.

Approximately 1-3 x 10⁵ periosteal cells of each digestion between passages 0-5 were incubated in 1 ml of blocking buffer (phosphate buffered saline pH 7.4 0.01M (PBS) containing 2% (w/v) bovine serum albumin (BSA), 0.1% (v/v) foetal bovine serum, and 0.1% (v/v) normal mouse serum (all Sigma-Aldrich) for 30 minutes at room temperature to limit non-specific binding. For direct staining, cells were then incubated with conjugated mAbs for 45 minutes at 4°C, washed in PBS containing 2% BSA (w/v) + 0.1% (w/v) sodium azide (flow buffer), centrifuged, and resuspended in 300 µl of flow buffer prior to analysis. For indirect staining using primary mAb Stro-1, the cells were first incubated with the primary mAb (45 min at 4°C), washed, and incubated with a FITC-conjugated fragment F(ab')₂ goat anti-mouse secondary Ab (Jackson ImmunoResearch, Suffolk, UK) for 45 minutes at 4°C. Matched isotypes were used as negative controls and prepared at the same concentrations as their matched primary Ab. Cell fluorescence was evaluated by flow cytometry using a FACSCalibur instrument (BD Biosciences, Oxford, UK) and data analysed using FlowJo software (Tree Star Inc, Ashland, USA). At least 20,000 events were collected per sample and percentage positive expression for each mAb was defined as the level fluorescence >99% of the isotype-matched control antibodies.

Cells from cultures from all cell digestions displayed little or no reactivity (≤1%) with antibodies CD31 (endothelial cells), CD45 (leukocytes), and α-SMA (248) and are therefore not included in our analysis.
6.2.8 Statistics

Analysis of colony number and size data was performed using a one-way ANOVA, with a Tukey post-hoc test to establish significance ($p < 0.05$). For histological and cell morphology analysis, data were analysed using the non-parametric Mann-Whitney U test to examine statistical differences between groups. Data were expressed as the mean ± SD and all tests were performed using SPSS (Chicago, IL, USA) at a significance level of 0.05.

6.3 Results

6.3.1 Assessment of thickness and cellularity of human periosteum

![Figure 6.2](image)

**Figure 6.2** Histological analysis of cambium and fibrous layer thicknesses in human periosteal explants. Explants were harvested from 7 female donors with ages ranging from 16 to 91 years. Fibrous layer (▲) and cambium layer (○) data is presented from each donor to determine potential age-related changes in tissue thickness of the respective layers. The letter superscripts indicate the results of non-parametric analysis of significance using the Mann-Whitney U test. Results that have the same letter are not significantly different from one another ($p>0.05$).

Statistical analysis of full-thickness periosteal explants harvested from 7 female donors ranging from 16-91 yrs of age, revealed no age-related trends in the thickness or cellularity of the cambium or fibrous layers. Linear regression analysis of cambium layer and fibrous layer thickness measurements in Figure 6.2 revealed $R^2$-values of 0.13 and 0.35 respectively (trend line not shown). As seen in Figure 6.2 and Table 6.1, the thicknesses of the total explant and each layer demonstrated some donor-to-donor variation. Cambium layer thicknesses varied between 84-111
µm, with the mean ± SD for all donors found to be 95.5 ± 13.4 µm. Fibrous layer thicknesses demonstrated larger donor-to-donor variation, with mean values ranging from 1061.7-1525.5 µm. Unlike the donor differences noticed in layer thickness, the cell densities measured for each layer was statistically comparable for each donor except one (Table 6.1). Across all donors, the cambium layer was found to be an average of 12.0-times more cellular than the fibrous layer (Table 6.1).

<table>
<thead>
<tr>
<th>Donor Age</th>
<th>Total Thickness</th>
<th>Cambium Thickness</th>
<th>Fibrous Thickness</th>
<th>Cambium cell density (x10^3/µm²)</th>
<th>Fibrous cell density (x10^3/µm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>16</td>
<td>1315.0 ± 107.9</td>
<td>84.32 ± 10.33a</td>
<td>1230.70 ± 110.28a</td>
<td>2.5 ± 0.4a</td>
<td>0.28 ± 0.09a</td>
</tr>
<tr>
<td>20</td>
<td>1525.5 ± 66.4</td>
<td>100.09 ± 10.09b</td>
<td>1425.88 ± 68.28</td>
<td>2.9 ± 0.5a</td>
<td>0.18 ± 0.03</td>
</tr>
<tr>
<td>24</td>
<td>1075.8 ± 66.8a</td>
<td>83.68 ± 11.22a</td>
<td>992.45 ± 69.17b</td>
<td>6.0 ± 0.7</td>
<td>0.31 ± 0.08b</td>
</tr>
<tr>
<td>33</td>
<td>1233.8 ± 117.5b</td>
<td>100.25 ± 10.10b</td>
<td>1129.02 ± 115.83c</td>
<td>2.7 ± 0.5a</td>
<td>0.29 ± 0.05b</td>
</tr>
<tr>
<td>48</td>
<td>1235.8 ± 126.5b</td>
<td>102.59 ± 10.90b,c</td>
<td>1202.41 ± 140.14ac</td>
<td>2.9 ± 0.6a</td>
<td>0.30 ± 0.04b</td>
</tr>
<tr>
<td>74</td>
<td>1061.7 ± 91.4ac</td>
<td>110.53 ± 7.70c</td>
<td>951.68 ± 92.80b</td>
<td>2.5 ± 0.3a</td>
<td>0.28 ± 0.07a</td>
</tr>
<tr>
<td>91</td>
<td>1117.7 ± 114.8c</td>
<td>90.27 ± 9.98a</td>
<td>1027.50 ± 111.93b</td>
<td>2.7 ± 0.6a</td>
<td>0.35 ± 0.09b</td>
</tr>
<tr>
<td>All</td>
<td>1248.1 ± 183.8</td>
<td>95.54 ± 13.39</td>
<td>1164.2 ± 185.2</td>
<td>3.35 ± 1.37*</td>
<td>0.28 ± 0.09*</td>
</tr>
</tbody>
</table>

Table 6.1 Histological analysis of the cambium and fibrous layer of human periosteal explants. Data is presented as mean ± SD. The letter superscripts (a,b,c) indicate the results of non-parametric analysis of significance using the Mann-Whitney U test. Results within a column that have the same letter are not significantly different from one another (p> 0.05). The row “All” is the cumulative mean ± SD of data measured across all donors. *The large standard deviations noticed in cambium and fibrous layer cell densities are a result of significantly different data derived from the 24 yr old and 20 yr old donors, respectively.

6.3.2 Cell isolation

The preliminary, 7 donor pilot study of full thickness digestion times revealed that an average time of 7.5 ± 0.5 hours was needed to fully digest a periosteal explant in a 6 mg/ml collagenase solution. Given the average thickness of the periosteal explants (1248 ± 184 µm), the average digestion rate over the 7.5 hours was 166 ± 24 µm of tissue/hour. However, digestion times over 5.0 hours resulted in a 50% decrease in cell viability as determined by trypan blue dye exclusion assay (data not shown). Based on the results of cell viability, average enzymatic reaction rate, and periosteum layer thickness, the temporal profile of the sequential digestion steps was designed to allow for the isolation of viable populations enriched for cambium or fibrous layer cells (Figure 6.1). Histological evidence of the success of this isolation strategy is shown in Figure 6.3. After 1.5 hours of enzymatic digestion, most of the cambium layer cells have been liberated and only the fibrous layer of the periosteum explant remains intact. Most of the fibrous layer cells were liberated during the last 3.0 hours of digestion (Steps 3+4). Congruent with this, the first 2 sequential digestion steps revealed substantially higher cell yields and associated cell release rates compared to the last 2 steps (Figure 6.1 and Table 6.2). The differences in cell yields between initial (Steps 1+2) and final (Steps 3+4) digestion steps correlates well with the differences in cell
density between the cambium and fibrous layer. This shows that the initial cell isolates (Digestion Steps 1+2) contain more cambium layer cells whereas final cell isolates (Digestion Steps 3+4) consist of mostly fibrous layer cells.

**Figure 6.3** Histology of representative human periosteal explant during the sequential enzymatic digestion method. Image on left is of explant prior to collagenase digestion (0 hours) showing the presence of 2 distinct layers; the highly cellular cambium layer and the less cellular fibrous layer. After 1.5 hours of digestion (Steps 1+2) most of the cambium layer has been digested from the explant, leaving just the fibrous layer to digest during steps 3+4.

To further confirm the quality of this enrichment strategy we compared the phenotype and features of cells isolated from digestion step 1+2 (termed initial digestion cells) against those isolated from final digestion step 4 (termed final digestion cells). As an additional comparison, a full thickness digestion (termed full digestion cells) was performed on a separate periosteal explant harvested from an age- and sex- matched, 74 yr old, donor. This digestion demonstrated a cell release rate between what was found in initial and final sequential digestions and resulted in a completely mixed population of cells from the fibrous and cambium layers.

<table>
<thead>
<tr>
<th>Enzymatic Digestion Characteristics</th>
<th>Passage 0 Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time in hrs (total)</td>
<td>Cells Released</td>
</tr>
<tr>
<td>Step 1</td>
<td>0.5 (0.5)</td>
</tr>
<tr>
<td>Step 2</td>
<td>1.0 (1.5)</td>
</tr>
<tr>
<td>Step 3</td>
<td>1.5 (3.0)</td>
</tr>
<tr>
<td>Step 4</td>
<td>1.5 (4.5)</td>
</tr>
<tr>
<td>Full</td>
<td>4.5</td>
</tr>
</tbody>
</table>

Table 6.2 Characterisation table of the different enzymatic isolations compared in this study. Sequential digestion steps 1+2 (initial digestion cells), step 3, and step 4 (final digestion cells) from one donor (71 yr old female) are cross-compared to each other and cultures derived from a full thickness digestion from a different donor (74 yr old female) according to total cells isolated and cell release rate. The different cell isolations are then placed into culture and assayed for expansion rates, colony forming ability, and colony size. * = Values significantly different from initial digestion values (Digestion step 1 + 2) (p<0.05). ‡ = Values significantly different from final digestion values (Digestion step 4) (p<0.05).
6.3.3 Cell proliferation

To evaluate differences in growth kinetics and total cell yields, freshly isolated (passage 0) periosteal cells from initial, final, and full digestions were plated at a standard density of 5000 cells/cm² and were cultured for 5 passages. Passage 0 initial digestion cells expanded approximately 37-fold in 14 days, whereas final and full digestion cells plated at the same density expanded only 6.1- and 7.2-fold respectively (Figure 6.4A and C). Over 5 passages, the total cell yield was highest in initial digestion cultures (2.8 x 10⁸ cells) compared to both final and full digestion cultures, which yielded similar numbers of 5.8 x 10⁷ and 3.1 x 10⁷ cells respectively. The increased cell numbers seen in initial digestion cultures over 5 passages, is comprised of 2 distinct proliferation trends; an initial rapid proliferation rate at passage 0 \( (m_{p0}=0.37, R^2=1.00) \) and a slower rate from passage 1 to 5 \( (m_{p1-5}=0.21, R^2=0.99) \) (Figure 6.4B). The latter of which is similar to the proliferation rates found in both final and full digestion cultures, which maintain steady growth rates \( (m=0.19-0.20, R^2=0.99) \) over the 5 passages. These growth trends are further highlighted by examining the fold increase in cell number at each passage for each digestion (Figure 6.4C). Here we find that the significant difference in early growth rates between initial and final digestion cultures diminishes to nominal differences after passage 0 and that final and full digestion cultures maintain similar growth profiles over 5 passages. This indicates that the difference in cell yields between the cultures seen over 5 passages is due to initial rate of expansion seen in cambial cell culture at passage 0.

To study the effects of plating density on cell proliferation, isolated cells from each digestion were plated at a low plating density of 50 cells/cm² and grown for a period of 14 days. As seen in passage 0 standard density cultures, initial digestion cells proliferated at a significantly greater rate than final digestion cells over the 14 day period (Figure 6.4A). Initial digestion cells expanded 5.3-fold over 14 days, whereas final digestion cells demonstrated negligible growth and expanded only 1.1-fold. At low seeding density, full digestion passage 0 cultures expanded 2.0-fold, with a growth profile residing between initial and final digestion profiles.
Figure 6.4 Population growth characteristics of periosteal cells isolated from the different digestions. (A) Primary, passage 0 cells were seeded at standard (5000 cells/cm²) and low (50 cells/cm²) density on tissue culture plastic and cultured for a period of 14 days in standard growth medium. Total cell yields and fold increase were calculated at day 7 and day 14 by harvesting and counting a total of 3 wells per digestion (n=3). (B) To examine expansion potential, cells from each of the digestions were seeded at a standard density (5000 cells/cm²) and expanded for 5 passages (~63 days). The cumulative population doublings achieved are plotted for each culture, with linear regression curves overlaid. The slopes (growth rates) highlight the presence of two distinct growth trends found in initial digestion cultures during expansion. (C) The fold increase (initial cell number/final cell number) is also calculated per passage to further demonstrate these trends.
6.3.4 Colony forming efficiency

The differences seen in the proliferative potential of passage 0 cells from the different isolates was further demonstrated in the ability of isolated cells to form distinct cell colonies. In preliminary experiments, we found that the optimal protocol for obtaining distinct and reproducible colony forming units (CFUs) was to culture isolated periosteal cells at a cell density of 10,000 cells/petri dish (78 cm²) for 12 days (Chapter 4). Likewise, to establish significance between cell layers, 3 petri dishes were counted for each assay. During culture, the enzymatically liberated cells experienced an initial lag-phase of roughly 3 days, during which small colonies were seen to arise from single cells. After 12 days in culture, initial digestion cells produced significantly more CFUs (32.0 ± 2.2) compared to both final digestion cells (5.1 ± 1.0) and full digestion cells (11.6 ± 2.2) (p<0.05) (Table 6.2). CFUs sizes were determined as an additional estimation of the proliferative potential of the periosteal cells. In agreement with previous work (453), average CFU sizes determined here, positively correlated with the true growth rates noted in each of the respective digestion cultures. Initial digestion cells showed a mean CFU area of (4.4 ± 1.9 mm) which was significantly greater than both final digestion (2.2 ± 1.0) and full digestion (2.9 ± 0.8) cell colonies (Table 6.2). The areas of colonies derived from final and full digestion were found to statistically similar (p= 0.344).

6.3.5 Cell morphology

To characterise initial cell morphologies, plastic adherent cells from each periosteal digestion were plated at a standard density of 5000 cells/cm² and grown in monolayer. After 10 days - a time-frame we chose which constituted the log phase of growth - phase-contrast images revealed 3 morphologically distinct cell types in all cultures: small spindle shaped cells, elongated fibroblast-like cells, and large flat cells (data shown in Chapter 5). To quantify the differences in cell morphology between the cells derived from each digestion, the mean cell area and population distribution were calculated. Compared to final and full digestion cells, initial digestion cells contained a more homogeneous population of cells with significantly smaller cell areas (p<0.05) (Figure 6.5A and C). Measures of mean cell circularity revealed initial digestion cells to also be significantly more rounded compared to the thinner and more elongated morphology of the final digestion cells (Figure 6.5B). In examining the distribution of areas in the different cultures, we identified a subpopulation of cells, representing 35.8% of the cells measured, to be smaller than 2000 µm² (Gate R1 in Figure 6.5C). This subpopulation was unique to initial digestion cultures as no cells were found to be ≤ 2000 µm² in either final or full digestion cultures. This data also revealed cells in final and full digestion cultures to have similar mean cell area and circularity (p> 0.05).
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Figure 6.5 Characterisation of cell morphologies present in passage 0 human periosteal cell cultures from each digestion. After 10 days in culture, cell area (A) and circularity (B) was measured digitally using ImageJ software based on cell spreading. Data is shown as the mean ± SD, with > 50 cells being measured in each culture (* = p < 0.05). (C) The distribution of cell areas is also shown. Gate R1 demonstrates the presence of a subpopulation of small, spindle-shaped, cells ≤ 2000 µm² in area, and represents 35.8% of initial digestion cultures.

To further assay for morphological changes and homogeneity of the respective cultures, cells from each isolate were placed in suspension and examined for their light scattering properties in a flow cytometer. This method excludes the effects of cell spreading on cell size. FSC is a complex function related to cell diameter, while orthogonal light scattering signals (SSC) reflects the granularity of the cells. After 14 days in primary culture (passage 0), analysis of forward-light scattering intensity indicated that initial digestion cultures contained a higher frequency of smaller cells compared to both final and full digestion cultures (Figure 6.6). Gating on the distribution histogram (Gate R2, Figure 6.6) revealed that this distinct population of small cells (55-300 FSC-H) comprised 35.8% of the total initial digestion culture. An observation consistent with evaluation of cell areas gated in R1 (Figure 6.5C). Upon passaging, however, this unique subpopulation of smaller diameter cells quickly disappeared and the differences seen in the original passage 0 cell size frequencies between the different digestions became negligible. Differences in side-light scatter (cell granularity) were also apparent, and revealed that at passage 0, initial
digestion cultures contained less granular cells compared final digestion and full digestion cultures (Figure 6.6). However, during subculture, a progressive shift to more granular populations was noted in initial and full digestion cultures. Whereas, the opposite trend to less granular populations was found in final digestion cultures.

Figure 6.6 Flow cytometry analysis of the cell size (FSC) and granularity (SSC) of cultured human periosteal cells isolated from the different cell digestions. Distribution histograms of initial (black line), final (grey line), and full (dotted line) digestion cells at passage 0, 1, and 5 demonstrate the morphological differences between the different cultures and the changes that occur during subculture. Gate R2 highlights a subpopulation of small, agranular cells unique to initial digestion cultures and represents 35.6% of the total cell population analysed.

6.3.6 Immunophenotype

To identify specific cell phenotypes, surface epitopes were examined in initial, final, and full digestion cultures over 5 passages using commercially available antibodies. Cultures derived from all the digestions contained a heterogeneous population of cell phenotypes, with varying levels of expression of each of the 6 antibodies examined (ALP, Stro-1, CD34, CD45, CD31, and α-SMA) (data not shown, see Chapter 5). In passage 0 initial digestion cultures, CD34⁺ (20.9%) and ALP⁺/Stro-1⁻ (17.7%) cells represented the largest populations of stained cells, whereas Stro-
1+/ALP⁻ (4.2%) and Stro-1⁻/ALP⁺ (2.3%) were expressed on a considerably smaller percentage of cells (Figure 6.7A). In contrast, passage 0 final digestion cultures contained much larger populations of ALP⁺/Stro-1⁻ (47.9%) Stro-1⁻/ALP⁺ (6.9%) and smaller populations of CD34⁻ (3.9%) and Stro-1⁻/ALP⁻ (1.5%) cells (Figure 6.7A). Full digestion cultures, as one might expect, contained expression levels between what we found in passage 0 initial and final digestion cultures, for all populations except the Stro-1⁻/ALP⁺ subpopulation (9.9%).

Figure 6.7 Expression of cell specific mAbs Stro-1, ALP, and CD34 on passage 0 (A) and subcultured (B) periosteal cells isolated from initial, final, and full digestions. Line graphs are displayed to highlight the changes in phenotype that occur during monolayer culture on tissue culture plastic. Expression levels were determined by recording at least 20,000 events using flow cytometry and gating using appropriately match isotype controls. Results are expressed as percentages of the total cell population measured.

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When placed in secondary culture and examined over 5 passages, the number of initially examined phenotypic populations changed substantially (Figure 6.7B). In final digestion cultures, both Stro-1\(^+\) populations (Stro-1\(^+/\)ALP\(^+\) and Stro-1\(^+/\)ALP\(^-\)) were found to rise slightly at passage 1 and decrease thereafter to very low levels (\(\leq 2\%)\) at passage 5. ALP\(^+/\)Stro-1\(^-\), on the other hand, maintained relatively consistent levels during the measured culture period, showing slightly less expression at passage 5 (32.4%) compared to passage 0 percentages (47.8%). Examining the lineage development in initial digestion cultures, we notice different trends. We found a drastic increase in both ALP\(^+\) subpopulations (ALP\(^+/\)Stro-1\(^-\) and Stro-1\(^+/\)ALP\(^+\)) over 5 passages, with the largest rise occurring at 1 passage. The Stro-1\(^+/\)ALP\(^-\) subpopulation was found to maintain expression levels between 2-5% over the 5 passages. In full digestion cultures we noticed consistent expression levels over the 5 passages, with subpopulations neither greatly increasing nor decreasing during subculture (Figure 6.7B). The increase from 27% at passage 0 to 43% at passage 5 of ALP\(^+/\)Stro-1\(^-\) expression represents the largest change seen in full digestion cultures. Similar to initial digestion cells, the Stro-1\(^+/\)ALP\(^+\) population was maintained at levels between 2-5% over 5 passages, and the CD34\(^+\) population was found to decrease with time in culture. In final digestion cultures, the decrease in Stro-1\(^+\) subpopulations occurs in concert with an increase in CD34\(^+\) percentages, accounting for a majority of cells examined at passage 5. CD34 expression in initial digestion cultures, in contrast, found at higher levels initially, was shown to decrease to roughly 7% of the total cell population at passage 5.

### 6.4 Discussion

The continued development and success of periosteum-based therapeutic strategies will be contingent upon the ability of researchers to safely and effectively harness the full regenerative potential of the periosteum. Further, this ability necessitates that researchers establish a simple and reproducible method for identifying and enriching target cells with the best reparative characteristics for the given clinical application. From previous studies conducted by us and others, it is known that a great biological variation exists between different periosteal cell populations. Therefore, in considering the rich heterogeneity of the periosteum both in terms of cellular and extracellular composition, this capacity to identify and enrich specific cell populations becomes even more important. It is well documented that cells derived from human periosteal tissue comprise a heterogeneous population that includes cells with the capacity to extensively self-renew, form colonies and differentiate into multiple lineages; indicative of the presence of stem cells (160;422). However, prior to this study, less focus had been given to targeting stem cell types or understanding the underlying differences in periosteal cell biology (398). In this study we devised an enrichment strategy based on a series of enzymatic digestions to [1] optimise selection protocols for the isolation of precursor stem cells from human periosteum and [2] to characterise
the differences in cellular phenotypes and \textit{in-vitro} behaviour between populations isolated from the distinct tissue layers of the periosteum.

To develop an accurate and reproducible isolation method, the thickness and cellularity of the individual periosteal layers was measured and compared across a number of donor explants. \textit{In-vivo}, the distinct cellular and structural differences in the cambium and fibrous layers have been detailed previously and are found to significantly vary with species, donor site, and age (359;384;387;446;454-457). Here, the metaphyseal portion of the medial proximal tibia was selected as the optimal donor site for periosteal explant extraction due to its easy accessibility during elective knee surgeries and characterised therapeutic potential. When examining both donor and regional donor site variations in the periosteum, explants from the proximal tibia have been shown experimentally to have a greater osteogenic and chondrogenic potential than periosteum harvested from the middle and lower tibia, calvarium, or skull (359;367). The regional differences in potential have been positively associated with the cellularity of the cambium layer, and have recently been shown to vary with age (394). During aging, the size and number of cells in the periosteum has been found to decrease along with the absolute and relative thicknesses of each layer (39;456;458;459). This reduction in cell number is thought to contribute to the apparent atrophy and thinning of the cambium layer that occurs with age (387). However, in our analysis of 7 sex-matched human donors we found that age had no apparent influence over the thickness or cellularity of either the cambium or fibrous layer. In fact, the cellularity of each layer was statistically similar across 6 of the donors tested and the thicknesses measured showed marked donor-to-donor variation with no direct correlation with age. The discrepancies noted between our histological analysis and previous studies may reflect the biological differences in human periosteum, used here, and animal periosteum investigated in the earlier studies. As the regenerative potential of the periosteum has been linked to the cellularity of the cambium layer, our histological analysis of cell density may further help to explain why the capacity for chondrogenesis is not lost upon aging (201).

The capacity to reproducibly harvest human periosteal explants of consistent size, structure, and cellularity, was important for the design of an effective sequential digestion strategy to isolate periosteal layer specific cells. The time scale for each digestion was based on the average thickness of the cambium and fibrous layers and rate of enzymatic tissue dissociation. This design allowed for the isolation of cell populations consisting of mostly cambium or fibrous layer cells. Although this cell separation was shown histologically (Figure 6.3), further characterisation of the individual digestions was necessary to confirm the success of this enrichment technique. Therefore, the morphology, phenotype, colony forming efficiency and growth profiles of initial
(Digestion Step 1+2) and final (Digestion Step 4) cell digestions were characterised and cross-compared.

Periosteal populations isolated from both initial and final digestions contained a multitude of cell types at varying numbers, including progenitor cells, osteoblasts, smooth muscle cells, lymphocytes, red blood cells, and adipocytes (all phenotypes shown previously in chapter 5). For this study, however, we focused on cells from osteogenic and hematopoietic lineages as they were the most prominent cell types present in the cultures. When grown in monolayer, initial digestion cells exhibited the rapid proliferation and subsequent sequential expression of phenotypic markers associated with the early stages of osteogenesis that has been characterised in previous calvarial and periosteal cell cultures (296;460). Our group (Chapter 5) and others (242;244) have shown independently that by employing dual staining techniques with developmental markers Stro-1 and ALP it is possible to identify subpopulations, that based on functional and phenotypic criteria, appear to represent cells at different stages of osteogenic differentiation; namely early progenitors (Stro-1+/ALP−), preosteoblasts (Stro-1+/ALP+), and maturing osteoblasts (ALP+/Stro-1−). Based on this assessment, cells of the final digestion initially contain more developmentally mature osteogenic cell populations than initial digestion cells. However, passage 0 initial digestion cultures demonstrated greater osteogenic potential, with larger populations of Stro-1+/ALP osteoprogenitor cells. The major and rapid increase in ALP expression in initial digestion cultures over time indicate that some of the cells, which initially did not express the ALP protein, have the ability to rapidly mature and differentiate, as would be expected for osteoprogenitor cells. Perhaps equally as interesting, however, final digestion cultures were also found to contain a small, yet consistent, population of Stro-1− osteoprogenitor cells. An observation that is consistent with previous immunohistological studies, where Stro-1− cells are found to be broadly expressed in the fibrous layer (394), particularly at the cambium/fibrous layer interface (461).

Upon plating, passage 0 initial digestion cells proliferated more quickly and reached a higher cell density (total cell number) than final digestion cells when cultured for 14 days. In addition, two cell densities were investigated to examine the impact of microcellular environment on the short-term self-renewal capabilities of the different cell isolates. In both low density (50 cells/cm²) and standard density (5000 cells/cm²) cultures, initial digestion cells displayed growth rates that were ~5x faster than final digestion cells over a period of 14 days. This trend is opposite to what was found in cultures of rabbit periosteal cells, where fibrous layer cells were shown to proliferate faster than cambium layer cells (398). This further highlights the cellular differences between human and rabbit periosteal cells, and suggests that rabbit models may not accurately reflect the true behaviour of human periosteal cells in-vitro (384). However, all isolated populations cultured at standard density exhibited elevated growth rates and population expansion compared to low
density cultures. These results are in direct opposition to features found in human bone marrow stromal cells in which the rate of expansion and yields of progenitor cells are shown to be inversely related to plating density (225). This discrepancy may be reflective of the cellular differences found between progenitor cell populations derived from the bone marrow and periosteum, and may emphasize the role that community effects or cell-cell interactions/secretable factors have on the maintenance and control of periosteal progenitor cell populations [(178;429)and Chapter 5].

Interestingly, this is only the second study to quantify the morphological heterogeneities present in human periosteal cultures, and the first attempt to define these differences in terms of periosteal layer. We have shown that initial digestion cultures contained, on average, significantly smaller and rounder cell populations compared to final digestion cell populations. These significant differences found are due to the presence of a morphologically distinct population of small, round, agranular cells that comprises roughly 35% of initial digestion cultures as confirmed by gating on flow cytometry histograms (R2=35.8%) and area distribution profiles (R1=35.6%). The size of this population is in contrast to a previous study detailing the physical attributes of passage 0 human periosteal cells, in which they found only 6% of the cells to be <2000 µm² in area, i.e. in gate R1 (Fig. 4C) (384). This stark contrast is most likely related to the fact that the previous cultures were derived from complete digestions of full thickness explants. This is supported in our analysis of full digestion cultures, in which no cells are found to be <2000 µm² in area.

Cell morphology has been strongly related to proliferative activity, with small round cells being associated with highly-proliferative, progenitor cells within bone marrow and periosteal tissues (225;384;462). Therefore, the increased growth rates, number of colonies, and colony sizes found in passage 0 initial digestion cultures are directly related to the presence of this unique cell population. To establish the phenotype of this distinct population we employed backgating techniques on flow cytometry histograms to reveal 78.8% of the Stro-1+/ALP- population to be present within the population of small cells (gate R2, Figure 6.5). However, this population of Stro-1+/ALP- cells represents only a small percentage (7.0%) of this unique population, and argues for the presence of a separate, Stro-1-, stem cell like subpopulation. Although this Stro-1- population has not been fully characterised here, two different hypotheses can be proposed to explain its existence. The first, and most orthodox hypothesis, is that the separate Stro-1- and Stro-1+ populations represent the hierarchical organisation of stem cells, where the Stro-1+ population represents the progenitor cell with restricted plasticity and capacity for self-renewal (227;463). This argument is congruent with the presence of functionally distinct osteoprogenitor and chondroprogenitor populations and even more primitive multipotent stem cells found previously in clonal studies of cultured periosteal cells (160;184). In her eloquent limiting dilution studies of rat bone marrow stromal cultures, Jane Aubin postulates a second hypothesis (178). The presence of
two different pools of progenitor cells: ones that differentiate in the absence of exogenously applied stimulus and ones that do so only in its presence and represent the majority of immature progenitors. Within the context of this study, the presence of more primitive stem cells or different populations of committed progenitors within this unique initial digestion population has not be fully elucidated and warrants further analysis. Unfortunately, the use of human monoclonal antibodies are restricted in their ability to delineate between these uncommitted cell pools, and currently enable the identification and enrichment of progenitors but not, more primitive stem cells (464). Yet in this study, which utilises standard media void of differentiation supplements, the combination of a consistent Stro-1+/ALP− population, rapid loss a of large population of small, highly-proliferative Stro-1− cells, and equally rapid increase in expression of developmentally mature ALP+ cells suggests that we too have isolated two unique periosteal progenitor cell populations from our initial sequential digestion. However under our culture conditions, we indirectly selected for the expansion of only the Stro-1+ progenitor population in initial digestion cultures. This secondary, culture-based, enrichment step identified here could potentially be limiting the differentiation and proliferative capacity of human periosteal cultures. The identification of a microcellular environment that maintains both, Stro-1− and Stro-1+, progenitor cell populations may work to enhance the therapeutic potential of periosteal cell cultures.

During monolayer expansion, the population of small, agranular, highly proliferative cells present in initial cell cultures is quickly lost and replaced by larger more granular cell populations comparable to populations found in final digestion cultures. Linear regression analysis of cell population doublings over 5 passages, clearly demonstrates the change in the initial digestion culture after passage 1 and the similarities in expansion potential with final digestion cells thereafter. The strong correlation found between growth rates and morphology in initial digestion cultures seems likely to be related to the rapid shift in population phenotype, from small, rapidly-proliferating developmentally primitive cells to large, slowly cycling, and more mature ALP+ cells. Equally, the consistent slower expansion rates seen in cultures derived from final and full periosteal digestions also demonstrated a large initial and consistent ALP+ population throughout the 5 passages. The relationship between cell size and growth capacity has been seen previously in full thickness periosteal cell isolates (Chapter 5) and cambium layer cells (398) and can be explained by the fact that a cell loses its proliferative potential during the differentiation and enlargement stage (465).

In comparing the sequential digestion cultures to cultures derived from fully digested periosteum, we found freshly isolated full digestion cultures to be more similar to final digestion cultures in terms of growth capacity, morphology, and phenotype. Upon subculture, however, we observed a mixture of trends and behaviours found in both sequential cultures and therefore can not make clear
comparisons to either. Importantly, however, full digestion cultures did not contain the morphologically distinct subpopulation composed of small, agranular, highly-proliferative cells with high osteogenic potential. In support of this, previous work performed is this thesis (Chapter 5) and by others (295;384;390;398) characterising human periosteal cells derived from fully digested periosteal explants also failed to observe this distinct population of cells. The absence of this population in full digestion cultures suggests that, as yet unidentified, cells and/or biological molecules intrinsic to the fibrous layer of the periosteum may act to suppress or change the characteristics and propagation of this unique cell population of the cambium. The existence of specific cells and biological factors resident to each tissue layer may also help to explain the time dependent increase in CD34+ cells in final digestion cells. Since the periosteum fibrous layer contains an extensive vascular network, it is likely that it would contain intrinsic environmental factors to support the propagation of numerous haematopoietic cell types in culture. Equally, the loss of the unique highly-proliferative population in initial digestion cultures suggests that this population requires a particular microcellular environment, not provided in this study, to maintain its ability to self-renew.

The differential cell characteristics and population behaviours noted between each digestion further demonstrates the capacity of our sequential digestion method to enrich cells according to periosteal tissue layer. In initial digestion cultures, composed mostly of cambium cells, we observed characteristics consistent with the enrichment of precursor cells, including significantly more and larger CFUs, increased proliferation rates and cell yields, and a rapid increase in osteoblastic phenotypes. These characteristics are directly related to the presence of a subpopulation of small, agranular cells, containing both Stro-1+ and Stro-1− phenotypes, unique to initial digestion cultures. Conversely, the lack of this unique subpopulation from final and full digestion cultures indicates that enzymatically digesting full periosteal explants may prove unsuitable for the isolation of all developmentally immature stem/progenitor cell populations known to reside in the cambium layer.

6.5 Conclusions

In conclusion, we have devised a simple enrichment method utilising sequential digestions to isolate distinct populations of human periosteal cells from the cambium and fibrous layer. To our knowledge, this is first study to detail the distinct morphological, phenotypic, and growth characteristics of cells from the individual tissue layers of human periosteum in a monolayer culture environment. Similarly, this is also the first study to isolate a morphologically unique subpopulation of small, rounded, agranular, highly-proliferative cells with an increased capacity for osteogenesis from human periosteum tissue. The fact that this subpopulation was absent from final digestion and full digestion cultures demonstrates the underlying differences in the microcellular environment present in each of the cultures and highlights the influence that accessory cells and
associated biomolecules have on stem and progenitor cell populations. Further analysis of this unique cell population and its surrounding microenvironment may lead to the development of improved periosteal-cell based treatment for orthopaedic injuries. Lastly, the ability to further dissect the differential characteristics and cellular behaviours in the periosteal layers may also help us to develop a deeper understanding of the fracture healing process and the regulation of bone growth and resorption during developmental, homeostatic, and diseased states.
Chapter 7

Identification of Rare Progenitor Cells from Human Periosteal Tissue using Droplet Microfluidics

7.1 Introduction

The ability to identify and isolate single cells from heterogeneous populations is an important procedure in a variety of experimental and clinical applications. Segregated cells, representing subpopulations, can provide important information about a wide range of cell and population parameters and thus extend the understanding and control of biological processes. In particular, the development of single-cell tools would allow the detection of rare tumour cells in blood for disease prognosis (235), provide an assessment of disease progression rates in human immunodeficiency virus (HIV) infection (466), and allow selective targeting of malignant cells (467). Moreover, enrichment steps will be necessary when creating novel stem cell and gene therapies, which rely upon the selection and characterisation of rare stem cell populations identified in numerous adult tissues (227-232). In the context of this thesis, enrichment steps are also needed to simultaneous identify and isolate reparative periosteal progenitor cell types for further characterisation and therapy development.

To date, a variety of isolation strategies have been employed for this task, including fluorescent activated cell sorting (FACS), limiting dilution, density centrifugation, magnetic sorting, and adherence to glass and plastic surfaces (233;234). Most popular amongst these techniques is FACS, which is a specific type of flow cytometry that allows each cell to be characterised and sorted according to a range of distinct biochemical and biophysical characteristics. Exploiting this strategy, rare cell types such as stem cells, antigen-specific B and T cells and circulating tumour cells have been identified and isolated (235-239). These successes are, in part, due to developments in fluorophore and antibody production and availability, which have led many researchers to focus their purification efforts on immunophenotypic assays utilizing cocktails of monoclonal antibodies (240-247). In this way, rare stem/progenitor cell populations are targeted based on the expression of intra-/extra- cellular molecules.

Although these popular techniques have enjoyed widespread use over recent years and have been employed in this thesis, new analytical technologies offer the promise of improved analytical performance, enhanced functionality and robust versatility in a variety of cell-based assays (250-
Amongst these technologies, droplet microfluidic systems offer the possibility of rapid, specific and detailed analysis of cell populations. In these systems, picolitre-sized droplets generated from immiscible phases can be produced at high frequencies (in excess of 1 KHz) and can act as discrete and isolated reaction compartments to study a number of biological and chemical processes (256). In this context, droplet microfluidic systems have been used as an analytical tool to examine enzyme kinetics (257), libraries (258), and assays (259), protein crystallisation (260;261) and biological assays (262;263). More recently, droplet microfluidic systems have been successfully demonstrated to effect single cell encapsulation and manipulation (255;262;264). Accordingly, such droplet systems have the potential to define a new high-throughput screening platform that can sample, encapsulate, manipulate, process and detect single cells and more importantly the presence of rare cell phenotypes. For cell biologists and tissue engineers alike, the compartmentalization provided by the droplets is particularly valuable for precisely analyzing multiple cellular characteristics simultaneously. An initial demonstration of this biological potential was recently provided by Huebner et al (262) and Koster et al (265), where droplet-based microfluidic systems were able to quantify protein expression and cell viability and metabolism.

The studies presented in this chapter significantly extend these proof-of-principle experiments and describe the use of droplet microfluidics to identify and quantify the presence of rare mammalian cell phenotypes within heterogeneous populations of human periosteal cells. Using fluorescent-conjugated antibodies against distinct cell-surface proteins, alkaline phosphatase and Stro-1, we were able to target cells at different stages of differentiation and lineage commitment. Based on the time-integrated fluorescence signal of these markers, the identification and enumeration of three developmentally distinct cell phenotypes was demonstrated. Additionally, to test the accuracy and diagnostic capability of the microfluidic approach, results were compared to expression levels obtained using traditional flow cytometry techniques. For this study, adult human periosteal tissue represents an ideal cell source due to its rich cellular heterogeneity and its potential for therapeutic applications in the repair and regeneration of musculoskeletal tissues (30;31;159;361;362;401). As demonstrated previously, periosteal cells can be easily derived from small periosteum tissue explants harvested from adult donors and extensively expanded ex-vivo. Phenotypically, periosteal cells are composed of a heterogeneous population of mesenchymal and hematopoietic cell types at different stages of maturation and lineage commitment, including a small subset of stem cell like cells (160;186;295). Secondly, droplet microfluidics can be used to validate the precision and accuracy of flow cytometry-based human periosteal cell immunophenotype studies performed in Chapter 5 and 6.
Chapter 7: Identification of rare progenitor cells from human periosteal tissue using droplet microfluidics

7.2 Materials and Methods

7.2.1 Harvest of periosteal tissue and isolation of cells

Periosteal tissue specimens were obtained from patients (providing informed consent and between the ages of 31-73 years) undergoing elective knee surgery at Chelsea and Westminster Hospital, London. Explants were harvested from the proximal tibia using a periosteal elevator, and transported in Earl’s Balanced Salt Solution at 37°C. Explants were weighed, finely minced, and enzymatically digested with 3 mg ml⁻¹ collagenase D (Roche Applied Science, West Sussex, UK), 3 mg ml⁻¹ collagenase type II (Sigma-Aldrich, Poole, UK) and 5 mM CaCl₂ in growth medium (high glucose Dulbecco’s Modified Eagle Medium containing 10% v/v foetal bovine serum, 1% v/v L-glutamine, 100 units ml⁻¹ penicillin, 100 µg ml⁻¹ streptomycin) (all from Invitrogen, Paisley, UK). After incubation at 37 °C for 4.5 hours, the cell suspension was washed, passed through a 70 µm cell strainer (Falcon, Becton Dickinson, Oxford UK), and counted. Cells were then placed into monolayer culture and allowed to attach to tissue culture plastic for a period of four days. The media was changed to remove non-adherent cells and the attached cells were further cultured as passage 0 until confluence. All characterisation experiments were carried out using serially expanded periosteal cells between passage 0 and 10.

7.2.2 Antibody staining

Antibodies raised against Stro-1 and human bone/liver/kidney alkaline phosphatase (ALP) were tested for their specificity, titrated, and used at concentrations varying from 1-2.5 µg/10⁶ cells. Stro-1 is a yet unidentified antigen present on the surface of developmentally primitive cell types with osteogenic potential. Under certain culture conditions Stro-1⁺ cells are able to give rise to an array of stromal cell types, including smooth muscle cells, adipocytes, chondrocytes, and osteoblasts (240;248;249). The bone/liver/kidney isoform of ALP, although not specific to the osteogenic lineage, is a well-documented marker of committed osteoblasts (468). Together, these two independent antibodies were used to dissect human periosteal cell populations according to surface protein expression.

Approximately 1-3×10⁵ periosteal cells of passages 0-10 were incubated in 1 ml of blocking buffer (phosphate buffered saline pH 7.4 0.01M (PBS) containing 2% (w/v) bovine serum albumin (BSA), 0.1% (v/v) foetal bovine serum, and 0.1% (v/v) normal mouse serum) (all from Sigma-Aldrich) for 30 minutes at room temperature to limit non-specific binding. For direct staining, cells were then incubated with APC (define)-conjugated ALP for 45 minutes at 4°C, washed in PBS containing 2% BSA (w/v) + 0.1% (w/v) sodium azide (flow buffer), centrifuged, and resuspended in 300 µl of flow buffer prior to analysis. For indirect staining using primary mAb Stro-1, the cells were first
incubated with the primary mAb (45 min at 4°C), washed, and incubated with a fluorescein isothiocyanate (FITC)-conjugated fragment F(ab’2) goat anti-mouse secondary Ab (Jackson ImmunoResearch, Suffolk, UK) for 45 minutes at 4°C. Matched isotypes were used as negative controls and prepared at the same concentrations as their matched primary Ab. To facilitate an even supply of cells to the microdevice, 25% Percoll (Sigma-Aldrich), filtered using a 5 μm syringe filter, was added into the final cell suspension. Percoll is a suspension of colloidal silica particles (15-30 nm diameter) coated with polyvinylpyrrolidone (PVP) that alleviates cell sedimentation during the transferral of cells into the microfluidic device (262).

7.2.3 Flow cytometry

Cell fluorescence was evaluated by flow cytometry using a FACSCalibur instrument (BD Biosciences, Oxford, UK) with data analysis performed using FlowJo software (Tree Star Inc, Ashland, USA). At least 20,000 discrete events were collected per sample and the percentage positive expression for each mAb was defined using unstained and isotype-matched controls. Based on the light scattering characteristics of each sample population, dead cells, cell debris, and aggregates were eliminated from further phenotypic analysis. For dual-color phenotypic analysis and enumeration, the gating combinations were defined as Stro-1+/ALP-, Stro-1+/ALP+, and ALP+/Stro-1-.

7.2.4 Microfluidic device fabrication

Microfluidic devices were fabricated by standard soft lithographic processing of poly(dimethylsiloxane) (PDMS). SU-8 masters on 4 inch diameter silicon wafers (IDB Technologies, UK) were lithographically patterned to serve as PDMS moulds (469). A 10:1 (w/w) mixture of base (tetra(trimethylsiloxy)silane) and curing agent (tetramethyltetravinyl cyclo tetrasiloxane) from a SYLGARD 184 Silicone Elastomer Kit (Dow Corning Limited, UK) was degassed and poured onto a master and then cured at 65°C for between 4-5 hours, after which PDMS replica was peeled off. For cell delivery, large access holes (~1 mm in diameter) were punched into PDMS channel ends using a syringe needle. To complete, the structured PDMS substrate was contacted with a 1 mm-thick microscope slide that acted as a support structure. Access holes in the microscope slide were drilled using a 1-mm-diameter diamond dental drill bit (Diama International, Inc., London UK). These holes coincided with inlet and outlet reservoirs in the PDMS layer. Fused-silica capillaries (375 μm o.d., 150 μm i.d., Composite Metal Services LTD, Hallow, UK) were then inserted through the holes of the microscope slide and glued using a two-part epoxy (Araldite 2014, RS Components, Corby, UK). These capillaries served as a fluidic interface between the microfluidic device and macroscale reservoir. The microscope slide with capillary connection was then bonded to the PDMS replica using an oxygen plasma treatment. To
allow efficient optical interrogation using high numerical aperture objectives, PDMS microchannels were enclosed permanently with a 160 μm microscope cover slip by oxygen plasma bonding. The completed PDMS device consisted of two inlets and an outlet, having a 50 μm deep, 50 μm wide and 4 cm long channel.

### 7.2.5 Droplet formation

Droplets were generated using a T-junction microfluidic flow geometry incorporating 1 aqueous inlet as shown in Figure 7.1A. The cell suspension with 25% Percoll loaded into a 250 μl gas tight syringe (SGE Europe Ltd, UK) was motivated into the perpendicular inlet at a flow rate of 1.0 μl min⁻¹, while an oil solution, a 1:2 v/v mixture of an electronic coating liquid (EGC-1700, 3M) and 3M fluorinated fluid FC-3283 (3M) with a 1:10 v/v dilution of 1H,1H,2H,2H-perfluorooctanol, was delivered at a flow rate of 1.5 μl min⁻¹ using a 0.1 ml gas tight syringe (SGE Europe Ltd, UK). The syringes were connected to the microfluidic device (via the capillaries) using Teflon tubing (0.356 mm i.d., 1.55 mm o.d., Upchurch Scientific) and PEEK fingertight unions (1/16", 10/32 threads, VICI AG International, Schenkon, Switzerland). All solutions were pumped into microfluidic channels using precision syringe pumps (PHD 2000, Harvard Apparatus, UK).

Figure 7.1 (A) A schematic of the microfluidic device having a 50 μm deep, 50 μm wide and 4 cm long channel. The experiment was carried out by pumping the oil and cell suspension at 1.5 μl min⁻¹ and 1.0 μl min⁻¹, respectively. The inset depicts an image of a cell which is encapsulated within a droplet moving along the microchannel. (B) A schematic diagram of a confocal fluorescence setup used for droplet detection. This setup consists of a two colour excitation system using 488 nm diode and 633 nm He/Ne lasers. APD – avalanche photodiode detector, DC – dichroic mirror, EM – emission filter, PH – pinhole and L – lens.

### 7.2.6 Optical detection system

A custom built confocal spectrometer in a combination with an Olympus IX71 microscope was used for droplet detection. This confocal spectroscopic setup, schematically shown in Figure 7.1B,
consists of a two colour excitation system using 488 nm diode (Coherent UK Ltd., UK) and 633 nm He/Ne (Spectra Physics) lasers. Beam steering optics are used to align the laser beams within the microscope. A dual-band dichroic mirror (z488/633rdc, Chroma Technology Corporation, USA) is installed to allow reflection of 488 and 633 nm laser beams into a 60x water immersion objective lens (Olympus). This objective lens focuses the excitation radiation into the microfluidic channel, with the entire device being placed on a controllable stage (ProScan II™, Prior Scientific) of the microscope. The two laser beams are aligned to generate completely overlapped spots that ensure coincident excitation of droplets. This is achieved by aligning two laser spots using an image from a CCD camera (Cascade II, Photometrics).

Fluorescence emission is collected by the same objective, passed through the dichroic mirror and then filtered by a dual-band emission filter (z488/635, Chroma Technology Corporation, USA) to remove residual excitation light. After that, the fluorescence signal is focused onto a 75 μm pinhole (Melles Griot, UK) using a plano-convex lens (+50.2F, Newport Ltd.). Another dichroic mirror (630dxcr, Chroma Technology Corporation, USA) splits the fluorescence emission into two paths to be simultaneously detected by two avalanche photodiode detectors (APDs) (AQR-141, EG&G, Perkin-Elmer). The reflected fluorescence signal is filtered by an emission filter (hq540/80m, Chroma Technology Corporation, USA), and then focused onto the first detector (green channel) using a plano-convex lens (f = 30.0, i.d. 25.4 mm, Thorlabs, UK). The transmitted fluorescence emission is filtered by another emission filter (hq640lp, Chroma Technology Corporation, USA) and then focused by another plano-convex lens onto the second detector (red channel). The output of the detectors is concurrently sent to a multifunction DAQ device for data logging (PCI 6602, National Instruments). This enables both detectors to have sub-microsecond time resolution per channel.

### 7.3 Results and Discussion

#### 7.3.1 Cells trajectories within droplets

A suspension of human periosteal cells in PBS containing 1% BSA and 25% Percoll (~500000-1000000 cells ml⁻¹) was immunostained using FITC and APC conjugated mAbs and loaded into the microfluidic device to generate droplets. Importantly, the encapsulation and confinement of cells in droplets removes the possibility of cell-surface interaction. Moreover, the inclusion of a coating liquid (EGC-1700) in the oil solution renders a clear and thin uniform film (~0.1 μm thick). The thin film coating the microchannel walls prevents any adsorption phenomena that will bias the analysis. This on-line coating serves as an alternative to silane pre-treatment (470). On-line droplet detection was performed at 4.0 mm from the droplet forming region. As droplets (containing single cells) travel past the detection region (4 mm from the droplet forming region), the contained cells
are coincidently excited by the two laser probe volumes at the centre of the channel. Based on the fluorescence emission spectra and intensity of each cell-droplet, we are able to confirm the expression levels of cell-surface antigens Stro-1 and ALP. The fact that these antigens are present on distinct cell types allowed us to target three different developmental phenotypes of the osteoblast lineage. Two previous studies and previous results shown in the thesis [(242;244) and Chapter 5 & 6] have independently shown that by employing dual staining techniques with developmental markers Stro-1 and ALP, it is possible to identify sub-populations, that based on functional and phenotypic criteria, appear to represent cells at different stages of osteogenic differentiation; namely early progenitors (Stro-1+/ALP−), preosteoblasts (Stro-1+/ALP+), and maturing osteoblasts (ALP−/Stro-1−). Progenitor cells stained with Stro-1-FITC were only excited by the 488 nm excitation radiation and emit ‘green’ fluorescence, while ALP-APC stained pre- and mature- osteoblast cells were only excited by the 633 nm excitation radiation and emit ‘red’ fluorescence. Due to dual labelling, pre-osteoblast cells are excited simultaneously by both lasers and thus emit both ‘green’ and ‘red’ fluorescence. Fluorescence bursts corresponding the transit of single cells through the detection volume were recorded at 50 μs resolution for a 60 s acquisition time. It was noticed that single cells inside the droplets were preferentially located in the middle of the channel width, as seen in Figure 7.1A, ensuring that every cell passage was detected.

An example of a fluorescence burst scan recorded over time period of 5 seconds for both green and red detection channels is shown in Figure 7.2. Droplet signatures from the green and red channels were coincident and approximately constant in height. It should also be noted that the fluorescence background present in all experiments is due to cell autofluorescence and the presence of unbound antibody left in solutions after staining. Attempts to reduce this non-specific fluorescent background resulted in an inability to clearly define positive and negative staining in droplets. In fact, the background fluorescence proved advantageous in some respects and was utilised to define droplet location. The transient ‘spiked’ peaks located on top of the background droplet signal correspond to the transit of a cell through the detection volume. Variation in the fluorescence intensity of such peaks was observed in both the green and red channels. This disparity is likely to be a consequence of a variable number of fluorophores being excited, which in turn is defined by a distribution of residence times of cells within the optical detection volume.
Chapter 7: Identification of rare progenitor cells from human periosteal tissue using droplet microfluidics

Figure 7.2 An example of fluorescence burst scan of 5 s traces recorded. Droplet signatures obtained from green and red channels are coincident and have almost the same height. Spiked peaks on top of the droplet signals signify the presence of cells. The inset illustrates an expanded 300-ms part of the main trace. The spiked peaks in the green and red channels represent stem cells and osteoblast cells, respectively. Co-incident events from both channels correspond to pre-osteoblast cells.

7.3.2 Cell analysis

All data originating from the droplet system were analysed using MatLab version R2007a (Mathworks, Cambridge, UK). The number of specific cells from each channel was counted to quantify each cell subpopulation of interest. MatLab algorithms, adapted from our previous work in single molecule and particle detection(262), were used to differentiate between background counts and droplet signatures to define droplet localisation. To determine single cell events, a threshold (Thr) was set using 2 standard deviations of the droplet mean height. This threshold is characterised as rectangular-shaped signal, as shown in Figure 7.3A. Accordingly, every peak above this threshold value was considered to originate from a single cell event. Due to the existence of autofluorescent proteins and molecules on and/or within cell membranes, the cell peaks observed were not solely representative of stained cells. Accordingly, appropriately matched-isotype control antibodies were used in parallel with positively stained cell samples to determine the level of background fluorescence and the number of unstained cells. The number of events calculated from the control samples was subtracted from the number of events originating from the stained samples to quantify the absolute numbers of stained cells in each subpopulation. Surprisingly, many ‘small’ peaks, having intensities just above the threshold, were observed in the positively stained samples and not in isotype control samples. These peaks will cause an error in the number of positive cells calculated in each subpopulation after subtraction. These small peaks are most likely due to
unbound antibodies left in solution and not a result of cell autofluorescence, since they were only found in stained samples. In addition, the stained cell peaks should be significantly higher than the threshold intensity. Consequently, a secondary threshold, namely ‘search above’ (SA), was established to allow discrimination of these small peaks. The SA value is defined according to,

\[
SA = Thr + \frac{3}{2}Thr
\]  

By using this additional threshold parameter, only peaks above the SA value are called and defined as cell peaks. A probability distribution of cell occupancy (per droplet) is illustrated in Figure 7.3B. Under the flow rates operated and sample cell density, single cell occupancy per droplet was achieved. Specifically the instantaneous occupation probabilities are 75% (for an empty droplet), 20% (for single occupancy) and 5% (for double occupancy). It should be noted that the cell occupancy in each droplet can be easily controlled by changing flow rates and cell loading. Data obtained from the current experiments were recorded over approximately 1000 droplets, which is enough for ensemble cell quantification. Additionally, average cell location within droplets was extracted from the temporal variation in fluorescence intensity. Inspection of Figure 7.3C illustrates that most cells are localised towards the front of a droplet (in the direction of flow).

Figure 7.3 Cell characterisation. (a) An example of threshold setting in the green channel. A threshold was established above the droplet height to define cell peaks. The threshold was represented as rectangular-shaped signal. (b, c) Probability distributions of cell occupancy per droplet and cell localisation within droplets, respectively.
7.3.3 Analysis of co-incident events

To quantify each cell population (in terms of the relative proportion of progenitor cells, pre-osteoblasts and osteoblasts), co-incident events between the green and red channels were evaluated. Co-incident peaks are defined by an exact temporal match between peaks in both green and red channels. If peaks are offset by more than 1 ms, they are not considered to be co-incident. An example of such co-incident data is shown in Figure 7.4. In this sample, 70 co-incident events were identified, indicating the number of preosteoblast cells possessing both ALP and Stro-1 markers (Stro-1+/ALP+) present. Subtraction of these co-incident events from the total number of cells called in the green and red channels provides the true number of only green and red cells, i.e. progenitor cells and osteoblasts, respectively.

![Figure 7.4](image)

**Figure 7.4** Co-incident events between green and red channels. The number of co-incident peaks denotes pre-osteoblast cell populations.

7.3.4 Flow cytometry analysis

When detecting rare cells within heterogeneous populations, it is crucial that one be able to objectively discriminate between true staining of cells by immunofluorescence methods and experimental staining artefacts, which includes dead cells, aggregates, non-specific staining, and autofluorescence. This is particularly important for flow cytometry-based methods where accuracy and interpretation of the results are defined in large part by the expertise of the user. Accordingly, numerous steps were taken to improve the accuracy and reproducibility of the analytical data relating to the cell populations under study. First, mouse serum was used to block non-specific Fc receptor binding sites present on the surfaces of cells, thereby reducing the number of false positives present in each analysis. Second, cell debris and staining aggregates were eliminated from further analysis based on their distinct light scattering characteristics (Figure 7.5A). Third, to
reduce high levels of autofluorescence exhibited by periosteal cells, the APD operating voltages were decreased so that >85% of the signals from the unstained cells appeared in the first decade of each fluorescence channel (Figure 7.5B). Attempts to further decrease the voltage resulted in a significant reduction in instrument sensitivity and an inability to discern positive staining. Lastly, isotype controls matched to each primary mAb were used to set the gates used in determining the true proportions of cells positively expressing each antigen (Figure 7.5C). For each antibody or antibody combination, positive expression was defined as the percentage of stained cells with fluorescence levels ≥ 99% of the corresponding isotype-matched control antibody stained cells.

Flow cytometric analysis of periosteal cell light scattering characteristics revealed the presence of a heterogeneous mixture of cells with varying sizes (FSC) and granularity (SSC) (Figure 7.5A). This observation confirms previous cell characterisation studies in this thesis which detailed the presence of morphologically and phenotypically heterogeneous cell populations. The immunophenotypic analysis, presented here, revealed three distinct cell phenotypes based on the differential expression of antigens Stro-1 and ALP. A representative example of the gating schemes and resulting expression levels of each target phenotype present in periosteal cells derived from one donor is shown in Figure 7.5. Across all donors, periosteal cells demonstrated a heterogeneous
pattern of expression for both the Stro-1 and ALP antigens. The majority (>70%) of periosteal cells demonstrated no detectable levels of Stro-1 or ALP at the surface (Stro-1⁻/ALP⁻). A high proportion of the remaining periosteal cell populations expressed the osteoblastic maker ALP exclusively (14.1 ± 11.2%, mean ± SD), while only minor populations were found to express the Stro-1 antigen (3.4 ± 3.6%). While inter-donor variations in phenotype were found, this experimental set-up and gating system enabled us to objectively and reproducibly identify and enumerate distinct cell phenotypes in heterogeneous populations. This proves that flow cytometry provides an analytical platform to compare droplet microfluidics against.

### 7.3.5 Droplet-based microfluidics versus flow cytometry

To prove the accuracy and biological capability of the droplet-based microfluidic system, cell characterisation results from the microfluidic system were correlated with flow cytometry data. Phenotypic results were compared in periosteal cell cultures derived from 4 different donors aged 31-73 yrs. For both analytical techniques the same sample population was used, so as to allow for direct comparison and reduce the likelihood of population differences. Comparative results are presented in Table 7.1, in which Stro-1⁺/ALP⁻, ALP⁺/Stro-1⁻ and Stro-1⁺/ALP⁺ symbolise progenitor cells, osteoblasts and pre-osteoblasts respectively.

<table>
<thead>
<tr>
<th>Donor</th>
<th>Stro-1⁺/ALP⁻ (% total)</th>
<th>ALP⁺/Stro-1⁻ (% total)</th>
<th>Stro-1⁺/ALP⁺ (% total)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FC</td>
<td>Droplet</td>
<td>FC</td>
</tr>
<tr>
<td>31 yrs male</td>
<td>4.31</td>
<td>4.08</td>
<td>3.78</td>
</tr>
<tr>
<td>73 yrs male</td>
<td>10.12</td>
<td>8.23</td>
<td>9.35</td>
</tr>
<tr>
<td>32 yr male</td>
<td>0.70</td>
<td>0.75</td>
<td>13.71</td>
</tr>
<tr>
<td>43 yrs female</td>
<td>0.29</td>
<td>0.38</td>
<td>29.64</td>
</tr>
</tbody>
</table>

Table 7.1 Identification of stage-specific cell phenotypes using flow cytometry and droplet microfluidics. Data are displayed as percentages of total cell populations examined. N/a = no measurements due to lack of cells.

When comparing the results generated using flow cytometry (FC) with droplet-based microfluidics, we found that both systems detected almost identical expression levels of each phenotype in each donor. Similarly, these population percentages, particularly Stro-1⁺ cells, are consistent with expression levels measured in other stem cell sources; such as the trabecular bone explants, bone marrow, synovium, adipose tissue, and muscle (242;422;471). The small differences (± 1.9 %) noted in the expression levels between the two analytical techniques were most likely due to discrepancies between the user-defined gates and thresholds. Furthermore, the droplet-based
microfluidics approach was able to detect both minute and substantial donor dependent differences in each target phenotype; particularly the ALP⁺/Stro-1⁻ population, which was expressed in <3% to >29% of the total population. This wide range in sensitivity is important to many biological based assays, which are reliant on the ability to detect both small- and large- scale changes in cell characteristics and functions. These include the identification of phenotypic changes in cells due to donor age, sex, and health, and cell culture microenvironment (226;359;368;376;386-388;472).

7.4 Conclusion

This study demonstrates that droplet-based microfluidics is an accurate and reproducible strategy to simultaneously isolate and detect typical (ALP⁺) and atypical (Stro-1⁺) cell types present in heterogeneous cell cultures. By controlling fluid streams in microchannels, we were able to compartmentalise single cells and direct droplet-laser interactions to measure cell surface properties. These data (measured for each individual cell) when correlated with other cell characteristics, allowed us to easily obtain information on how a targeted parameter is distributed in a cell population. Moreover, single-cell measurements performed using the droplet-based microfluidics platform were found to be in excellent agreement to results generated using conventional flow cytometry. This confirms the biological capacity of droplet-based microfluidics and further verifies this technology as an accurate and reliable alternative to flow cytometry-based assays for cell characterisation.

For therapy development, droplet microfluidics represents a potentially fast and efficient clinical platform technology for purifying and further characterising heterogeneous populations. The ability to target cell surface markers expressed selectively by distinct cell types is particularly useful for the enrichment of stem cells, which are found to reside in a variety of tissues at extremely low copy numbers (e.g. about 0.01-0.001% of total bone marrow cells(163)). The isolation of rare cells is, of course, entirely dependent on their identification. Accordingly, this study is the very first step towards the development of a novel microfluidics-based cell-sorting platform. As shown here, droplet microfluidics provides a simple route to physically encapsulating and simultaneously characterising single-cells. Future experiments will now focus on the use of droplet-based microfluidic systems combined with external electric fields to safely and effectively separate viable cells from heterogeneous populations. This will define the next generation of high throughput single cell assays using droplet-based microfluidics.

Ultimately, droplet microfluidics is a robust tool that has the potential to simplify complex biological processes so that we may better comprehend their interactions, functions, and role in biology. Apart from stem cell research, this technology would be hugely useful in numerous application fields, including immunology, microbiology, parasitology, and oncology.
Chapter 8

Dynamic Compressive Strain Influences Chondrogenic Gene Expression in Human Periosteal Cells

8.1 Introduction

Various tissue engineering and regenerative medicine strategies, have exploited the therapeutic potential of periosteal cells through use of tissue grafts (147;189), explants (190;362), or isolated cells with or without an associated 3D scaffold (30;186;201;473). As shown in this thesis and previous studies, periosteal cells hold promise in osteochondral repair applications due to their ease of isolation, expansion potential in-vitro, and ability to differentiate into multiple mesenchymal lineages (160;295). All these approaches would benefit from a thorough exploration and optimisation of the governing factors controlling periosteal cell osteogenesis and chondrogenesis.

Periosteal cells play a role in the development and maintenance of the musculoskeletal system (47;395;474), including long-bone growth, bone fracture repair, and bone remodelling. In embryonic and postnatal bone development, radial growth of long bones involves the direct conversion of mesenchymal precursor cells in the periosteum to osteoblasts (433;475;476). In fracture healing, the periosteum is responsible for bridging the callus formation and participating in endochondral and intramembranous ossification (477;478). The migration, proliferation, and differentiation of periosteal precursor cells during these processes are coordinated by a variety of systemic, local, and mechanical factors. The effects of systemic hormones (404;405;479;480) (e.g. growth hormone, parathyroid hormone, bisphosphonates, sex steroids) and local growth factors and cytokines (395;406;407;481;482) (e.g. FGF, TGF, BMP, IGF, VEGF, interleukins, tumour necrosis factor) on periosteal cell proliferation and differentiation have been well-studied and characterised both in-vivo and in-vitro. Mechanical control mechanisms over periosteum function, however, have received considerably less attention.

In-vivo, it has been suggested that mechanical loading environment is a primary modulator of bone apposition rate (409;454;483) and secondary chondrogenesis (484) on periosteal surfaces during joint formation and post-natal growth. Attempts to characterise the biological responses of the periosteum to mechanical loading have led researchers to develop small-animal models using externally applied loading devices (408;485-489). By applying mechanical forces in-vivo, the
expression of a variety of osteogenic and chondrogenic genes in the periosteum can be induced (408;490) and rapidly transforms quiescent periosteal surfaces to those on which bone formation occurs (491). Based on this link between local physical milieu and periosteum function in-vivo, it is speculated that mechanical forces could be used as a means to induce and control periosteal cell differentiation. Based on experimental studies and corresponding finite element models of the bone repair process and articular cartilage development, Carter et al proposed a mechanobiological theory that states low stresses and strains cause bone formation, hydrostatic compressive stresses stimulate chondrogenesis, and high tensile strains produce fibrous tissue in developing/regenerating skeletal tissues (279;409;492). Indeed, previous studies have suggested that compressive loading regimes can stimulate chondrogenic differentiation of MSCs (297;493;494) and enhance cartilage-specific matrix formation of differentiated chondrocytes (495-497). To date, however, there are no comparable studies published on the effects of mechanical stimulation on human periosteal cells in-vitro.

This study aims to determine whether compressive mechanical strains provide early chondro-inductive effects in isolated human periosteal cells. This required the development of a 3D culture system and determination of appropriate conditions for applying controlled external loading regimes. Accordingly, expression patterns of early response genes will be examined in periosteal cells cultured in 3D agarose constructs under dynamic compression strain in the presence or absence of TGF-β.

8.2 Materials and Methods

8.2.1 Human periosteal cell culture

For all optimisation and mechanical stimulation experiments, human periosteal cells were enzymatically isolated from periosteum explants and cultured in monolayer as described in detail in Chapter 5. Briefly, explants were harvested from the proximal tibia of donors using a periosteal elevator, and transported in Earl’s Balanced Salt Solution (EBBS, Invitrogen, Paisley, UK) at 37 °C. Explants were weighed, finely minced, and enzymatically digested in a collagenase solution consisting of 3 mg/ml collagenase D (Roche Applied Science, West Sussex, UK), 3 mg/ml collagenase type II (Sigma-Aldrich, Poole, UK), and 5 mM CaCl2 in growth medium (high glucose Dulbecco’s Modified Eagle Medium containing 10% v/v foetal bovine serum, 1% v/v L-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin [all from Invitrogen, Paisley, UK]). After incubation at 37 °C for 4.5 hours, the cell suspension was washed, passed through a 70µm cell strainer (Falcon, Becton Dickinson, Oxford UK), and counted. The cells were expanded in monolayer at the seeding density of 5000 cells/cm² for up to 10 passages in growth medium. Upon confluence periosteal cells were recovered from monolayer culture by enzymatic treatment with
0.25% (v/v) trypsin and 1mM EDTA and replated at the optimised density of 5000 cells/cm² in a T75 flask containing 15 mls of growth medium. Viable cell number was determined using trypan blue dye exclusion and a haemocytometer. To examine the phenotypes present in each culture during expansion, periosteal cells were stained with monoclonal antibodies against Stro-1 and ALP and analysed using flow cytometry (for methodology see Chapter 5). All experiments were carried out using isolated periosteal cells serially expanded from passage 1-6 from 2 different donors.

8.2.2 Preparation of periosteal cell-agarose constructs

A 6% (w/v) low gelling temperature agarose (type VII; Sigma) solution was prepared in EBSS and sterilised by autoclaving. The agarose was then cooled to 37°C and an equal volume of a periosteal cell suspension was added to the agarose, to yield a final concentration of 3% (w/v) agarose. To prepare 3-dimensional constructs, the cell/agarose suspension was added by Pasteur pipette to cylindrical wells of a stainless steel casting mould of 6 mm diameter and 4 mm depth. This casting mould was bound at the top and bottom surfaces by glass slides and allowed to gel for 10 minutes at 4°C.

8.2.3 Effect of seeding density on cell viability

The effect of nutrient diffusivity on cellular viability was examined in order to mitigate the challenges associated with the preparation and culture of 3-dimensional cell-seeded constructs. This was done by comparing the effects of cell-seeding density on cellular viability. Cell constructs were produced as described above with final cell densities of 0.5, 2.0, and 8.0 x 10⁶ cells/ml in 3% (w/v) agarose. The resultant constructs were washed 2X in EBSS and cultured flat in individual wells of a 24-well plate (Nunc; Fisher Scientific, Loughborough, UK) and supplied with 1 ml of growth medium. The constructs were incubated in a humidified environment at 37°C and 5% CO2 for 10 days with media replacement every 2 days. At days 2, 4, and 10, three constructs per density were removed from culture, sectioned vertically, and incubated for 1 hr at 37°C with 5 µM calcein-AM and 5 µM ethidium homodimer-1 (Molecular Probes; Paisley, UK) in 2 mls of complete media. The constructs were laid cut-side down on a cover slip and placed on a stage of an inverted fluorescent microscope (TE2000-S; Nikon Instruments, Surrey, UK.). Fluorescently stained cells were visualised using filter attachments allowing for the separation of green (live) and red (dead) cells. A systematic sampling procedure, as described in a previous study (498), was adapted and applied to this microscope set-up. Briefly, images were taken (Olympus DP70 Camera; Olympus Ltd, Middlesex, UK) in a continuous series running from the top to the base of the construct and the number of live and dead cells and a threshold was applied using Image J software to count cells. The 0.875 x 0.660 mm sampling area was calibrated by a 1 mm stage graticule as viewed under the 10x objective. A minimum of 4 replicate sampling series were performed per specimen, with an n=3 per density per time-point. The live-dead cell counts
were subsequently converted to percentage viability and the average viable percentages (mean ± SD) were determined for each density throughout the whole construct and peripheral and core regions. To determine the influence of cell proliferation on viability and other results exploring the functionality of periosteal cells in agarose, total cell numbers were calculated per image across the depth of each construct. These total cell numbers were used to assess cell proliferation and cell-agarose homogeneity as a function of initial cell density, time in culture, and distance from the construct surface.

**8.2.4 Determination of optimum strain level**

To determine the optimal conditions for effective strain translation from mechanical apparatus to cells, cell constructs were subjected to a range of physiologic strains and the cell displacements measured. Periosteal cell agarose constructs were produced with a final density of 2.0 x 10⁶ cells/ml in 3% (w/v) agarose. Cell constructs were selected based on their integrity and were immediately incubated in a solution of Calcein-AM (5µM; Molecular Probes, Paisley, UK) and Hoechst 33258 (5 µM; Sigma, Poole, UK) prepared in PBS for 30 min at 37° C. The constructs were then rinsed twice and left in PBS at 37° C until loading into the mechanical straining apparatus. The test rig used in this study was previously designed at Queen Mary University London (QMUL) to facilitate the compression of individual cell-agarose specimens and to allow for the simultaneous visualisation of cells using an inverted microscope. A schematic cross-section of the test rig is shown in Figure 8.1. A cell-agarose construct was placed on a coverslip within the test rig and was kept hydrated in PBS throughout the experiment. The rig was then mounted on the stage of an inverted fluorescent microscope and subjected to gross compressive loads. Compression was applied to the construct via a sliding platen attached to and controlled by a micrometer. Uniaxial, unconfined compressive strains of 1.0, 5.0, 10, 15, and 20% were applied statically and analysed using microscopy techniques.

![Figure 8.1 Schematic cross-section of the mechanical straining apparatus used to measure the translation applied to local strains in human periosteal cell-seeded agarose constructs.](image)

Cell nuclei within the cell-agarose specimen were visualised using fluorescence microscopy with a 10x objective lens coupled to a lens extender (Nikon, UK). High-contrast images were recorded via
an Olympus DP070 digital camera and analysed using Olympus DP Controller software (Olympus Imaging, London, UK). Prior to the application of compression, an image was captured of an easily distinguished cluster of cells in the construct. Static compression was then applied sequentially from 0 to 20% strain by twisting the micrometer nob. Two images were taken at each applied strain; one directly after the application of strain and one after the rough centring of the image. This centring was done by sequentially moving the microscope stage to allow for the following and focusing of the same cell populations in the construct. The subsequent images were manipulated and analysed using ImageJ software (NIH, Bethesda, MD). Briefly, a threshold was applied to the image which allowed the cells to be outlined and assigned Cartesian coordinates (Appendix F). After assigning coordinates, we were able to quantify cell displacements and stage translations to determine the local strains in the construct.

### 8.2.5 Chondrogenic culture of cell-agarose constructs in static conditions

Periosteal cell agarose constructs were produced as described above with a final density of 2.0 x 10^6 cells/ml in 3% (w/v) agarose. The periosteal cells used for this study were harvested from a 43 year old female (donor 2) and cultured for two passages prior to gel encapsulation. The cell-agarose constructs were distributed, one per well, into a 24-well plate and bathed overnight in 1 ml of chondrogenic media, consisting of high glucose DMEM, 1µM dexamethasone, 1mM sodium pyruvate, 0.17 mM ascorbic acid-2-phosphate, 0.35 mM praline and ITS + premix (defined chondrogenic media without TGF-β3, Lonza, Slough, UK). The following day, designated time 0, the constructs were bathed in 1 ml of chondrogenic media with or without 10 ng/ml TGF-β3 and incubated for periods of up to 16 days in static conditions. Media replacement and TGF-β3 supplementation were performed every other day. Samples were taken at 0.5, 1, 2, 4, 8, and 16 days for analysis of gene expression using real-time RT-PCR (n=3 per time point per condition).

### 8.2.6 Intermittent dynamic mechanical stimulation of periosteal cell-agarose constructs

All mechanical stimulation experiments were performed at QMUL and were set-up using methods described previously (297;298). Cell-seeded agarose constructs (3% w/v) with a cell density of 2.0 x 10^6 cells/ml were prepared using periosteal cells derived from two different donors (32 year old male-Donor 1 and 43 year old female-Donor 2). Constructs were incubated overnight in defined chondrogenic media in the absence of TGF-β3. Individual constructs were arranged in the centre of each 24-well plate, and positioned within the custom made cell-straining apparatus (Zwick-Roel, Redditch, UK) (Figure 8.2A). This apparatus was positioned into a custom made cell-straining rig and individual flat-ended indenters at the base of stainless steel pins platens were lowered onto the top surface of the cell-seeded constructs to provide a tare load of 0.028 N (Figure 8.2B). A syringe
fitted with an angled gauge 22 needle was used to deliver 1 ml of defined chondrogenic media, with or without 10 ng/ml TGF-β3, into each of the wells. Each mechanical experiment incorporated 12 constructs (6 unloaded + 6 dynamically loaded) which were bathed in control chondrogenic media without TGF-β3 supplementation and a further 12 constructs bathed in chondrogenic media with TGF-β3 supplementation. In this context, there were 4 separate conditions per plate. Experimental time course conditions were selected as 0.5, 1, 2, and 4 days, with or without dynamic compressive strain. The assembled straining apparatus with cell-seeded constructs was transferred to an adapted incubator at 37 °C and 5% CO₂, where the loading frame was secured to the loading actuator. Dynamic unconfined compression with amplitude of 15% strain and frequency of 1 Hz was applied to the experimental constructs with repeated cycles of loading and recovery of 1.5 hours and 4.5 hours, respectively. In this way, 4 complete loading/unloading cycles of 6 hours per 24 hour period were performed. The medium in each condition was changed every 2 days.

**Figure 8.2** Schematic diagrams of the apparatus used to apply compressive strain to agarose-periosteal cell constructs (A) and locked and floating loading pins applying strains of 0% and 15% to loaded and control (unloaded) constructs (B).
8.2.7 **Real time RT-PCR**

For gene expression experiments, cellular RNA was extracted from cell-agarose constructs by incubating constructs for 10 min at RT in a modified agarose disruption buffer, consisting of a chaotropic salt buffer, Buffer QG, (Qiagen, UK) supplemented with 0.1% β-Mercaptoethanol. The released RNA was then isolated, purified, and reverse transcribed into cDNA as done previously in monolayer cultures (Chapter 5). Single-plex real-time RT-PCR reactions were carried out in 20 µl volumes containing 10 µl Taqman® universal mastermix (Applied Biosystems, CA, US), 7µl 0.1% v/v diethylpyrocarbonate (DEPC) water (Invitrogen Ltd, Paisley, UK), 2µl extracted cDNA and 1µl Taqman® probe (Applied Biosystems, CA, USA). TaqMan® probes used to target genes are detailed in Table 8.1, and include Runx-2, Sox-9, bone-specific ALP, and collagen I, II, and X. 18S ribosomal RNA was used as a housekeeping gene. Each reaction was carried out in triplicate. The PCR reaction was initiated by a 2 minute 50°C and a 10 minute 95°C step to optimise thermal cycling conditions for the ABI Prism 7700 sequence detection system (Applied Biosystems, CA, USA) used to detect relative quantification of gene expression. This was followed by PCR amplifications performed for 40 cycles in a Corbett Rotor-Gene 6000 (Corbett Life Science, Sydney, Australia) at 95°C for 15 seconds and 60°C for 1 minute. A measurement of quantitative expression of target sequences, normalised to the abundance of the endogenous reference gene (18s) and expressed relative to day 0 control constructs was carried out using the –ΔΔCT method, as previously described (414). The target signal was plotted against the number of cycles and the threshold level was set at 0.05. Comparison of all data was taken at the intercept, where sample reactions crossed this phase of amplification. No template and no-amplification controls were run to check for evidence of contaminating external and internal sources, respectively. Fold changes in gene expression are presented as mean ± SD change relative to day 0 control constructs.

8.2.8 **ICP analysis of metal dissolution ions**

The presence of metallic ions within cultures subjected to mechanical loading regimes was measured using inductively coupled plasma atomic emission spectroscopy (ICP-AES) (Varian Vista-Pro; Varian Scientific Instruments,Yarnton, UK). After 1 and 4 days under intermittent mechanical loading, culture media was aspirated from the periosteal cell-agarose constructs and analysed for the presence of metallic ions Al, Fe, Cr, and Ni. For analysis, 3 different media samples from each time point were diluted 1/10 with 2% nitric acid and the apparatus was calibrated with synthetic standards at concentrations 0-200 ppm.
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**Table 8.1** Taqman™ gene probes used in this study, with details of their sources and specificities.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Symbol</th>
<th>Catalogue number</th>
<th>Accession number</th>
<th>Chromosome location</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaline phosphatase</td>
<td>ALP</td>
<td>Hs00758162</td>
<td>NM000478</td>
<td>Chr. 1 21,769,385 - 21,772,879</td>
</tr>
<tr>
<td>Collagen Type 1, alpha 1</td>
<td>COL1A1</td>
<td>Hs00164004</td>
<td>NM000088</td>
<td>Chr. 17 45,632,113 - 45,633,974</td>
</tr>
<tr>
<td>Runt-related transcription factor 2</td>
<td>Runx-2</td>
<td>Hs00298328</td>
<td>NM001015051</td>
<td>Chr. 6 45,622,542 - 45,626,797</td>
</tr>
<tr>
<td>SRY (sex determining region-y)-box 9</td>
<td>Sox-9</td>
<td>Hs00165814</td>
<td>NM000346</td>
<td>Chr. 17 67,630,455 - 67,634,156</td>
</tr>
<tr>
<td>Collagen Type II, alpha 1</td>
<td>COL2A1</td>
<td>Hs00156568</td>
<td>NM001844</td>
<td>Chr. 12 46,679,969-46,684,528</td>
</tr>
<tr>
<td>Collagen Type X</td>
<td>COL10A1</td>
<td>Hs00166657</td>
<td>NM000493</td>
<td>Chr. 6 116,546,814-116,553,363</td>
</tr>
<tr>
<td>Eukaryotic 18S rRNA</td>
<td>18s</td>
<td>Hs99999901</td>
<td>X03205</td>
<td>N/A</td>
</tr>
</tbody>
</table>

8.2.9 **Statistics**

For cell viability and cell density measurements a Mann-Whitney U test was used to assess significance between data from the construct periphery and core across time. Mann-Whitney U tests were also used to examine the differences in loaded and unloaded constructs in the absence or presence of TGF-β3. In all cases, a level of 5% was considered statistically significant (*p ≤ 0.05).

8.3 **Results**

8.3.1 **Encapsulation and culture of periosteal cells in agarose**

The encapsulation of periosteal cells in 3% (w/v) agarose resulted in a significant change in cell morphology. Periosteal cells in monolayer exhibited spindle-shaped, and elongated fibroblastic phenotypes with long membrane extensions, whereas corresponding encapsulated cells were smaller and more rounded (Figure 8.3).
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Figure 8.3 Phase contrast (A) and fluorescent imaging (B) of human periosteal cells (43 yr old female) in monolayer culture (A) and 3D agarose culture (B). To visualise cells within the agarose gels, periosteal cells were stained with 5 µm Calcein-AM. The images were taken prior to gel encapsulation (A) and 24 hours post encapsulation (B). Scale bars = 200 µm

At 24 hours post-cell encapsulation, periosteal cells in agarose exhibited significant increases in Runx-2, Sox-9, collagen I, and collagen X mRNA (Figure 8.4). In particular, hypertrophic chondrocyte marker collagen X, increased 19.6-fold, osteogenic transcription factor Runx-2 increased 9.5-fold, and chondrogenic transcription factor Sox-9 increased 4.6-fold.

Figure 8.4 Real time RT-PCR quantification of Sox-9, Runx-2, ALP, Collagen X, and Collagen I gene expression for periosteal cells encapsulated in 3% (w/v) agarose and incubated for 24 hours in standard growth medium. Fold changes in mRNA expression are expressed relative to patient matched monolayer cultured cells. A Mann-Whitney U test was used to assess significant differences between gene expression levels in periosteal cells in monolayer and agarose gels (* p< 0.05).

In 3D culture, encapsulated periosteal cells were well dispersed throughout the construct. The cell encapsulation method used in this study resulted in a homogenous distribution of cells throughout the construct, independent of initial cell-seeding density. During culture, no significant differences in absolute cell number or density were found between the core and periphery of the constructs of each seeding density (Figure 8.5).
Figure 8.5 Differences in cell density between the core and periphery of periosteal cell-constructs seeded at different densities and cultured over a period of 10 days. The peripheral region represents the initial and final 1/3 of the construct and the core region represents the central 1/3 of the construct. Each value represents the mean ± standard deviation of 20 replicate samplings in each region for 3 constructs per seeding density. No statistically significant differences in cell number were noted between the two construct regions for all seeding densities (Mann-Whitney U test, *p<0.05).

Additionally, there were no significant changes in overall construct cell density over 10 days in culture at initial seeding densities of 0.5 and 2.0 x 10^6 cells/ml (Figure 8.6). Constructs seeded at 8.0 x 10^6 cells/ml experienced a significant decrease in cell density over 10 days in culture. Under these static culture conditions, in the absence of local growth factors and mechanical stimuli, agarose embedded periosteal cells were found not to proliferate.
Figure 8.6 Average cell density in full-thickness constructs seeded at 0.5, 2.0, and 8.0 \( \times 10^6 \) cells/ml and cultured for a period of 10 days. Values are recorded as the mean viability ± standard deviation of 2D cross-sectional scans across the whole of the construct (n=3). Statistical differences in construct density over time are assessed at each seeding density using a Mann-Whitney U test was used (*\( p < 0.05 \)).

8.3.2 Cell viability in 3% (w/v) agarose culture

Periosteal cell viability profiles were analysed to determine the optimal seeding density for the established model parameters of 3% (w/v) agarose and 1 ml media changes. Prior to seeding the constructs, post-trypsinisation viability exceeded 97%, as assessed by trypan blue dye exclusion assay, and was maintained at 1 day post-construct production. During culture, however, there was a reduction in cell viability, particularly in the construct core, for all seeding densities. A representative sequence of live-dead staining images are shown to highlight the spatial profile of viability across the thickness of cell constructs (Figure 8.7). To statistically assess the spatial reduction in viability, construct data was dissected into peripheral and core regions based on distance from the construct surface (Figure 8.8). The spatial decrease in viability from the periphery to the core of the constructs was found to be statistically significant within constructs seeded at low-density (500,000 cells/ml) at all time points (\( p < 0.05 \)). However, these differences between construct regions were found to become less significant with increased cell-density.
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The average viability across the whole cell-construct was also calculated to determine the density-dependent effects on viability during extended 3D culture (Figure 8.9). After 4 days in culture, construct viability as a whole significantly decreased for each cell density examined. In the case of constructs seeded at 500,000 cells/ml, the percentage of viable cells was found to further decrease from 4 (87.6 ± 5.5%) to 10 (82.4 ± 9.2%) days in culture. Constructs seeded at 2 x 10^6 cells/ml maintained the greatest level of cell viability over the 10 day period (86.3 ± 5.5%), and were found to have statistically similar full-thickness viabilities at day 1 and day 10 in culture. The temporal profiles for viability revealed greater rates of viability loss in constructs seeded at high (8.0 x 10^6 cells/ml) and low (0.5 x 10^6 cells/ml) densities, as compared to the middle seeding density of 2 x 10^6 cells/ml.

Figure 8.7 Representative overlay of micrographs indicating fluorescently labelled viable cells (green) and nonviable cells (red) across a cell-seeded agarose construct (2.0 x 10^6 cells/ml) at day 10. The corresponding graph indicates percentage viability versus position in the same construct.
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Figure 8.8 Spatial and temporal profiles throughout 3% (w/v) agarose constructs seeded with 0.5, 2.0, and 8.0 x 10^6 periosteal cells/ml. Profiles were determined on day 1 (open circles), day 4 (closed squares), and day 10 (open squares). Individual data points represent the mean viability ± standard deviation of 3 constructs with 2-4 replicates per construct. The depth of construct has been normalised such that 0 is the upper surface of the construct and 1 is the lower surface. The bar graphs display the change in viability profiles in peripheral and core regions of constructs over time. The peripheral region represents the initial and final 1/3 of the construct and the core region represents the central 1/3 of the construct. Each value represents the mean ± standard deviation of 20 replicate samplings in each region. Statistical significance was assessed between the two construct regions using a Mann-Whitney U test. (*p<0.05).

For our model parameters, this data revealed that seeding density had a profound effect on the rate of full-thickness and core viability loss in periosteal cell-seeded agarose constructs. Further, consistent cell density measurements of seeded-constructs over the 10 day culture period ruled out the influences of seeding density dependent cell distribution and cell division heterogeneities on spatial and temporal viability profiles. Thus, the mid-level seeding density of 2x10^6 cells/ml represented the critical seeding density necessary for maintaining the largest percentage of viable periosteal cells in the core and peripheral regions of cell-seeded 3% (w/v) agarose constructs whilst in culture.
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Figure 8.9 Cell viability over 10 days within 3% (w/v) agarose constructs seeded with 0.5, 2.0, 8.0 x 10⁶ periosteal cells/ml. Values are recorded as the mean viability ± standard deviation of the construct as a whole (n=3). To statistically assess significant differences in construct viability within each seeding density over time, a Mann-Whitney U test was used. (*p<0.05)

8.3.3 Temporal profiles of gene expression in free-swelling agarose constructs

The temporal profiles for osteogenic (Runx-2, ALP, and Col Iα1) and chondrogenic (Sox-9, Col IIα1, Col X) gene expression by periosteal cells in 3D agarose static culture are illustrated in Figures 8.10. For this study, periosteal cells were encapsulated in 3% (w/v) agarose at a density of 2.0 x 10⁶ cells/ml and were cultured in defined chondrogenic media in the absence or presence of TGF-β3 for a period of 16 days. In general, the addition of 10 ng/ml of TGF-β3 during culture was found to increase mRNA levels of each gene examined, as compared to untreated constructs (-TGF-β3). Peak expression levels of Sox-9, Col X, and Runx-2 were found at 16 days in the presence of TGF-β3. In the absence of TGF-β3, Runx-2, Col I, and Col X mRNA levels were upregulated after 12 hours in culture and subsequently decreased to levels similar to or below baseline day 0 control expression levels at 48 hours.

Expression of osteoblast marker ALP progressively declined during culture in both TGF-β3 treated and untreated constructs (Figure 8.10). Conversely, periosteal cells in agarose exhibited a progressive increase in hypertrophic chondrocyte marker, Col X, expression from 0.5 to 16 days in the presence of TGF-β3 (Figure 8.10). Periosteal cells exhibited transient up-regulation of transcription factors Runx-2 and Sox-9 and type I collagen during culture. Peak collagen I mRNA levels were found at day 8, whereas Runx-2 and Sox-9 peaked at day 16, with fold increases of 3.5- and 26.5-fold respectively. Importantly, Col II mRNA expression only became detectable in cultured constructs at day 16 in the presence of TGF-β3. Collagen II data was not shown on graphs.
due to an inability to express Day 16 mRNA levels relative to day 0 control samples, which expressed negligible levels of mRNA.

![Figure 8.10](image)

**Figure 8.10** The relative gene expression of Sox-9 (A), Collagen Type X (B), Runx-2 (C), ALP (D), and Collagen Type I (E) in periosteal cell-agarose constructs cultured for a 16 day culture period in chondrogenic media with or without 10 ng/ml TGF-β3. Expression levels are normalised to internal housekeeping gene 18s and data are presented relative to day 0 expression levels (>1 = increase and <1 = decrease in expression). Each value represents the mean ± SD of 3 constructs.
8.3.4 Validation for cell-construct straining

Prior to studying the effects of mechanical stimulation on periosteal cell differentiation, the effective translation of external applied strains into the local cellular environment needed to be validated in this agarose culture system. For this, a previously designed mechanical testing system (QMUL) was adapted to microscopically visualise cell displacements within cell seeded-agarose constructs subjected series of compressive strains. Figure 8.11 displays the results of overall cell displacement from a common origin versus the distance away from the moving platen at five different physiologically relevant applied strain levels.

![Cell displacement versus distance from moving platen for periosteal cells in agarose constructs at 5 different applied compressive strains (1.0%, 5.0%, 10.0%, 15.0%, and 20.0%). Linear fits were used to find the slopes at each applied strain rate. The slope of the line indicates the average local strain that the periosteal cells experienced inside the agarose construct.](image)

This graph shows a trend of higher displacements near the moving interface with the platen, decreasing as we move towards the centre of the specimen, as evidenced by the negative slopes. These localised strain concentrations are suggestive of physically and mechanically heterogeneous constructs exhibiting properties of a viscoelastic material. This result is in support of the fact that tissue strain in response to an applied load is greatest in the superficial regions (499). Based on the slopes of the cell displacement data (Figure 8.11), corresponding local cellular strain for each applied external strain were plotted (Figure 8.12). Figure 8.12, revealed that increases in applied strain resulted in increases in local cell strain in the construct after a strain level of ~5.0%. Thus 5.0% strain represented a threshold value, exceeding which, allowed for a near linear translation of strain until the construct’s physical integrity began to be compromised (≥ 19.0%). At strain levels
of 10.0 and 15.0% the ratio of local strain to gross applied strain were found to be 0.9 and 1.01, respectively, resulting in near-perfect translation of mechanical strains to the cellular environment.

![Figure 8.12](image)

**Figure 8.12** Local strain versus applied strain for a randomly selected area in periosteal explant-agarose constructs. This graph shows an initial non-linear toe region, followed by a near-linear increase as gross strain is increased. At ~19% the physical integrity of the construct becomes compromised and results in local strain levels exceeding applied levels. In this graph 15.0% applied strain is seen as the ideal strain level in this culture model.

This non-linearity noticed in the strain translation, resembles strain profiles of other viscoelastic biological materials such as ligaments, arterial walls, and skin (35). In this culture system, agarose chain entanglements result in a linear elastic effect, and fluid streams from the media absorbed in and surrounding the construct result in a non-linear viscous effect. Additionally, linear deviations could arise from the fact that we are trying to analyse cell displacements in one-direction, using 2-dimensional images, of a 3-dimensional environment. Since we are working with a 3-dimensional construct, with viscoelastic properties, the cells can not be expected to serially move in one direction.

Importantly, the 3% (w/v) agarose gel allowed for direct translation of gross, applied, strains into the cellular construct at a strain level of 15%. The optimisation and validation of this periosteal cell-agarose culture model in a mechanical environment was a critical step for the investigation of mechanical stimulation on periosteal cell differentiation.

### 8.3.5 Influence of dynamic stimulation on periosteal gene expression in agarose constructs

Periosteal cells harvested from two different donors (32 year old male and 43 year old female) were encapsulated in 3% (w/v) agarose at densities of $2.0 \times 10^6$ cells/ml and subjected to intermittent dynamic compressive strains of 15.0% for periods of up to 4 days. All constructs remained intact
throughout the in-vitro dynamic mechanical stimulation procedure. Total RNA was successfully isolated from cells embedded in agarose constructs, quantified (ng/µl), and analysed for purity by ratio measurement of absorbances for nucleic acid (A 260) against protein (A 280). The average absorbance ratio (A 260/ A 280) of isolated RNA from periosteal cell-constructs was 2.08 ± 0.11. This average absorbance ratio falls within the range of values for pure RNA (A 260/ A 280 = 1.9-2.1) and demonstrated that RNA preparations were free of contaminating proteins, DNA, and other cellular material.

To determine the effects of mechanical stimulation on periosteal cells, constructs were exposed to intermittent stimulation blocks of 1.5 hrs loading followed by 4.5 hrs unloading for initial durations of 12, 24, and 48 hours in chondrogenic media with or without TGF-β 3. At a loading frequency of 1 Hz, this translated to construct loading cycles of 10800, 21600, and 43200 at 12, 24, and 48 hours respectively. Quantitative, real-time RT-PCR analysed the expression of early transcription factors Sox-9 and Runx-2 for each experimental condition (loaded + TGF-β 3, unloaded + TGF-β 3, loaded - TGF-β 3, loaded - TGF-β 3) at each time point in each donor. In each case (n=6 per condition per time point), mRNA expression levels were normalized to individual sample 18s ribosomal RNA and displayed relative to day 0 control periosteal cell constructs.

The effects of dynamic compression varied greatly between medium conditions, genes, and donor. Overall, Runx2 and Sox9 gene expressions were highest in constructs treated with TGF-β 3 and subjected to intermittent compressive strains over a period of 24 hours (Figure 8.13). RT-PCR analysis performed on loaded and unloaded cell constructs from both patients demonstrated significant increases in Sox-9 and Runx-2 expression with the addition of TGF-β 3 at all time points as compared to untreated (-TGF-β 3) constructs (Table 8.2). In both donors, the largest TGF-β 3 induced increases in gene expression were found to occur at 24 hours of culture. The effects of mechanical loading on periosteal cell gene expression levels, however, were found to be much more donor dependent.

In periosteal cell-constructs derived from donor 1 (32 year old male), loading did not have a positive effect on Sox-9 or Runx-2 mRNA levels compared to unloaded constructs at all time points. In the absence of TGF-β 3, periosteal cells responded to mechanical compression by significantly down-regulating Sox-9 expression at 12 (1.6-fold ↓) and 48 (1.7-fold ↓) hours. A similar decrease (1.6-fold) was found in Runx-2 expression after 48 hours of loading in constructs treated with TGF-β 3.
Figure 8.13 The relative gene expression of Sox-9 (A and C) and Runx-2 (B and D) by human periosteal cells encapsulated in 3% (w/v) agarose subjected to 15% intermittent dynamic compression at 1 Hz for 12, 24, and 48 hours, in chondrogenic medium with or without 10 ng/ml of TGF-$\beta_3$. Expression levels are presented from periosteal cells derived from 2 different donors; 32 year old male (A and B) and 43 year old female (C and D). For each condition mRNA levels have been normalized to endogenous 18s rRNA levels and are expressed as the mean $\pm$ SD relative to day 0 control constructs. Non-parametric Mann-Whitney U tests were used to assess significant differences between loaded and unloaded constructs (*$p \leq 0.05$).

Conversely, periosteal cells from donor 2 (43 year old female), were much more responsive to compressive loading cycles (Figure 8.13). In general, constructs responded to mechanical loading by up-regulating Sox-9 expression. In cultures without TGF-$\beta_3$, loading regimes of 12 and 24 hours significantly up-regulated the expression of Sox-9 compared to unloaded constructs. Oppositely, Runx2 mRNA levels in loaded and unloaded constructs cultured without TGF-$\beta_3$ were found to be statistically similar at all time points. Importantly, in this donor the addition of TGF-$\beta_3$ significantly changed the mechanoresponsive behavior of the cells (Table 8.2). The combined effects of loading and TGF-$\beta_3$ on gene expression levels are demonstrated at all time points for both genes of interest. In particular, Sox-9 mRNA levels in periosteal cells were increased by 4.0-, 59.0-, and 4.0-fold, respectively, following loading for 12, 24, and 48 hours in the presence of TGF-$\beta_3$. These levels were significantly higher than levels found in unloaded constructs ($p< 0.05$). A synergistic effect was also seen in Runx-2 expression levels in constructs subjected to loading in the presence of TGF-$\beta_3$. 
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<table>
<thead>
<tr>
<th>Donor</th>
<th>Gene of interest</th>
<th>Presence of TGF-β3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Unloaded</td>
</tr>
<tr>
<td>1 (32-m)</td>
<td></td>
<td>12 hrs 24 hrs 48 hrs 96 hrs</td>
</tr>
<tr>
<td></td>
<td>Sox-9</td>
<td>1.7↑ 4.7↑** 1.7↑* 2.4↑* 5.0↑** 2.4↑*</td>
</tr>
<tr>
<td></td>
<td>Runx-2</td>
<td>3.7↑** 6.6↑** 6.3↑* 3.3↑* 9.8↑** 5.3↑**</td>
</tr>
<tr>
<td>2 (43-f)</td>
<td></td>
<td>1.3↑ 7.0↑* 1.3↓ 1.1↓ 2.2↑* 22.1↑* 6.0↑* 1.5↑</td>
</tr>
<tr>
<td></td>
<td>Sox-9</td>
<td>2.6↑* 3.8↑** 1.1↓ 3.1↑* 4.2↑* 11.9↑* 4.7↑** 3.9↑**</td>
</tr>
</tbody>
</table>

Table 8.2 Trends in gene regulation with the addition of TGF-β3 in the presence or absence of intermittent dynamic compression at 12, 24, 48, and 96 hours in periosteal cells derived from two different patients. Numbers represent fold increase or decrease in gene expression as compared to corresponding constructs without TGF-β3 under similar loading conditions. *Indicates statistical significance (\( p \leq 0.05 \)).

To determine the effects of more prolonged exposure to mechanical compression, periosteal cells from donor 2, were dynamically loaded for a period of 4 days (86,400 loading cycles) under identical conditions to the previous studies. RT-PCR analysis revealed no additional increase in mRNA levels in constructs mechanically stimulated over a period of 4 days (Figure 7.14). Loaded constructs with and without TGF-β3 demonstrated no significant differences in Sox-9 or Runx-2 mRNA expression compared to corresponding unloaded constructs. In the absence of TGF-β3, the mRNA levels of both genes were found to be similar to day 0 expression levels. Supplementation with TGF-β3 increased Runx-2 expression 5.3- and 4.7-fold in unloaded and loaded constructs respectively (Figure 8.14). Comparatively, TGF-β3 had no effect on Sox-9 mRNA levels at day 4 (Table 8.2).

**Figure 8.14** The relative gene expression of Sox-9 (A) and Runx-2 (B) by donor 2 human periosteal cells encapsulated in 3% (w/v) agarose subjected to 15% intermittent dynamic compression at 1Hz for 4 days, in chondrogenic medium with or without 10 ng/ml of TGF-β3. For each condition mRNA levels have been normalized to endogenous 18s rRNA levels and are expressed as the mean ± SD relative to day 0 control constructs. Non-parametric Mann-Whitney U tests were used to assess significant differences between loaded and unloaded constructs (*\( p \leq 0.05 \)).
8.3.6 Metallic ion dissolution

The constant contact between the metal loading pins and cellular environment during construct loading led us to look at the presence of metallic ions within the culture media. It is well known that metallic dissolution ions will influence the behaviour of bone cells, by significantly altering cellular metabolism and differentiation (500;501). The presence of metal analytes Cr, Fe, Ni, and Al were therefore examined in the culture media to identify their presence and elucidate their potential influence on periosteal cell gene expression patterns. ICP-AES analysis revealed no detectable levels of Cr, Fe, Ni, or Al ions in any of the culture samples examined at both 1 and 4 days under loading (data not shown). This result eliminates the potential role that stainless steel or aluminium corrosion elements or dissolution ions may have on the results obtained from mechanical loading studies performed in this chapter.

8.4 Discussion

In this study, a model system in which human periosteal cells were embedded in agarose was developed and optimised to assess whether periosteal cell gene expression profiles are influenced by the application of mechanical strains. Although in-vitro culture systems are inherently non-physiological, the agarose-cell culture model is ideally suited for this study. This system allows for the simple and reproducible capacity to apply physiological levels of strain to cells whilst maintaining cell viability and phenotypic stability. Intermittent dynamic strains were applied to periosteal cell-agarose constructs using a specially designed loading apparatus. However, due to design constraints associated with the straining apparatus, a number of design parameters had to first be optimised for this culture model, including construct seeding density, applied strain level, loading duration, and frequency.

The clinical and experimental success of any 3D cellular construct is dependent on the maintenance of cell viability throughout its entirety. Furthermore, to study the influence of mechanical stimulation on periosteal cell behaviour the cells must continue to be metabolically active and retain the ability to respond to the external environment. The restriction of cell viability and ECM formation to the periphery of 3D tissue-engineered constructs is frequently observed. This phenomenon can be attributed to a number of factors including a non-homogenous cell distribution and/or insufficient transfer of nutrients to the centre of the construct (498;502). The latter of which is directly influenced by initial cell seeding density, hydrogel density, and medium volume (498). An agarose density of 3% (w/v) was selected based on its capacity to permit optimal strain translation whilst allowing for efficient nutrient diffusion to cells. Medium volume, however, was limited to 1 ml based on the design of the mechanical straining apparatus. Therefore, to establish the optimal culture parameters for sustained cell viability throughout the full construct, 3 different
cell-seeding densities (low, medium, and high) were examined. In agreement with reports of other authors (297;498), loss of periosteal cell viability was observed at the centre of 3D constructs (Figure 8.8). The decrease in core viability was inversely related to initial seeding density, with constructs seeded at 500,000 cells/ml demonstrating significant reductions in core viability during culture. This loss of viable cells across the agarose constructs can be attributed in part to inadequate mass transfer of key nutrients to the cells at the center of the construct. Similarly, cell density had an effect on cell viability during the 10 day culture period. However, periosteal cell viability reductions (2-15% during 10 day culture period) were not as profound as decreases seen in mesenchymal stem cell- (~10-30%) and chondrocyte- (~2-70%) seeded hydrogels (297;498). Indeed, average periosteal cell viability levels remained >75% during culture for all densities examined. However, for our model parameters 2 x 10^6 cells/ml represented the optimal seeding density for sustained periosteal cell viability during \textit{in-vitro} culture.

Critically, cell density calculations performed on periosteal cell constructs during culture revealed; (1) a homogenous distribution of cells throughout the construct thickness regardless of cell seeding density and (2) agarose encapsulated periosteal cells did not actively proliferate during 10 days in culture. In the context of this study, these two observations are significant, as heterogenous cell distributions due to seeding method and/or cell proliferation can lead to considerable construct-construct variations which can ultimately affect experimental outcomes.

The level of gross strain used (15%) resulted in local cell strain values of approximately 15%, which lies in the 0-30% physiological range of cell strains found in intact cartilage subjected to physiologic loads (499;503). This is in support of previous studies which found that compression of cell/agarose constructs caused cell strains of approximately the same magnitude as that applied to the agarose (504;505). Further, Lee and Guilak found that local cell strains of 15% resulted in changes to cell area and nuclear deformation, prior to increases in proteoglycan and collagen production in chondrocyte constructs (504;506). Deformations at the cellular and subcellular level lead to intracellular signalling events that finally lead to altered effector-cell response. It is through these mechanotransduction pathways that we aimed to induce and study periosteal cell chondrogenesis at the molecular level. Previous \textit{in-vitro} loading studies using cell and explant culture models have demonstrated that the method of application, as well as the duration, frequency, and duty cycle of loading can influence genetic and biosynthetic response (496;507-510).

The loading frequency of 1 Hz was selected based on the viscoelastic properties of agarose and results of previous chondrocyte loading studies, where frequency-dependent matrix metabolism was noted (496;511). Due to the viscoelastic nature of agarose, dynamic loading regimes result in both dynamic and increasing (cumulative) compressive strain components [unpublished results of
QMUL researchers and (511)]. Cumulative displacement (analogous to creep strain under static loading conditions) is defined as the portion of starting thickness not recovered upon return to minimum applied stress. Importantly, low loading frequencies (<0.33 Hz) result in significantly higher cumulative strain components which are known to have an inhibitory effect on chondrocyte differentiation and chondrogenesis (512). Conversely, reduced cumulative strains were found in chondrocyte constructs and explants loaded at higher physiologically relevant frequencies (0.4-1.0 Hz) and resulted in enhanced matrix production and chondrogenic gene expression levels (496;513). Lee and Bader found that 15% dynamic compression of chondrocytes embedded in agarose was inhibitory at 0.3 Hz, stimulatory at 1 Hz, and did not change proteoglycan synthesis at 3 Hz (496).

In the second phase of this work, chondrogenic differentiation was initiated in free-swelling agarose cultures. Prior to chondrogenic induction, the encapsulation of periosteal cells in agarose gels caused a change in morphology, which coincided with a significant up-regulation in Sox-9, Runx-2, and Coll I and X (Figure 8.4). Previous studies have reported phenotypic modulation caused by an enforced spherical morphology within hydrogels. For example, Coll II and aggrecan gene expression was less evident in marrow stromal cells cultured in collagen gels where cells could assume a more stellate morphology, as opposed to alginate and agarose hydrogels (514). Although the mechanism behind this change in periosteal gene expression was not explored here, Hardingham et al. found that cell rounding in alginate gels, causes cytoskeletal reorganisation which leads to induction of Sox-9 expression through the p38 MAPK stress-activated protein kinase signalling pathway (515).

Chondrogenic differentiation was induced through TGF-β and dexamethasone supplementation, as described in previous bone marrow stromal cell cultures (241;297;516). In periosteal cell-constructs treated with TGF-β3 the expression of transcription factors Runx-2 and Sox-9, and Col I and X all increased over the 16 day culture period as compared to day 0 controls (Figure 8.10). Increased expression of Coll I is a feature common with the early stages of chondrogenesis, where mesenchymal precursor cells produce type I collagen (517). The occurrence and accumulation of Coll X mRNA over the 16 day period would tend to indicate differentiation toward the hypertrophic phenotype. The corresponding levels of Runx-2 and ALP found during culture would, however, suggest that cells are present as a pre-hypertrophic phenotype at day 16. Runx-2 serves as a positive regulatory factor in chondrocyte maturation to hypertrophic phenotype and is expressed mainly in pre-hypertrophic and less in late hypertrophic phenotypes (518). Similarly, increased ALP expression, not seen in this study, usually accompanies Coll X up-regulation during the late phases of chondrocyte hypertrophy (518). This pre-hypertrophic expression pattern
analysed here correlates with previous human mesenchymal stem cell studies that report the up-regulation of collagens type I and X during the early stages of chondrogenesis (519-521).

Additionally, the presence of pre-hypertrophic phenotypes in periosteal cell-constructs during free-swelling culture was further substantiated by the heightened expression levels of both Runx-2 and Sox-9 at day 16. Both transcription factors are highly expressed in osteochondroprogenitor cells and pre-hypertrophic chondrocytes. Sox-9, however, is dominant over Runx-2 function, acting as a transcriptional repressor for osteoblast differentiation (522). In this context, the 3-fold increase in Sox-9 expression compared to Runx-2 levels at day 16 suggests that cultures are progressing down the chondrogenic lineage. In agreement with this, Hardingham et al demonstrated, in retroviral transduction studies, the importance of Sox-9 expression for maintaining a chondrogenic phenotype and promoting cartilage matrix production (520;523). Indeed, a change is noticed in collagen expression at day 16. Coll I expression levels which peaked at day 8 were found to decline at day 16. Accordingly, Coll II mRNA became detectable at day 16. This apparent switch in collagen expression suggests that day 16 represents the beginning of the next phase of chondrogenesis, namely chondrocyte differentiation (517). Longer culture studies, however, are needed to examine the complete temporal expression profile of periosteal cells during chondrogenesis and to compare this marker pattern to what is seen in vivo (524). Importantly, the results shown in this study, demonstrate the capacity of this agarose culture model to support chondrogenic phenotypes during periosteal cell chondrogenesis and supports similar results reported previously (525).

The in-vitro culture and loading conditions, detailed above, permitted mechanical stimulation to be applied to periosteal cells with precise control and reproducibility. Interval-based loading was based on the enhanced effects of intermittent compressive loading regimes on metabolic activity and extracellular matrix production in chondrocytes and cartilage explants compared to static loading (507;526-528). Further, chondrogenic differentiation has been demonstrated in MSC constructs under dynamic compressive loading conditions (297;493;494;509).

Extending upon these studies, we aimed to impose a new level of control over the differentiation potential of human periosteal cells through the combined use of biomechanical and biochemical stimuli. This study represents a necessary first step towards this aim, by providing an initial proof-of-purpose detailing the effects of short-term dynamic mechanical loading regimes on periosteal cell phenotype. For this purpose the expression levels of two important early transcription factors, Runx-2 and Sox-9, were examined in periosteal cells subjected to different loading cycles and culture conditions for periods of up to 4 days. The application of dynamic compressive strains at 15%, 1.0 Hz resulted in a donor-dependent response in human periosteal cells. In cells from donor 1, loading had a negative effect on Runx-2 and Sox-9 expression, as demonstrated by decreased mRNA levels compared to unloaded constructs at nearly all time points. Conversely, in donor 2
cells, loading significantly increased gene expression levels, particularly in the presence of TGF-β3. Utilising an intermittent dynamic compression profile, of 1.5 hours loading/4.5 hours unloading, we were able to establish a short-term temporal response profile over a period of 4 days. It is well known that the duration of loading and the number of loading cycles applied play a role in the downstream response of cells (507;509;529). In particular, periodic applications of low pressure dynamic fluid pressures at 4 hours/day enhanced cartilage formation in periosteal explants (510). Similarly, we found that periosteal cell response to dynamic compressive strains was dependent on the schedule of loading. In responsive constructs (43-f), expression levels peaked in the presence of TGF-β3 at 24 hours, which consisted of 2 separate loading blocks of 1.5 hours (5400 cycles). This peak in expression was much more evident in the profile of Sox-9 mRNA, which demonstrated a 59-fold increase at 24 hours. The profile of Runx-2 expression under each condition, however, remained relatively consistent over the 4 day study. Notably, in periosteal cells from donor 2, significant increases in Sox-9 mRNA levels occurred in the absence of TGF-β3, suggesting that mechanical signals alone may be enough to begin induction of chondrogenesis. To confirm this, longer loading studies (≥ 2 weeks) with more donor populations are needed to examine the production of Coll II and aggrecan. Miyanishi et al (494), for example, only detected an increase in Coll II and aggrecan after 14 days in human MSCs undergoing loading without the addition of TGF-β3.

In agreement with results from free-swelling constructs, TGF-β3 treatment alone was found to increase expression levels of Runx-2 and Sox-9 mRNA in periosteal cells from both donors. In donor 2 cells, however, the combination of mechanical stimulation and TGF-β3 was found to significantly up-regulate mRNA levels at all time points compared to either factor alone. This is particularly evident at 24 hours, where a 59-fold increase was seen in loaded constructs treated with TGF-β3, compared to loading (2.7-fold ↑) and TGF-β3 treatment (7.7-fold ↑) alone. The synergistic effects of mechanical stimulation and TGF-β treatment are consistent with recent studies. In the presence of TGF-β and cyclic compressive strains (10%), Terraciano et al. demonstrated increased chondrogenic differentiation and cartilage-related matrix production in human MSCs after 14 days of exposure (299). Similarly, Huang et al. found an increase in TGF-β receptor gene expression of rabbit MSCs after 1 hour of stimulation, which contributes to a greater amount of TGF-β binding (529).

The interactions between TGF-β signalling and mechanical stimulation could be due to a wide range of potential mechanisms. Stimulated TGF-β signalling pathways could modulate mechanotransduction either directly or indirectly by increasing the sensitivity of periosteal cells to loading. For example, TGF-β treatment may lead to the activation of mechanosensitive proteins, such as focal adhesion kinase and paxillin, which might increase the mechanosensitivity of the cells...
(530;531). Alternatively, downstream targets of TGF-β signalling may be necessary components of mechanotransduction in periosteal cells. TGF-β has been shown to up-regulate Sox-9 gene expression in differentiating periosteal cells (Figure 7.10 and 7.13) and is known to modulate periosteal chondrogenesis (481;532;533). Prior to matrix deposition in the cartilage anlagen, nuclear transcription factor Sox-9 promotes the expression of the type II collagen and aggrecan mRNA by binding with the promoter of type II collagen and aggrecan genes (534;535). Thus by increasing transcription of downstream targets of mechanical stimulation, TGF-β signalling may indirectly amplify the effects of mechanotransduction.

Conversely, mechanical stimulation may modulate TGF-β signalling. One mechanism may involve the production of TGF-β or its receptors through upregulation of mRNA expression by mechanical loading. Raab-Cullen et al found that in-vivo controlled four-point bending promoted c-fos and TGF-β mRNA in rat periosteum within 4 hours of loading (490). In addition, Huang et al found that dynamic compressive loading promoted gene expression and protein production of both TGF-β receptors (TβR-I and II) in rabbit-MSCs in agarose (529). This is important because TGF-β receptor 1 can initiate intracellular TGF-β signal transduction after phosphorylation (536), which may mediate chondrogenic differentiation of chondroprogenitor cells and mesenchymal stem cells (537).

Although both TGF-β treatment and physical strain represent strong transcriptional stimuli for early chondrogenic differentiation in periosteal cells cultured in agarose, this response is markedly donor dependent. Similar donor-to-donor variability was also seen in the molecular response of human mesenchymal stem cells subjected to dynamic strains (538). In general, the intrinsic heterogeneity of periosteal cells, in terms of lineage hierarchy and differentiation potential, represents a major challenge towards studying their cellular responses. As shown in this thesis and previous studies, differentiation processes in human periosteal cultures are not synchronised throughout the cell populations (160;295). In fact, phenotypic and genotypic analysis of periosteal cells and other adult stem cell cultures suggest that early differentiation may be regulated, at least in-vitro, by stochastic mechanisms (539;540). This is thought to represent the developmental flexibility found in undifferentiated stem cells, which is lost during the commitment to more mature phenotypic state.

To elucidate if donor-dependent responses to loading were due to differences in population phenotypes, an immunophenotypic analysis was performed on cells from both donors (For methodology see Chapter 5). Flow cytometry, however, did not reveal any significant differences in the number of progenitor cell (Stro-1+/ALP+), preosteoblast (ALP+/Stro-1+), or osteoblast (ALP+/Stro-1+) phenotypes present in periosteal cell cultures from both donors (Table 8.3). Although we were unable to establish a relationship between cell phenotype and molecular
response to loading, further attempts should be made to understand the effects of loading at different stages of periosteal progenitor differentiation. This information, for example, could be used for the development of pre-treatment regimes for cultured cells prior to introduction into cartilage defects.

<table>
<thead>
<tr>
<th>Phenotype Profile (mean ± SD % of total population)</th>
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<tbody>
<tr>
<td>Donor</td>
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<tr>
<td>Donor 1 (32-m)</td>
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<tr>
<td>Donor 2 (43-f)</td>
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</tbody>
</table>

Table 8.3 Immunophenotypic profile of human periosteal cells from the two donors used in this study. Monoclonal antibodies against Stro-1 and ALP and flow cytometry were used to examine the relative sizes of 3 developmentally distinct cell populations present in human periosteal cell cultures. A two-tailed Student t test was used to assess significant differences in population phenotypes between the two donors (*p ≤ 0.05). No significant differences were found.

8.5 Conclusion

In conclusion, a periosteal cell-agarose culture model has been successfully developed and optimised, allowing for the study of cell differentiation in a biomechanical environment. This study demonstrated temporal expression patterns of early response genes (Sox-9 and Runx-2) in human periosteal cells under dynamic compressive loading. Based on the significant up-regulation of Sox-9, the application of 15% dynamic compressive strain was found to promote early chondrogenic differentiation in human periosteal cells in-vitro. This chondrogenic effect was dependent upon the applied temporal loading profile, complex interaction between TGF-β3 and dynamic loading, and donor. This established model can now be used to elucidate the specific mechanotransduction pathways involved in periosteal cell response to short-term loading and to determine whether chondrogenesis can be further induced with sustained loading. Studies, similar to that of Chowdhury et al (507), are required to more accurately examine the optimum loading conditions needed to stimulate chondrogenic differentiation in human periosteal cells. Ultimately, these studies will be useful in understanding the influences of loading on human periosteal cell differentiation and in enhancing the in-vitro development of tissue engineered constructs for cartilage regeneration.
Chapter 9

Conclusions

Tissue Engineering is a thriving area of multi-disciplinary research that has the potential to revolutionise the treatment of diseased and damaged tissue. In orthopaedics, the ultimate challenge of repairing and regenerating joints compromised by injury or disease is massive and should not be underestimated. Even small chondral defects may necessitate surgical intervention and would benefit from the development of new material- or cell- based therapies that fill the defect and integrate with the surrounding tissue; preventing further degeneration and consequent osteoarthritis. The size and complexity of the challenges faced by Tissue Engineers over the last 20 years is reflected by the relatively few tissue engineered products clinically available to the public. Although it is difficult to identify where the largest clinical hurdles are, I believe the greatest need for research input resides at the cellular and molecular level. The successful design of scaffold materials and cellular therapies requires an intimate understanding of the complex molecular interactions that occur within tissues, between cells and the extracellular environment. This level of understanding is particularly necessary in stem cell research, where remarkable new developments have shown adult stem cells to reside in numerous tissues throughout the body. These multipotent cell types, recently identified in human periosteal tissue, have the capacity to form multiple tissues in-vitro and in-vivo, and will likely fuel the next wave of tissue engineered therapies.

In this thesis, broadly based research teams were brought together to form interdisciplinary collaborations, with input received from cell biologists, biomaterial scientists, biomedical engineers, and orthopaedic physicians. Utilising these collaborations, successful in-vitro therapeutic models were developed and optimised to characterise, understand, and control orthopaedic tissue regeneration using key elements of the tissue engineering tool kit; namely differentiated and undifferentiated skeletal cell types with particular tissue function, and polymeric biomaterials to support the culture of cells in-vitro and form 3D scaffolds for tissue regeneration in-vivo.

In a commercial research venture with Polynovo Biomaterials Pty. Ltd., I helped to develop and characterise a novel two-component polyurethane platform that can be administered arthroscopically, polymerise at biological temperatures in-situ, and provide appropriate bonding strength and mechanical support for orthopaedic applications. These novel polymers provided a favourable in-vitro environment for initial cell adhesion, maintained cell viability, and normal rates of proliferation of human osteoblasts. Physicochemical characterisation techniques revealed that
heterogeneous topographies and chemical moieties present on the polymer surfaces created favourable hydrophilic environments to support human osteoblast attachment and growth. Based on the encouraging structural, mechanical, and biological properties, I was able to identify suitable orthopaedic applications for this platform technology, particularly as bone glues/cements.

For cell-based tissue engineering strategies, human periosteum was selected as the therapeutic cell source due to its intimate involvement in bone and cartilage formation during embryogenesis, fracture healing, and bone remodelling \textit{in-vivo} and proven osteochondral potential \textit{in-vitro}. The feasibility in therapeutic applications for isolated periosteal cells and explants has been demonstrated previously in small animal models, where certain cells are capable of multilineage differentiation, forming bone and cartilage tissue as well as participating in skeletal myogenesis (160). Moreover, the clinical feasibility and safety of the periosteum for cartilage repair has been shown in numerous clinical models using periosteal tissue grafts alone or in combination with autologous chondrocytes (17;358). However, a major concern for periosteum-based therapies has been the large functional variability in experimental and clinical outcomes. This variability is at least partly due to donor-related and harvest site-specific factors, and inconsistencies in cell/explant preparation protocols. Previously, surgical skill (386), explant size (368), donor site (359) and donor age (387) have been found to significantly influence the quality and regenerative potential of periosteal grafts.

In building on the seminal periosteum studies of Stevens et al.(361;362;401), O’Driscoll et al.(31;189-191), and De Bari et al.(160;201), the research presented herein has successfully bridged the knowledge gap between periosteal cell phenotype and functionality. Through the development of \textit{in-vitro} culture models I have successfully investigated the underlying heterogeneities present in donor derived human periosteal cell populations. High-quality, viable, human periosteal explants composed of both tissue layers were reproducibly harvested from donors during both arthroscopic and invasive knee surgeries. Different enzymatic digestion protocols were designed and optimized to preferentially isolate heterogeneous periosteal cell populations from full tissue explants or distinct periosteal tissue layers. Initial cell isolates were found to be morphologically and phenotypically heterogeneous, consisting of distinct cell types at different stages of lineage commitment and developmental maturation. A monolayer culture system, without differentiation supplements, was designed to allow for the extensive expansion (>20 population doublings) of all adherent periosteal cell populations and simultaneous examination of population properties, such as growth, morphology, differentiation, phenotype, and genotype. Robust cell and molecular biology assays such as flow cytometry, real time RT-PCR, and immunocytochemistry, were combined with standard microscopy techniques to probe distinct cellular changes during culture and examine differences between donor-derived populations. Interestingly, the behavior and phenotype of human periosteal cells, particularly progenitor cell types, was heavily influenced by a combination
of environmental and cellular factors, including cell-seeding density, culture media, cell-cell interactions, and cell-secreted autocrine/paracrine factors. During culture, significant changes in population proliferation rates, growth potential, and colony forming efficiency were correlated with changes in cell morphology and linked to progressive changes in overall population phenotype. Although significant donor-dependent differences were initially identified in culture, the time-dependent trends in growth kinetics, morphology, and gene/protein expression were consistent across all donors.

Apart from the biological exploration of general periosteal population characteristics, considerable research efforts were made to identify, characterise, and discuss periosteal progenitor cell populations. The ability to continuously examine periosteal cell populations for 15 passages and preferentially enrich for cambium layer cells has enabled the identification of two distinct precursor populations based on the expression of Stro-1. In all donors, colony forming, Stro-1⁺, osteoprogenitor cell populations were consistently isolated from tissue explants, regardless of donor age. A separate Stro-1⁻, more primitive precursor cell subpopulation was only isolated and identified in cambium enriched cell cultures. This morphologically distinct subpopulation was found to consist of small, rounded, agranular, highly-proliferative cells with an increased capacity to form large cell colonies and differentiate into osteogenic cell types. This more primitive Stro-1⁻ subpopulation, however, was rapidly lost in culture, whereas Stro-1⁺ osteoprogenitor cells were cultured for up to 15 passages without a loss in population numbers or ability to form mineralized tissue. Gene expression analysis confirmed that in the absence of differentiation stimuli, monolayer cultured human periosteal cells were found to adopt a more osteogenic phenotype.

To induce periosteal cell chondrogenesis, a 3-dimensional culture environment combined with chondroinductive stimuli, in the form of chemical and physical factors, was required. For this, a periosteal-agarose cell culture model was created to control and monitor lineage specific differentiation in biomechanical and biochemical environments. As an initial proof-of-concept demonstration, dynamic mechanical loading of periosteal cell constructs was found to successfully promote early chondrogenic differentiation. This chondrogenic effect was dependent upon the applied loading profile, complex interaction between TGF-β3 and dynamic loading, and donor. Although more studies with more donor populations are needed, initial results demonstrate the usefulness of this model system for studying the complex cellular and molecular responses to biophysical and biochemical loading environments.

For the development of periosteal cell-based therapies, there is a considerable need to target and isolate specific cell types to allow for further analysis and amplification ex-vivo. In this thesis numerous cell enrichment techniques were performed to isolate rare progenitor cell phenotypes present in heterogeneous human periosteal cell cultures. These techniques included selective
adherence to tissue culture plastic, FACS, MACS, sequential tissue digestion, and droplet microfluidics. The selective adherence of periosteal cells to tissue culture plastic resulted in the propagation of numerous mesenchymal cell types and was unable to selectively enrich progenitor cell types. The physically demanding sorting processes of FACS and MACS were continuously plagued by viability loss and compromised sterility and were also unable to accurately isolate pure Stro-1^+ progenitor cell populations. Due to these challenges, alternative strategies were explored. The design of a sequential digestion protocol allowed for the preferential isolation, expansion, and characterisation of periosteal cell populations consisting mostly of cambium or fibrous layer cells. Although this technique was found to be successful in its ability to identify and enrich for highly proliferative Stro-1^+ and Stro-1^- precursor cell types, these liberated periosteal cell populations were phenotypically heterogeneous. In moving forward to more complex strategies, droplet microfluidics was identified as a promising alternative technology for the rapid, specific and detailed analysis of periosteal cell populations due to its robust functionality and versatility. By controlling fluid streams in microchannels, we were able to compartmentalise single cells and direct droplet-laser interactions to measure cell surface properties. Single-cell immunodetection measurements were correlated with other phenotypic characteristics, allowing information to be easily gathered on how progenitor cells were distributed in heterogeneous periosteal cell populations. Based on these results, droplet-based microfluidics was found to be an accurate and reproducible strategy to simultaneously isolate and detect typical (ALP^+) and atypical (Stro-1^-) cell types present in human periosteal cell cultures. Moreover, the biological utility and reliability of this system for cell characterisation was further confirmed by comparing results with conventional flow cytometry. In the context of this thesis, droplet microfluidics was found to be a robust tool, with the potential to simplify complex biological processes present in human periosteal cell cultures so that we may better comprehend their interactions, functions, and role in biology. Apart from stem cell research, this technology would be hugely useful in numerous application fields, including immunology, microbiology, parasitology, and oncology. Utilising this exciting platform technology, future work should focus on sorting potentially therapeutic periosteal cell types identified in this thesis (Stro-1^+ and Stro-1^- precursor cells) so that they may be further characterised and exploited in tissue engineering applications.

It is hoped that the promising results and well-defined and optimised cell isolation and culture models presented in this thesis will encourage researchers to further explore the behaviour and characteristics of human periosteal cells. In building off of the protocols developed here, more robust analytical technologies such as microarrays (145) and mass-spectroscopy proteomics (541), may provide a definitive picture of the complex biological signals and cues that trigger natural development and repair processes in joints. In progressing into clinic, it is this knowledge that will be exploited in tissue engineering and regenerative medicine applications.
Appendices

Appendix A: Osteoblast phenotyping

An all important aspect for the study performed in Chapter 3 was the confirmation of the osteoblast phenotype of the primary human cells isolated from femoral heads. This was achieved by positive immunostaining of 2 bone specific proteins, alkaline phosphatase (ALP) and collagen type I (Col. Type I) after 7 days in culture (Figure A.1.). These two markers have been used extensively to identify differentiated osteoblastic phenotypes. ALP is a plasma membrane bound enzyme found in differentiated osteoblasts and has been implicated in the initiation of mineralization. Col type I is a protein synthesised and secreted by osteoblasts and is therefore one of the major proteins found in bone ECM. The images positive for Collagen Type I (figure 12b) and ALP (figure 12a) show strong positive cell cytoplasm staining, indicating that the cell population used in this study is composed of differentiated osteoblasts. This study was performed at 7 days to show that the osteoblasts used throughout this study maintain a stable phenotype in all the assays run. Thus, this quality control experiment confirms that we are in fact performing this study with differentiated human osteoblasts.

Figure A.1 Detection of ALP (A) and Col Type I (B) expression in adult human osteoblasts by immunofluorescent staining. Primary osteoblasts were grown on chamber slides for 7 days and stained with conjugated antibodies specific for bone ALP (Green) and Col Type I (Red). Cells were counter-stained with dapi (blue) to display cell nuclei. Images show a significant amount of positively staining, indicative of mature osteoblasts.
Appendix B: Cell-cycle analysis of periosteal cells

Figure B.1 Representative results of the step-wise gating method used to analyse the percentages of quiescent and proliferating cell populations in human periosteal cell cultures. Cellular debris and clumped cell clusters were excluded from the analysis by progressively gating on nucleated and single cells (Gates R1 and R2). From this filtered population a gate (R3) was set, based on the isotype control histogram, to identify the total population of cells expressing the proliferation marker, Ki-67. Additionally, the percentages of cells in G0/G1, S, or G2/M phase were determined in each sample based on the DNA histogram. Further, a gate (R4) was set for cells in G0/G1 phase based on DNA content. Double staining with Ki-67 was used to discriminate between cells in resting G0 and cells in activated G1 phase. Cells in the active, proliferating phase were defined as cells expressing Ki-67 (R5), whereas cells in G0 showed no expression of Ki-67.
Appendix C: Shape factor coefficient analysis of human periosteal cells

Figure C.1 Characterisation of cell shape in human periosteal cell cultures from 6 donors between passages 0-20. At each passage, area and perimeter of 50-100 cells were measured and used to calculate shape factor coefficient. By definition, a Sfc value of 1 corresponds to a perfect circle, whereas a straight line has a value of 0; hence the more elongated the cell shape is, the lower the Sfc value. Scatter plots display the changes in Sfc values (mean ± SD) during culture. Linear regression analysis of this data reveals no significant correlation (two-tailed t-test, $p > 0.05$) between Sfc and time in culture in 5 of the 6 donors examined, with Pearson’s product-moment correlation coefficients ranging from -0.33 to 0.98.
Appendix D: Antibody binding, titration, and specificity

To reduce the amount of cross-reactivity and non-specific mAb-protein binding, animal serums were tested for their ability to block non-specific binding sites (Figure D.1). Normal mouse serum was chosen as the optimal blocking agent for its ability to reduce non-specific background noise without significantly affecting positive staining. This allows for improved separation between negatives (isotype control stained cells) and positives (antigen specific staining).

![Bivariate dot-plots of flow cytometric analysis of serum blocking experiments](image)

*Figure D.1* Bivariate dot-plots of flow cytometric analysis of serum blocking experiments. Human periosteal cells were blocked with 0.1% (v/v) normal animal serum from a mouse or goat and further stained with APC-conjugated alkaline phosphatase and appropriately matched isotype-matched control antibodies. Gates were set on each sample to determine the effectiveness of the blocking agent to reduce background staining (isotype controls) without causing a decrease in positive staining. Control samples of unblocked cells were used to establish the baseline level of non-specific binding in periosteal cell cultures. Normal mouse serum was chosen as the optimal blocking agent for its ability to reduce non-specific background noise without significantly affecting positive staining.

Second, mAbs Stro-1, D7-fib, ALP, and CD34 were titrated with periosteal cells at concentrations between 0.25-3.0 µg/10⁶ cells using appropriately matched isotype controls. Based on the signal to noise ratio or %Fsp (specific fluorescence/total fluorescence x 100) the saturation point of each mAb was defined and further used to test to their specificity in various cell types (Figure D.2).
Figure D.2 Titration of antibody cell reactions by flow cytometry. Graphs A and E, B and F, C and G, and D and H represent four different antibodies with different affinities: ALP (A and D), D7-fib (B and F), Stro-1 (C and G), CD34 (D and H). Graphs A, B, C, D show mean fluorescence intensity of positively stained periosteal cells (Ftot, ■) with mAb concentrations between 0.25 – 3.0 µg/106 cells. The mean fluorescence of appropriately match Isotype controls (Fbkgd, ○) were used at the same concentrations as positive samples. These values were then used to find the staining sensitivity, or % Fsp, that is defined by the equation %Fsp = (Ftot – Fbkgd)/Ftot x 100. Graphs E, F, G, and H show % Fsp for each antibody. The peak value of % Fsp determines the optimal staining concentrations used in this thesis. At these concentrations the greatest distances between positive and negative fluorescent distributions are achieved.

Third, flow cytometry was used to define the level of mAb staining in human osteoblasts, fetal osteoblasts, human foreskin fibroblasts, and osteosarcoma cell lines Saos-2 and MG63 (Figure D.3). Results revealed that fibroblast marker, D7-fib, was broadly expressed on all cell types tested (>95% of cells) and was therefore not specific to fibroblasts. This level of non-specific binding was deemed unsatisfactory and the antibody was therefore not used for subsequent periosteal characterisation studies. Conversely, the cell specificity of mAb ALP was predictable, with a high level of expression in osteoblasts (>72%) and osteosarcoma cell lines (>90%) and low expression levels in fibroblasts (<2%). Stro-1, a progenitor cell marker, showed a high level of reactivity in MG63 cells (>98%) and low to undetectable levels of expression in Saos-2 cells and primary osteoblasts and fibroblasts (<2%).

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Figure D.3 Bivariate dot plots of the tissue specificity of mAbs D7-fib (fibroblasts), ALP (osteoblasts), and Stro-1 (progenitor cells). Flow cytometry was used to assess the level of positive staining in human osteoblasts (hOBs), fetal osteoblasts (fOBs), foreskin fibroblasts (HFFs) and osteosarcoma cell lines SaoS-2 and MG63. In all cases, aggregates and dead cells were filtered from the analysis by examining plots of population light-scatter characteristics. Additionally, in each analysis quadrant gates were set using appropriately matched isotype controls. The numbers shown in each gate represent the number of positively stained cells as a percentage of total cells examined. More than 20,000 cells were stained and examined in each plot.
Appendix E: Human periosteal cell phenotype

Figure E.1  Expression of SSEA-1 and α-smooth muscle actin in embryonic stem cells and periosteal cells cultured on 4-well chamber slides for a period of 4 days. Images show (A) Positive staining for SSEA-1 (red) in differentiating embryoid bodies present in passage 15 mouse embryonic stem cells. (B) SSEA-1 staining in passage 0 human periosteal cells, showing no expression of the antigen. (C and D) Positively stained cells within passage 0 human periosteal cell cultures isolated from a 32 yr old male, expressing α-smooth muscle actin (green) within the cell cytoplasm. (E and F) Positively stained cells identified within passage 13 periosteal cell cultures isolated a 32 yr old male. (In all images blue stain = cell nuclei).

Figure E.2  Oil-Red O staining of lipid droplets (dark red) within A549 and periosteal cells cultured for 4 days on 4-well chamber slides. (A) Positive control A549 cells showing a majority of cells expressing large deposits of lipids within their cytoplasm. (B & C) Passage 0 periosteal cells isolated from a 74 yr old female highlighting cells positive (B) and negative (C) for lipid droplets. (D) Positive staining of passage 5 periosteal cells isolated from a 74 yr old female. (E and F) Passage 12 periosteal cells isolated from a 74 yr old female (E) and 32 yr old male showing populations of cells void of cytoplasmic lipid accumulation. In all images a filter was applied to equalise shadows, midtones, and highlights by automatically redistributing the significant pixel values throughout the tonal range (Corel Photo-Paint; Corel Corporation, Berks, UK).
Figure E.3  Dual-color flow cytometric analysis depicting the cell surface expression of the Stro-1 and ALP antigens (A) and CD34 and CD45 antigens (B) on single-cell suspensions of periosteal cells. The gates were set to >98% of reactivity levels obtained with the isotype matched control antibodies for Stro-1 (IgM: FITC) and ALP (IgG1: APC) and CD34 (IgG1: PE) and CD45 (IgG1: APC), shown in histograms (C) and (D) respectively. In each instance, more than 20,000 events were collected to determine the percentage positive expression.
Appendix F: Strain translation methodology

Figure F.1 ImageJ software manipulation of a representative fluorescent image of cell-agarose constructs in compression. a) The original digital image taken after compression. b) Threshold applied to distinguish cell nuclei. c) Cells outlined and assigned numbers and (X, Y) coordinates, which are then used to determine displacements.
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