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Skeletal Stem Cells: phenotype, biology and environmental niches informing tissue regeneration

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Abstract

Advances in our knowledge of the biology of skeletal stem cells, together with an increased understanding of the regeneration of normal tissue offer exciting new therapeutic approaches in musculoskeletal repair. Skeletal stem cells from various adult tissues such as bone marrow can be identified and isolated based on their expression of a panel of markers associated with smooth muscle cells, pericytes and endothelial cells. Thus skeletal stem cell-like populations within bone marrow may share a common perivascular stem cell niche within the microvascular network. To date, the environmental niche that nurtures and maintains the stromal stem cell at different anatomical sites remains understood. However, an understanding of the osteogenic and perivascular niches will inform identification of the key growth factors, matrix constituents

and physiological conditions that will enhance the ex vivo amplification and differentiation of osteogenic stem cells to mimic native tissue critical for tissue repair. This review will examine skeletal stem cell biology, the advances in our understanding of the skeletal and perivascular niche and interactions therein and the opportunities to harness that knowledge for musculoskeletal regeneration.

Keywords: skeletal stem cell, perivascular, niche, tissue regeneration, osteoprogenitor,

1. Introduction

The cell responsible for bone formation, the osteoblast, is derived from a marrow stromal fibroblastic stem cell. These marrow stromal fibroblastic stem cells exist postnatally, are multipotent and have the ability to generate myelosupportive stroma, osteoblasts, adipocytes, chondrocytes, smooth muscle cells and astrocytes (Fig. 1). This population of cells are also referred to as osteogenic stem cells, marrow stromal fibroblastic cells, bone marrow stromal stem cells, mesenchymal stem cells, stromal precursor cells and, more recently, skeletal stem cells (Caplan, 1991; Bianco and Robey 2001; Barry and Murphy 2004; reviewed in Oreffo et al., 2005; Sacchetti et al 2007). Although mesenchymal stem cells (undifferentiated multipotent cells of the mesenchyme) appears to have gained wider acceptance in recent years, this term is however nonspecific and the term skeletal stem cell will be used throughout this review to restrict description to stem cells from bone marrow able to generate all skeletal tissues (Sacchetti et al 2007). Advances in our understanding of skeletal stem and progenitor cells, development of isolation protocols from a variety of tissues and differentiation strategies offer the potential to open a new frontier in regenerative medicine, which aims to replace cells and tissues in a broad range of conditions associated with damaged cartilage, bone, muscle, tendon and ligament. The term 'stem cell' can be applied to a remarkably diverse group of cells, which, regardless of their source, share two characteristic properties. A capacity for prolonged or unlimited self-renewal under controlled conditions, and the potential to differentiate into a variety of specialised cell types. However, while skeletal stem cells hold enormous promise for future cellular-based tissue engineering applications, their rarity and limited ex vivo growth potential has restricted their wide spread clinical use. In order to understand the biology of the skeletal stem cell an appropriate homogenous population is a prerequisite and, similarly, an understanding of their developmental environment or niche.

The attraction in the use and manipulation of skeletal stem cells lies in their apparent lack of immunogenicity, which has increased the attraction in the use of these cells in skeletal tissue repair. This review concentrates on the characterisation and properties of skeletal stem cells, their environmental niches and interactions therein and how this can inform their potential application using regenerative medicine strategies to the currently unmet needs of bone regeneration in an increasing ageing population.

2. Human bone marrow-derived skeletal stem cells: phenotype and characteristics

It is more than forty years since Alexander Friedenstein demonstrated that adult bone marrow contains rare (1 in 10^4 - 10^5 bone marrow mononuclear cells/ BMMNC) clonogenic precursors associated with the soft fibrous tissue of the marrow stroma (Friedenstein et al., 1966, 1970; Friedenstein 1980). These cells were originally described as colony forming units-fibroblastic/ CFU-F (Owen and Friedenstein, 1988). Under appropriate culture conditions, undifferentiated CFU-F differentiate into multiple stromal lineages (Fig. 1) including osteoblasts, chondrocytes, adipocytes, smooth muscle cells, astrocytes, endothelial cells, and more recently, beta-pancreatic cells and neural cells, although formation of beta-pancreatic cells and neural cells remains highly contentious (Azizi et al., 1998; Ferrari et al., 1998; Pittenger et al., 1999; Lin et al., 2000; Majumdar et al., 2000; Bianco and Robey, 2001; Reyes et al., 2001, 2002; Heng et al., 2004; Fellous et al 2007; Davani et al., 2007). While it is also generally accepted that skeletal stem cells give rise to a hierarchy of developmental cell populations including; the determined osteoprogenitor cell (DOPC), preosteoblast, osteoblast and ultimately, the osteocytes, a unique, specific single cell-surface marker that specifically identifies the skeletal stem cell has remained elusive. This, understandably, has led to a degree of conflicting data in the literature concerning the characteristics of the skeletal or mesenchymal stem cell. Nevertheless, it is generally accepted that adult human skeletal stem cell do not express haematopoietic markers CD45, CD34, CD14, CD11, adhesion molecules such as CD31 (PECAM-1), CD56 (NCAM-1), CD18 (LCAM), and co-stimulatory molecules such as CD80, CD86, CD40. However, these cells do express CD105 (SH2/ Endoglin), CD73 (SH3/ 4), CD44, CD90 (Thy-1), CD71, the STRO-1 antigen, as well as the adhesion molecules CD106 (VCAM-1), CD166 (ALCAM), ICAM-1 and CD29 (Haynesworth et al., 1992; Galmiche et al., 1993; Pittenger et al., 1999; Conget and Minguell, 1999; Le Blanc et al., 2003; Barry and Murphy, 2004) (Table 1).

To circumvent some of the issues around the characterisation of the skeletal stem cell, a number of groups have attempted to generate antibodies recognising subsets of human marrow stromal cells using conventional hybridoma technology (Bruder and Caplan, 1990; Simmons and Torok-Storb, 1991; Haynesworth et al., 1992; Yamaguchi and Kahn, 1993; Gronthos et al., 1994; Bruder et al., 1997; Joyner et al., 1997; Bruder et al., 1998; Filshie et al., 1998). Of the current available antibodies that recognise stage- and/ or lineage-specific stromal antigens, to date, STRO-1 is the most extensively used. STRO-1 is a monoclonal antibody that recognises a cell-surface trypsin-resistant antigen expressed by a subset of freshly isolated bone marrow mononuclear cell (BMMNC) that includes essentially all adherent, high growth-potential CFU-F (Simmons and Torok-Storb, 1991; Gronthos et al., 1994). Other antibodies reported to recognise antigens expressed by the CFU-F population include the endoglin/ CD105 - a type III TGF- β receptor (Robledo et al., 1996); HOP-26 (Joyner et al., 1997) recognising CD63 – a lysosomal membrane glycoprotein of the tetraspanin superfamily (Zannettino et al., 2003); SB-10 (Bruder et al., 1997), which recognises the activated leukocyte adhesion molecule (ALCAM) CD166 (Bruder et al., 1998); and the antibody recognising CD49a, the α -1 integrin subunit (Deschaseaux and Charbord, 2000). Letchford and colleagues reported a unique strategy involving the use of phage display to generate antibodies recognising stem cell-enriched bone marrow mononuclear cell populations resulting in the identification and preliminary characterisation of a novel antibody termed C15 (Letchford et al., 2006). Although C15 was not selective for skeletal stem cell/ CFU-F, C15 immunoreactivity was strongly associated with areas of active bone formation where it was shown to bind to osteoblasts and their putative precursors in the proximal bone marrow (Letchford et al., 2006). Recently, Gronthos and co-workers identified a novel monoclonal antibody, STRO-3, which was shown to bind to tissue nonspecific alkaline phosphatase (TNSALP), a cell-surface glycoprotein usually associated with cells of the osteoblast lineage (Gronthos et al., 2007). STRO-3 was found to identify a high proportion of bone marrow derived skeletal stem cells that possessed extensive proliferative and multilineage differentiative capacity (Gronthos et al., 2007).

The STRO-1 antibody was demonstrated to bind to approximately 10% of the BMMNC isolated from bone marrow aspirates of normal donors (Simmons and Torok-Storb, 1991). In suspensions of formalin-fixed human bone marrow cells, HOP-26 immunoreactivity was demonstrated in <10% of the nucleated population at day 1 of culture, in >95% of the cells at day 3 of culture and in <1% of the nucleated cells of day 28

cultures (Joyner et al., 1997). In a separate study by Majumdar and co-workers, the proportion of CD105/Endoglin+ cells in BMMNC populations was determined between 3% and 1.8% (Majumdar et al., 2000). It was also noted that over 60% of CD105+ BMMNC co-expressed the STRO-1 antigen (Majumdar et al., 2000). In our group, the proportion of STRO-1+ cells isolated from BMMNC populations using MACS (magnetic activated cell sorting) was determined at $7 \pm 3.8\%$ (Howard et al., 2002). Using flow cytometry however, the average proportion of STRO-1+ cells in BMMNC, freshly isolated from bone marrow samples of osteoporotic and osteoarthritic individuals, was determined at $11 \pm 3\%$ in our group (Fig. 2).

In BMMNC populations, freshly isolated from femoral reamings of osteoarthritic patients undergoing routine surgery, the proportion of antigen-positive cells for STRO-1, HOP-26, SB-10 and anti-CD49a was 27%, 46%, 19% and 5% respectively (Stewart et al., 2003). In this study, Stewart and co-workers also demonstrated co-expression of the STRO-1 antigen by the CD49a+ BMMNC fraction. The high level of HOP-26 immunoreactivity observed by Stewart and co-workers, in comparison to that demonstrated by Joyner et al. (1997), was attributed to the greater sensitivity of the flow cytometry protocol, and the finding that CD63 was ubiquitously expressed by bone marrow cells of both haematopoietic and non-haematopoietic origins (Stewart et al., 2003). Flow cytometric analysis by Letchford and co-workers determined the average proportions of STRO-1+, CD49a+ and C15+ fractions in BMMNC at 15%, 5.4% and 4.4% respectively (Letchford et al., 2006). A recent study by Zannettino and co-workers established that the STRO-1^{bright+} fraction of human bone marrow lacked expression of CD34, CD45 and glycophorin-A, markers associated with haematopoietic progenitor cells (Zannettino et al., 2007). *In vivo*, the STRO-1^{bright+}/CD34-, STRO-1^{bright+}/CD45- and STRO-1^{bright+}/glycophorin-A- fractions were all capable of forming lamellar bone structures and vascularised fibrous tissue supporting adipose formation and a haematopoietic active bone marrow (Zannettino et al., 2007).

While variations in cell/ tissue origin and analysis methods may account for the differences in average percentages of STRO-1+ cells reported by the various studies (Simmons and Torok-Storb, 1991; Howard et al., 2002; Stewart et al., 2003; Letchford et al., 2006) summarised in Table 2, it is also important to note that the STRO-1+ fraction of cells is a heterogeneous population containing other cell types of both stromal and haematopoietic origin (Simmons and Torok-Storb, 1991; Gronthos et al., 1994; Stewart et al., 2003; MacArthur et al., 2006). Similarly, expression of the STRO-1 antigen, CD63 and CD49a, and the proliferation

and differentiation capabilities of these enriched fractions *in vitro* have been reported to be donor-dependent (Gundle and Beresford, 1995; Walsh et al., 2000) although, remarkably, expression of CD166 appears to be donor independent and the molecule is expressed by $\geq 95\%$ of BMMNC (Bruder et al., 1997; Stewart et al., 2003). Until a selective antibody is derived, the application of cocktails of antibodies to enhance MSC enrichment remains a route currently under investigation in a number of laboratories.

3. Identifying the skeletal stem cell niche

The stem cell niche is the functional, anatomic location in which stem cells are maintained, typically in a quiescent state, prior to division and generation of proliferating, repopulating differentiated cell populations. Stem cell niches, identified in a number of different adult tissues, including bone marrow, skin and dental pulp, are often highly vascularised sites (Spradling et al., 2001; Shi and Gronthos, 2003). It has been previously hypothesized that one possible niche for precursors of osteoblasts may be the microvasculature networks of the bone marrow (Doherty et al., 1998). Pericytes are relatively undifferentiated cells associated with the walls of small blood vessels, and serve as blood-flow regulators in the microvasculature. Previous studies have proposed that pericytes may be precursors of endothelial and/ or smooth muscle cells, but the exact developmental relationship between all three cell lineages is obscure during angiogenesis (Nehls et al., 1992; Schor et al., 1995). The pericyte-associated cell-surface antigen, 3G5, is highly expressed by a large proportion of haematopoietic cells from human bone marrow, but only a minor proportion of bone marrow stromal cells are found to express 3G5 (Shi and Gronthos, 2003). Cultured bovine pericytes however are STRO-1+, indicating the fidelity of STRO-1 as a marker of primitive skeletal progenitors derived from diverse tissues (Doherty et al., 1998). Pericytes isolated from bovine retinal capillaries exhibit the potential to differentiate into a variety of cell types including fibroblasts, osteoblasts, chondrocytes and adipocytes (Brighton et al., 1992; Schor et al., 1995; Doherty et al., 1998). Mounting evidence therefore suggests that, in addition to participating in the maintenance of blood vessel homeostasis, pericytes may also represent multipotential MSC (Nehls and Drenckhahn, 1993; Schor et al., 1995; Doherty et al., 1998).

Bone marrow derived skeletal stem cells have been described to have a vascular smooth muscle cell-like phenotype *in vitro* (Dennis et al., 2002). In accordance with these findings, freshly isolated human bone marrow STRO-1+ cells were demonstrated to lack expression of von Willebrand factor (an endothelial

marker), but were positive for α -smooth muscle actin (smooth muscle cell and pericyte marker) and CD146 (endothelial, smooth muscle cell and pericyte marker) (Shi and Gronthos, 2003). The study by Shi and Gronthos (2003) also demonstrated ectopic bone formation when STRO-1+ cell populations co-expressing CD146 were transplanted with hydroxyapatite/ tricalcium phosphate ceramic powder into immunocompromised mice for 3 months. To aid this process it will be necessary to understand the chondrogenic and perivascular niches, critical in elucidating the fundamental conditions necessary to selectively maintain and expand primitive skeletal populations *in vitro* and thus harness their reparative capacity.

3.1 The chondrogenic niche

Chondrogenesis is the process involving skeletal stem cell differentiation, and proceeds through the determination of cells and their aggregation into prechondrogenic condensations, differentiation into chondrocytes, and chondrocyte maturation. These complex spatial and temporal sequences of events are co-ordinated by the effects of hormones, morphogens, and cytokines through their respective receptors, along with extracellular matrix components and cell-cell and cell-matrix interactions (Knudson, 1998; Djouad et al., 2007). Expression markers associated with chondrogenesis include transcription factors such as *Sox-9*, and extracellular matrix genes such as *aggrecan*, *collagen types II and IX* (Foster et al., 1999). Critical in the chondrogenic process, are Bone Morphogenetic Proteins (BMPs), members of the transforming growth factor- β (TGF- β) superfamily. Interplay between the BMPs and FGFs has been shown to inhibit *Ihh* expression, while promoting hypertrophic differentiation and suppressing the proliferative capacity of chondrocytes. Thus studies indicate BMPs bind to a type II receptor that is present on the cell membrane of their target cell and recruit a type I receptor forming a heterotetrameric-activated receptor complex. This phosphorylated type I receptor, in turn phosphorylates a set of intracellular substrate signalling proteins (SMAD family) and following translocation into the nucleus results, ultimately, in targeted gene regulation (Cao and Chen, 2005; Granjeiro et al., 2005; Wan and Cao, 2005). FGF signalling pathways induce the expression of *Sox-9* in primary chondrocytes and in undifferentiated cells. FGF2 has been recognized as a mitogen for cells of the chondrogenic lineage, and more recently FGF18 has been demonstrated to act as a trophic factor for articular chondrocytes and to have significant anabolic effects on chondrocytes both *in vivo*

and *in vitro*, including increased deposition of matrix proteoglycans and type II collagen and also stimulated cell proliferation (Ellsworth et al., 2002; Davidson et al., 2005). Furthermore, cartilage-derived morphogenetic proteins (CDMP, also known as growth/differentiation factor-5 (GDF-5)) play a pivotal role in joint formation and in the developing appendicular skeleton (Provot and Schipani, 2005), and the Wnt and Wnt-related family of signalling proteins are also involved in chondrogenic differentiation and in adult cartilage homeostasis (Tuan et al., 2002; Jorgensen et al., 2004). Essential for cartilage formation is Sox-9, one of the earliest markers of chondrogenesis and a key regulator for the expression of type II collagen and aggrecan as well as a number of other cartilage-specific matrix proteins (Kou and Ikegawa, 2004; Lefebvre et al., 1997; Goldring et al., 2006). Other factors such as the *Hox*, *Pax*, *Forkhead*, homeodomain-containing families have had roles identified in the chondrogenesis process but further characterization is required to understand their precise functions (Jorgensen et al., 2004). Alsalameh et al., (2004) and Fickert et al., (2004) have detailed the presence subpopulations of cells with characteristics of skeletal progenitor cells in normal and osteoarthritic cartilage. The percentage of skeletal progenitor cells present in cartilage was reported to represent 3.5% of the constituent cells (Alsalameh et al., 2004). The constituents of the cartilage matrix, which constitutes a specific niche composed of extracellular matrix proteins include, notably, proteoglycans, matrix metalloproteinases, chemokines, integrins, cadherins, lipids, inorganic components as well as collagens and fibrillar proteins are thought to be pivotal in the crosstalk between progenitor cells and the extracellular matrix and critical in the chondrogenic process (Foster et al., 1999; Djouad et al., 2007). The chondrogenic niche thus encompasses all these extracellular elements and soluble factors immediately surrounding the stem cell and progenitor populations, and it is this microenvironment, and the cell-cell and cell-matrix communication, that regulates the maintenance of the stem cell pool and specific commitment towards specific lineages through intrinsic and extrinsic factors (Djouad et al., 2007; Kolf et al., 2007). Interestingly, to date, there has been no evidence of an identifiable extracellular matrix protein capable of maintaining the skeletal stem cell in an undifferentiated and naïve phenotype. Further clues as to the composition of the chondrogenic niche can be drawn from *in vivo* observations on the chondrocyte. Thus, *in vivo*, chondrocytes are responsible for the maintenance of articular cartilage, by synthesizing and degrading all extracellular matrix components, and replace degraded matrix molecules through catabolic and synthetic activities in order to maintain the correct architectural structure and biomechanical properties (Temenoff and

Mikos, 2000; Aigner et al., 2006). Chondrocytes exist in a low oxygen tissue environment in the absence of a vascular supply or innervation within discrete cavities or lacunae embedded within a dense extracellular matrix. A further degree of complexity is that the functions of chondrocytes are dependent on their location. Thus, the main function of chondrocytes that occupy supporting structures is the secretion and maintenance of an extracellular matrix, whereas chondrocytes that are located in the epiphyseal plates are associated with growth (Archer and Francis-West, 2003). It is therefore the interactions, not fully elucidated, between the chondrocyte and the cartilage extracellular matrix in the cartilage niche that regulate and maintain native cartilage tissue.

3.2 The Perivascular Niche

The perivascular niche consists of endothelial and or pericyte cells which influence residing adjacent cell types during critical cellular and tissue development (Nikolova et al., 2006a). The close proximity to all cells and the high permeability of the endothelium allow for efficient metabolic exchange between blood and tissues (Cleaver & Melton, 2003). However, endothelial cells are found in direct contact with certain cell types indicating a role greater than merely suppliers of nutrients and oxygen (Palmer, 2000). During development, haematopoiesis and vascularisation occur simultaneously where; endothelial cells (EC) and hematopoietic stem cells (HSC) originate from the same progenitor cell, the hemangioblast (Robertson et al., 1999; Lacaud et al., 2001; Hristov and Weber, 2004). During vasculogenesis ECs develop into tubules surrounded by a vascular basement membrane. A lumen develops and attaches to the circulatory system activating a signalling cascade to attract mural cells (pericytes or smooth muscle cells) to stabilise the vessel (Foo et al., 2006) and to express factors which reproduce the effects of the vascular niche.

In development, proximal interactions of endothelial and progenitor populations in tissues such as the heart, brain, liver, bone and blood indicate that signals generated by the microvasculature affect these progeny cells (Cleaver & Melton, 2003). The primitive progeny of HSC is located adjacent to the endosteal surface of trabecular bone (Taichman, 2004) and hence linked to the adult bone and marrow. HSC that fail to express a calcium-sensing receptor fail to localise to the endosteal niche, indicating that the bone matrix mineral content plays an important role in the osteoblastic niche (Adams et al., 2006). Osteoblasts, reticular cells, fibroblasts and adipocytes all function to create a niche for HSC, controlling numbers and

differentiation (Balduino, 2005). However, HSC are known to interact with other stromal cells including ECs. The ablation of osteoblasts in transgenic mice leads to extra-medullary hematopoiesis (Visnjic et al., 2004) indicating the existence of HSC niche in other tissue. HSC were thought to reside in niches adjacent to the endosteum. However, identification of long term repopulating HSCs by CD-150⁺ (signalling activation lymphocyte molecule (SLAM) family) showed that CD-150⁺ positive cells were located at the osteoblastic surface of trabecular bone and co-localised to the sinusoidal ECs of vascular tissue (Kiel, 2005) a site that has been identified as the regulatory region for megakaryocyte progenitors (Avecilla et al., 2004). HSC remain quiescent in the endosteal zone but in the central vascular niche differentiation and mobilization to the circulation occurs (Abkowitz et al., 2003). Indeed, identification of two cell populations, Human bone marrow stromal cells (BMSCs) and dental pulp stem cells (DSPCs) have been shown to reside in perivascular niches (Shi and Gronthos, 2003). In thrombocytopenia, upregulation of stromal-cell-derived factor (SDF-1) and VEGF activate matrix metalloproteinase-9 (MMP-9), converting the membrane-associated kit ligand into soluble kit Ligand (sKitL) resulting in the translocation of HSCs to the vascular niche where they undergo cell cycling, differentiation and translocation to the vascular system via sinusoids (Heissig et al., 2002; Visnjic et al., 2004). Conversely, MMP-9^{-/-} mice fail to release sKitL and HSC motility is impaired (Heissig et al., 2002).

Approximately two thirds of HSC in the bone marrow and spleen are adjacent to sinusoids (Kiel et al., 2005). Sinusoidal vessels are characterized by a highly permeable thin wall consisting of a single layer of endothelial cells which are supported in part by a vascular membrane, pericytes, and reticular cells (Tavassoli, 1981, Nikolova et al., 2006a). Sinusoids surrounded by reticular cells secrete high amounts of a chemokine, SDF-1 (CXCL12) which is required for the maintenance of HSC (Sugiyama et al., 2006). Deletion of CXCR4 (receptor for SDF-1/ CXCL12) results in a reduction of HSCs and increased sensitivity to myelotoxic injury (Sugiyama et al., 2006). Localisation of HSC to the endosteum or vascular regions is usually associated with SDF-1 secreting reticular cells. Perivascular cells express significantly higher levels of SDF-1 than osteoblasts or endothelial cells (Sugiyama et al., 2006). These two pieces of evidence indicate that the vascular and endosteal niches exploit a number of common mechanisms for maintaining HSCs.

During embryogenesis mice deficient in SDF-1 have a reduced capacity of hematopoietic colonization of BM by HSC from the peripheral circulation (Nagasawa, 2000). Equally, EC induced expression of SDF-1 can rescue the defective hematopoietic colonization of BM signifying that bone marrow EC are essential for HSC colonization of the fetal BM (Ara et al., 2003). HSC and Haematopoietic progenitor cell (HPC) mobilization is highly regulated by the osteoblastic and vascular niches (Adams and Scadden 2006). Controlled level changes of SDF-1 in the BM ensure that quiescently maintained HSC migrate from the osteoblastic niche to the vascular niche facilitating trans endothelial migration to the vascular circulation via the sinusoids. Equally, homing of HSC is simply the reverse of this process (Lapidot et al., 2005; Cancelas and Williams, 2006). Mobilization and recruitment of HPCs to the vascular niche requires endothelium FGF-4 as well as SDF-1. An elevated concentration gradient of O₂ and FGF-4 from the osteoblastic niche to the vascular niche may influence HSC/HPC recruitment, proliferation and trafficking (Ceradini and Gurtner, 2005; Yin and Li, 2006). The basement membrane surrounding sinusoidal EC may yet provide a niche to support proliferating stem and osteoprogenitor cells. As the membrane also expresses heparin sulphate proteoglycans, it may increase the growth factor levels of FGF-4 and SDF-1 which are critical to HSC (Kopp et al., 2005).

Bone marrow endothelial cells (BMECs) support proliferation and differentiation of HPCs *in vitro* (Rafii et al., 1994, 1995, 1997) and may be unique to the vascular niche in bone marrow. In contrast to other tissue-derived endothelial cells, BMECs are potent inducers of HPC adhesion and migration (Imai et al., 1999a; Yong et al., 1998). BMECs have a high surface expression of SDF-1, express lower levels of vWF and constitutively express adhesion molecules such as VCAM-1 and E-selectin. Their tissue specific heparin sulphate proteoglycan patterns and adhesion molecule profiles (VCAM-1, ICAM-1, E-selectin and P-selectin), functionally cause homing of the hematopoietic progenitor cell (expressing VLA-4, LFA-1 and hyaluronan binding cellular adhesion molecule) (Mazo et al., 1998) homing to the BM via a homeostatic mechanism ensuring a constant flux of such cells into the BM (Imai et al., 1998; Imai et al., 1999b; Rood et al., 1999; Peled et al., 2000; Netelenbos et al., 2001; Yu et al., 2003).

The interaction of endothelial cells, HSC and stromal lineages are required for the homeostasis of HSC and skeletal stem cells at specific sites in the 3D microenvironment of adult bone marrow (BM).

Recently, a functional interplay has been identified between the development of angiogenesis, skeletal progenitors in BM sinusoids and the formation of the hematopoietic microenvironment (HME) *in vivo* (Sacchetti et al., 2007). Melanoma-associated cell adhesion molecule (MCAM/CD146) expressing subendothelial cells in human BM stroma (upon transplantation) were able to transfer the HME to heterotopic sites concomitantly with the development of identical subendothelial cells within a small bone structure. These subendothelial stromal cells in the developing heterotopic BM occur with the inception of sinusoids (Sacchetti et al., 2007) and produce Angiopoietin-1 (Ang-1), a pivotal molecule of the HSC niche involved in vascular remodelling (Davis et al., 1996). Indeed, Ang-1 as well as VEGF, SDF-1 and FGF4 play a critical role in BM regeneration after myelosuppression (Han et al., 2006).

HSCs expressing Tie-2 have recently been shown to respond to Ang-1, inducing HSCs to tightly adhere to stromal cells preventing HSC cell division (Arai et al., 2005). Equally, *in vivo*, Ang-1 reduces the activity of HSCs allowing them to attach to the bone surface whereby they become protected in the HSC compartment from factors that reduce hematopoiesis (Arai et al., 2005). Additionally, human BM-derived skeletal stem cells express Tie-1 allowing them (under proinflammatory induction) to be chemoattracted by Ang-1, but not by VEGF, demonstrating a fundamental difference between endothelial cells and skeletal stem cells (Ponte et al., 2007). However, under mechanical stimuli, skeletal stem cells can initiate an angiogenic reaction via VEGFR and FGFR signalling cascades (Kasper et al., 2007).

TNF alpha induces MSC migration towards chemokines CCR2, CCR3 and CCR4 effecting the activation and homing to injured tissues (Ponte et al., 2007). Indeed, vascularisation was demonstrated by using cell guidance and homing of MSCs within 3D scaffolds with addition of VEGF, SDF-1 and BMP-6 without prior cell seeding (Schantz et al., 2007). Understanding the interactions of EC, HSC and skeletal stem cells and the “niches” that allow for this complex orchestration to take place will ultimately enhance the ways bioengineers develop constructs for bone regeneration. Importantly, the correct isolation of bone marrow progenitors will play a critical role in the functionality of future cell therapy and therapeutic tissue engineering strategies (Seeger et al., 2007).

4. Tissue Regeneration – clinical need and tissue engineering

An understanding of the osteogenic, chondrogenic and perivascular niches is critical in the identification of the key growth factors, matrix constituents and physiological conditions that will enhance tissue regeneration. This has gained prominence given the demographic challenges of an advancing aged population and the need for innovative approaches to augment and repair skeletal tissue. It is estimated that by 2020, 20% of the UK population will be over 65 and with this demographic challenge, the numbers of hip fractures worldwide are projected to increase from 1.7 million in 1990 to 6.3 million in 2050. Furthermore, in the UK there are over 50,000 primary hip replacement operations each year at a cost of £250million (Javaid and Cooper, 2001). From a patient's perspective, the ultimate goal is replacement of their damaged skeleton with autogenous material, harvested with minimal morbidity. With autogenous bone, a lack of sufficient material, the failure of complete remodelling and difficulties in shaping the bone graft to fill the defect, often precludes the universal use of autogenous bone for orthopaedic applications while allogeneic bone, carries potential risks of cell-mediated immune responses to alloantigens and of transmission of pathogens such as HIV / prion mediated contamination. Against this backdrop, tissue engineering using skeletal progenitor populations has emerged as a promising approach for skeletal tissue formation (reviewed in Oreffo et al., 2005). In principles this involves the combination of living cells within a natural or synthetic scaffold, to produce a three dimensional living tissue construct which is functionally, structurally and mechanically equal to, if not better than, that which it has been designed to replace (Stock and Vacanti, 2001; Logeart-Avramoglu et al., 2005). Human bone marrow stromal cells injected percutaneously for the treatment of tibial non-unions and avascular necrosis of the hip have met with some success, while, animal studies have shown that tissue engineering techniques using autologous skeletal populations seeded onto a scaffold can lead to skeletal tissue regeneration at a defect site (Bruder et al., 1998, Arinzeh et al., 2003; Dai et al., 2005; den Boer et al., 2003).

4.1 The Chondrogenic niche informing skeletal regeneration

Cartilage degeneration as a result of trauma or degenerative diseases such as osteoarthritis is a major healthcare problem and due to the aneural, avascular and alymphatic nature of articular cartilage spontaneous repair is limited. Furthermore, the potential for spontaneous repair is observed to decrease with age as mature cartilage shows a decrease in chondrocyte activity. Tissue regeneration strategies,

coupled with the ability to isolate human chondrocyte populations, offers a significant, yet achievable, challenge to engineer cartilage at the required site. The process of tissue engineering is influenced by inductive signals and the presence of appropriate growth factors. Therefore a number of growth factors can be used to stimulate the differentiation along a particular lineage or to enhance the production and deposition of extracellular matrix molecules that parallels *in vivo* physiology. Previous studies have demonstrated that TGF- β -induced chondrogenesis involves a rapid deposition of a cartilage-specific extracellular matrix (Barry et al., 2001) and that more specifically; chondrogenic differentiation of skeletal stem cells is rapidly induced in the presence of TGF- β 2 or β -3, with an increase in aggrecan and type II collagen. BMPs also induce differentiation of skeletal stem cells into chondrogenic lineage cells (Wan and Cao 2005). BMP-2 has been shown to induce extracellular matrix synthesis, whilst BMP-7 has been shown to restore proteoglycan synthesis by chondrocytes (Wan and Cao 2005). Furthermore, IGF-I has been shown as the main anabolic growth factor of normal cartilage, and FGF signalling pathways induce Sox-9 expression in primary chondrocytes and in undifferentiated cells (Goessler et al 2005).

Further requirements for tissue regeneration are a supportive scaffold and alginate polysaccharide capsules offer a 3D structure for the encapsulation and organization of cells exploiting this paradigm and can not only support a range of cell skeletal cell types but allow presentation of specific stimuli to direct the formation of a desired tissue as well as allowing mechanical integrity to a cell-tissue construct (Caterson et al., 2001; Alsberg et al., 2002; Green et al., 2005). We have used human skeletal progenitors encapsulated within alginate/chitosan polysaccharide templates to promote chondrogenesis and looked at the potential to begin to create artificial microenvironments (Pound et al., 2006, 2007). Following the additional encapsulation of types I and II collagen, large regions of extracellular matrix have been generated that stained positively for cartilage proteoglycans and type II collagen, demonstrating chondrogenic differentiation (Fig 3). Additionally, constructs developed regions of matrix that stained positively for type I collagen, bone sialoprotein and osteoid formation, demonstrating the potential to promote chondrogenic and osteogenic differentiation and extracellular matrix formation with implications for skeletal regeneration and application (Pound et al., 2006, 2007). Furthermore, encapsulation of Sox-9 transfected bone marrow stromal and chondrogenic cells, in alginate templates, generated enhanced cartilaginous extracellular matrix, positive for Sox-9 and type II collagen, in comparison with templates encapsulated with untransfected cells (Babister et al 2008). These

findings further emphasise the need to understand the interactions between cells and the extracellular matrix components of the chondrogenic niche and their importance in tissue regeneration strategies as specific factors regulating native tissue generation, maintenance and repair will provide invaluable insights into regeneration of cartilage defects.

4.2 Perivascular niche informing skeletal tissue regeneration

The interaction between the osteoblast/endothelial cell and perivascular niche and resultant effects on stem cell maintenance and production will ultimately improve the way new tissue engineered bone and vascular constructs are generated and implanted. EC signalling is a critical mechanism during the development and repair of bone (Gerber and Ferrara, 2000). VEGF, a potent angiogenic factor, couples hypertrophic cartilage remodelling, ossification and angiogenesis during endochondral bone formation (Gerber et al., 1999). In bone fractures the osteoblastic and vascular niches are severely compromised. Without the recruitment of osteoprogenitor and vascular cells and the development of a bone callus (this may in turn provide a unique osteoblastic/vascular niche) at the fracture site, bone repair will be severely impaired. Creating a tissue engineering construct that provides a niche with a combination of growth factors, stem cells and a biomimetic scaffolds would be a critical step in the regeneration of bone. While a variety of materials have been used for bone regeneration together with skeletal stem cells and osteoprogenitors, including ceramics or materials based on hydroxyapatite, ceramic forms of β -tricalcium phosphate and composites of both hydroxyapatite and β -tricalcium phosphate, there has been relatively little work on creating specific niche environments. There has been work on trying to exploit biological cues that are necessary to mimic the cell-bone matrix interactions with the generation of biomimetic scaffolds based on poly(lactic acid) (PLA) poly(-lactic-co-glycolic acid) (PLGA) as well as poly(glycolic acid) (reviewed in Pearson et al., 2002). In our studies we have used a simple strategy of combining select growth factors encapsulated within scaffolds to thus engineer scaffolds that release growth factors such as VEGF, BMP-2 or both in a spatial and temporal manner to stimulate resident skeletal osteoprogenitors to repair a critical sized defect (Fig 4). The continued development of smart designer scaffolds and materials with selection of skeletal stem cell and osteoprogenitor populations is needed to develop appropriate microenvironments that may ultimately deliver on the promise of skeletal tissue engineering using skeletal stem cell populations.

5. Summary

This review has centred on the osteogenic, chondrogenic and perivascular niches and the growth factors and matrix constituents that modulate and influence skeletal stem cell activity and fate. We have examined the derivation of skeletal stem and progenitor populations and examined the key regulatory factors, matrix constituents and signal molecules that influence activity, niche identity and function and the complex interplay across niches including the haemopoietic environment. Current evidence suggests biomimetic strategies that utilise the information from these environments and their control of cell function for osteogenic stem cell function will impact significantly on strategies to enhance skeletal tissue repair and offer exciting new research directions to harness that knowledge for musculoskeletal regeneration in an ageing population.

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Fig. 1. The differentiation of skeletal stem cells to generate stromal lineages including bone, fat, cartilage, connective tissue, muscle as well as nervous tissue and their corresponding regulation by transcription and growth factors. The postulated steps are highly schematic and the potential for neuronal tissue remains controversial.

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Fig. 2. Representative histograms (from 8 experiments) demonstrating mean % STRO-1+ cells (\pm SD) in BMMNC populations ($n = 8$), isolated from bone marrow samples of haematologically normal osteoporotic and osteoarthritic subjects (panel A). The average proportion of STRO-1+ cells in BMMNC populations was determined at 11% (± 3). Day 3 CFU-F of STRO-1+ cells (panels B and C), cultured in basal medium (α -MEM containing 10% FCS) for 9 days, exhibited STRO-1 (-FITC) immunoreactivity (panels D and E). STRO-1(-FITC) immunoreactivity was maintained in fibroblastic cells of day 6 cultures (panels F and G) and day 9 cultures (panels H and I). Scale bars = 100 μ m.

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Fig 3. Human bone marrow stromal cells encapsulated within alginate polysaccharide templates with the addition of 0.25mg/ml type I collagen (a) and type II collagen (b), demonstrating development of large regions of chondrogenic matrix. Regions of cell-generated extracellular matrix were positive for type I collagen (c) and type II collagen (d).

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Fig. 4. Representative μ CT scans 28 days post scaffold implantation in MF-1 nu/nu mouse femur defect model. Control defect without scaffold (panel A), VEGF encapsulated PLA scaffold seeded with HBMSC (panel B). Alcian blue/ Sirius red staining of tissue sections through the femur defect implanted with VEGF encapsulated PLA scaffold and seeded with HBMSC (panel C and D). Scale bars: 2mm for panel A & B; 100 μ m for panel C & D.

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Table 1
Cell-surface antigen expression profile of human bone marrow-derived skeletal stem cells

Cell-surface antigen	Expressed	Not expressed	Details
STRO-1 antigen	✓		Cell-surface trypsin-resistant antigen expressed by CFU-F Antibody: STRO-1
Tissue non specific Alkaline phosphatase (TNSALP)	✓		Cell-surface glycoprotein associated with osteoblast lineage cells Antibody: STRO-3
CD63	✓		Synonyms: Melanoma-associated antigen ME491, Tetraspanin 30, Lysosomal-associated membrane protein 3 Antibody: HOP26
CD105	✓		Synonym: Endoglin/ SH2
CD73	✓		Synonym: SH3/ 4
CD71	✓		Synonym: Transferrin receptor protein 1 (TfR1)
CD90	✓		Synonym: Thymocyte antigen-1 (Thy-1)
CD49a	✓		Synonym: Laminin and collagen receptor VLA- α 1
<u>Cell adhesion molecules</u>			
CD106	✓		Synonym: Vascular cell- adhesion molecule-1 (VCAM-1)
CD166	✓		Synonym: Activated leukocyte cell-adhesion molecule (ALCAM) Antibody: SB-10
ICAM-1	✓		Intercellular cell- adhesion molecule-1
CD29	✓		Synonym: β 1 subunit of the integrin family
CD31		✓	Synonym: Platelet/ endothelial cell-adhesion molecule-1 (PECAM-1)
CD56		✓	Synonym: Neural cell- adhesion molecule-1 (NCAM-1)
CD18		✓	Synonyms: Leukocyte cell- adhesion molecule, integrin β 2 (LCAMB), Lymphocyte function associated antigen-1 (LAD, LFA-1)

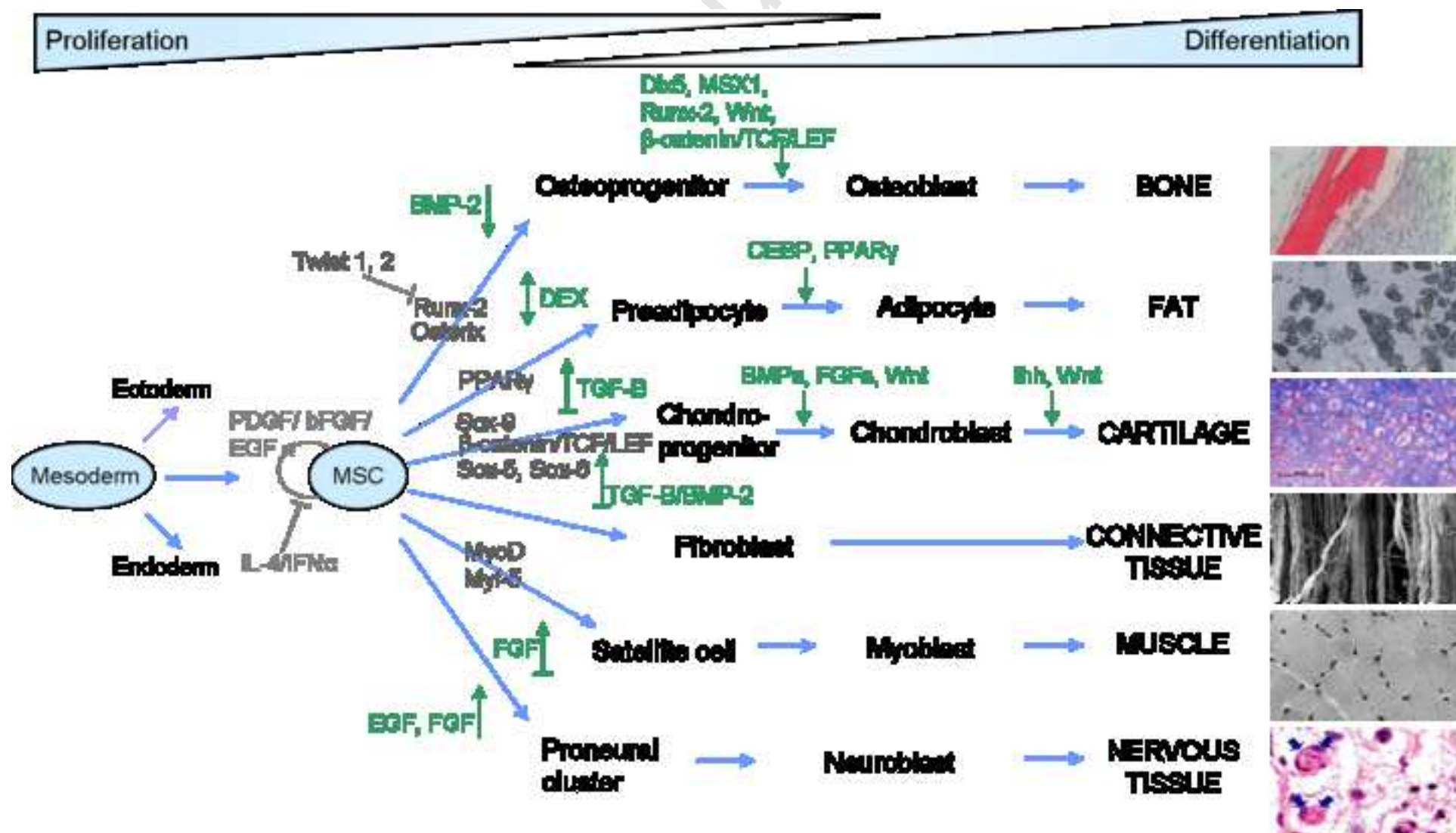
<u>Haematopoietic markers</u>		
CD45	✓	Synonym: Leukocyte common antigen Located on haematopoietic cells except erythrocytes and platelets
CD34	✓	Identified on haematopoietic progenitors and endothelial progenitors
CD14	✓	Preferentially expressed on monocytes/macrophages
CD11	✓	α component of various integrins, especially those in which the β component is CD18
CD44	✓	Expressed by haematopoietic as well as bone marrow mesenchymal stem cells
<u>Co-stimulatory molecules</u>		
CD80	✓	Synonym: B7.1, provides co-stimulatory signal necessary for T cell activation and survival
CD86	✓	Synonym: B7.2, provides co-stimulatory signal necessary for T cell activation and survival
CD40	✓	Expressed by all mature B lymphocytes, monocytes, dendritic, endothelial and epithelial cells

Table 2

Overview of isolation protocols utilising antibodies recognised by stromal antigens expressed on human bone marrow mononuclear cells

Reference	Cells/ tissue	Protocol used	% antigen-positive cells
Simmons and Torok-Storb, 1991	BMMNC from bone marrow aspirates of normal donors	Flow cytometry	STRO-1+ (10%)
Joyner et al., 1997	4% formalin-fixed cultures of bone marrow-derived cells from trabecular bone samples of haematologically normal individuals	Immunohistochemistry	CD63+ Day 1 of culture: <10% Day 3 of culture: >95% Day 28 of culture: <1%
Majumdar et al., 2000	BMMNC isolated from human bone marrow	Magnetic activated cell sorting (MACS)	CD105/ Endoglin+ Dual antibody procedure: 3±0.9% Single antibody procedure: 1.8±0.3%
		Flow cytometry	Over 60% of CD105+ cells co-expressed STRO-1 antigen
Howard et al., 2002	BMMNC from bone marrow samples of haematologically normal individuals	Magnetic activated cell sorting (MACS)	STRO-1+ 7.12±3.8%
Stewart et al., 2003	BMMNC from femoral reamings of osteoarthritic individuals	Flow cytometry	STRO-1+ (27%) CD63+ (46%) CD49a+ (5%) (CD49a+ cells also coexpressed STRO-1 antigen) CD166+ (19%)
Letchford et al., 2006	BMMNC from femoral reamings of osteoarthritic individuals	Flow cytometry	STRO-1+ (15±9%) CD49a+ (5.4±4.5%) C15+ (4.41±2.66%)

Figure 1 Lineage



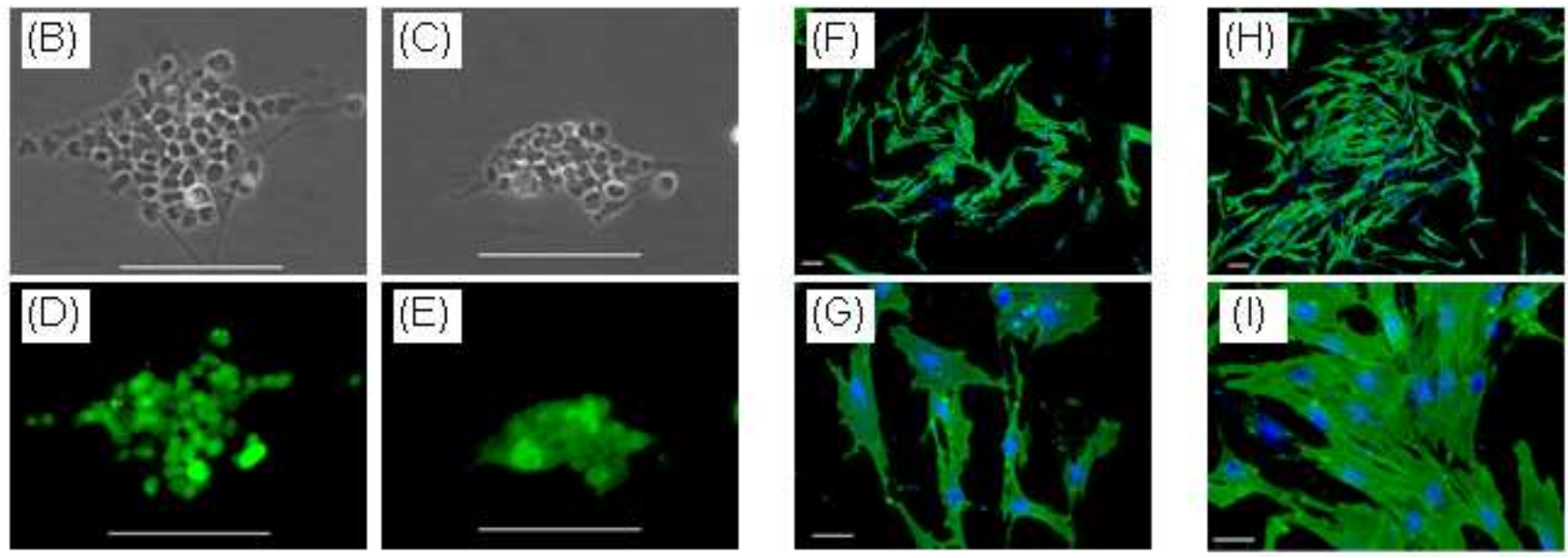
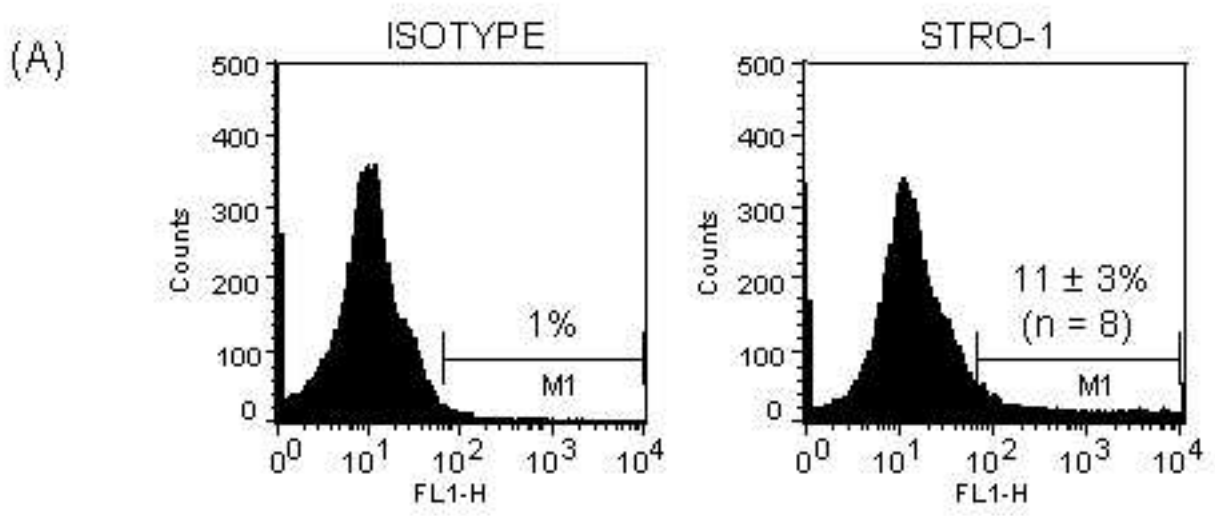
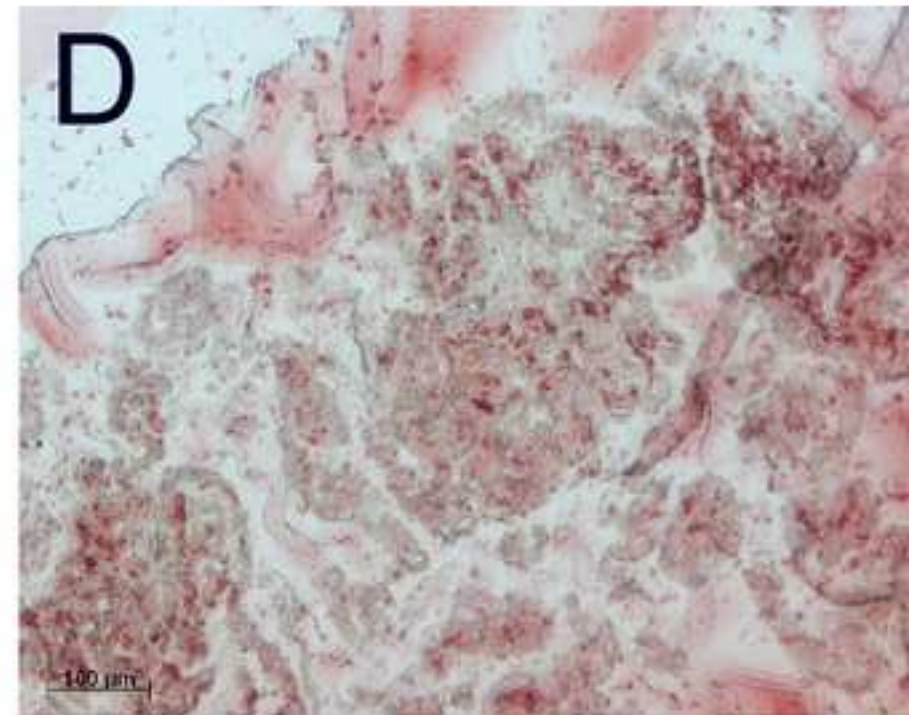
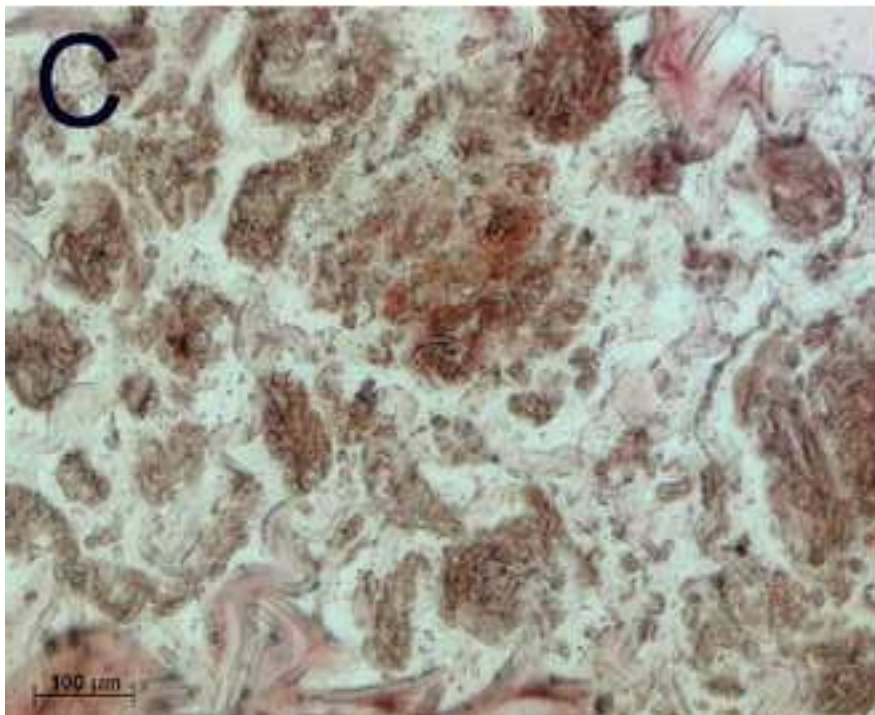
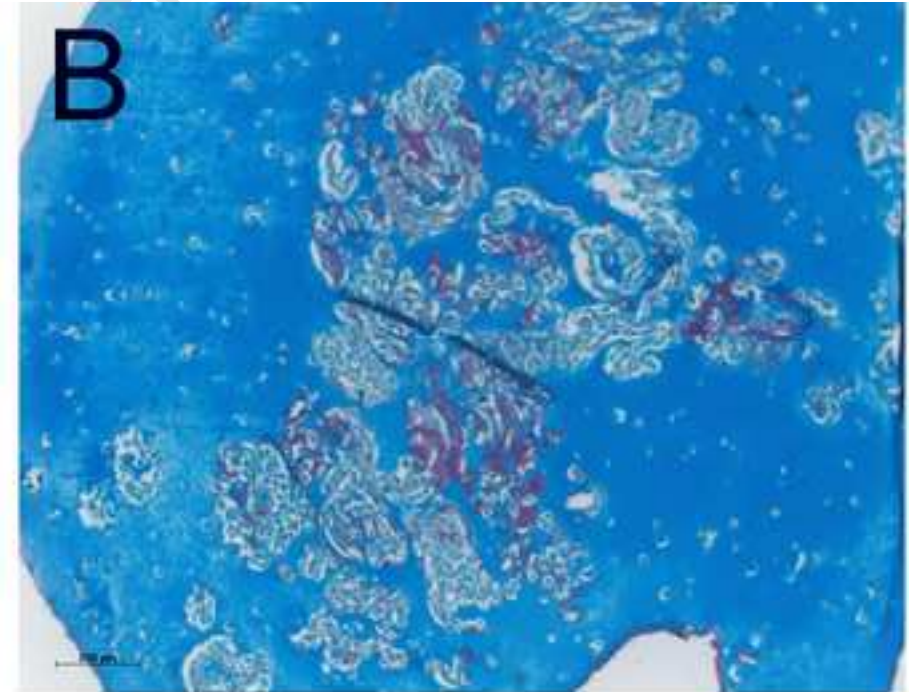
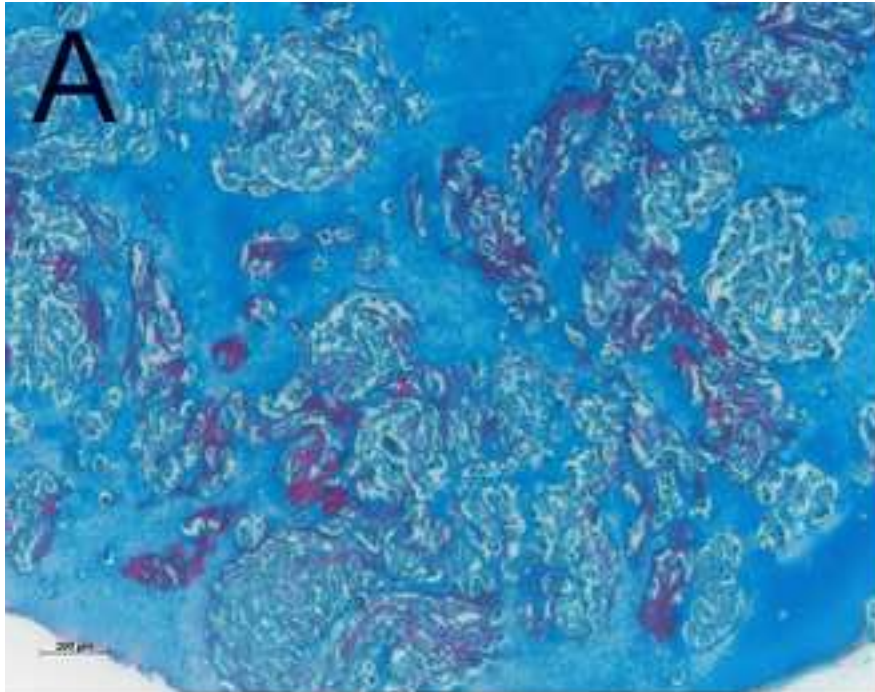


Figure 3



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