Mesenchymal Stem Cells in Regenerative Medicine: Opportunities and Challenges for Articular Cartilage and Intervertebral Disc Tissue Engineering

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Defects of load-bearing connective tissues such as articular cartilage and intervertebral disc (IVD) can result from trauma, degenerative, endocrine, or age-related disease. Current surgical and pharmacological options for the treatment of arthritic rheumatic conditions in the joints and spine are ineffective. Cell-based surgical therapies such as autologous chondrocyte transplantation (ACT) have been in clinical use for cartilage repair for over a decade but this approach has shown mixed results. This review focuses on the potential of mesenchymal stem cells (MSCs) as an alternative to cells derived from patient tissues in autologous transplantation and tissue engineering. Here we discuss the prospects of using MSCs in regenerative medicine and summarize the advantages and disadvantages of these cells in articular cartilage and IVD tissue engineering. We discuss the conceptual and practical difficulties associated with differentiating and pre-conditioning MSCs for subsequent survival in a physiologically harsh extracellular matrix, an environment that will be highly hypoxic, acidic, and nutrient deprived. Implanted MSCs will be exposed to traumatic physical loads and high levels of locally produced inflammatory mediators and catabolic cytokines. We also explore the potential of culture models of MSCs, fully differentiated cells and co-cultures as "proof of principle" ethically acceptable "3Rs" models for engineering articular cartilage and IVD in vitro for the purpose of replacing the use of animals in arthritis research.

The increase in life expectancy that has accompanied modern advances in medicine has lead to increased prevalence of a range of musculoskeletal disorders which are causing an ever greater socioeconomic burden on health systems around the world as the population ages. According to the World Health Organization (WHO) (http://www.who.int/en/) rheumatic, arthritic, and musculoskeletal conditions comprise over 150 diseases and syndromes, which are usually progressive and associated with pain. They can broadly be categorized as joint diseases, physical disability, spinal disorders, and conditions resulting from trauma. Musculoskeletal conditions are leading causes of morbidity and disability, giving rise to enormous healthcare expenditures and loss of work. Knowledge of the key determinants of disability in musculoskeletal conditions is critical for reducing their burden on the world's growing population (Weigl et al., 2008). Musculoskeletal pain affects one in four adults and is the most common source of serious long-term pain and physical disability. Consequently musculoskeletal conditions are now recognized by the United Nations, the WHO, World Bank, and numerous governments throughout the world through support of the Bone and Joint Decade 2000–2010 initiative (Walsh et al., 2008).

Contract grant sponsor: National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs);
Contract grant number: 28102007.
Contract grant sponsor: Biotechnology and Biological Sciences Research Council (BBSRC);
Contract grant numbers: BBSRC/S/M/2006/13141, BB/G018030/1.
Contract grant sponsor: WALTHAM Centre for Pet Nutrition.
Contract grant sponsor: The Wellcome Trust;
Contract grant number: CVRT VS 0901.
Contract grant sponsor: The Engineering and Physical Sciences Research Council (EPSRC).
Contract grant sponsor: Research Councils UK (RCUK).
Contract grant sponsor: The Ingenuity Programme (University of Nottingham Business School).

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The number of rheumatoid arthritis (RA) and osteoarthritis (OA) patients steadily rises as the elderly population grows in Western Europe, North America, and the rest of the developing world. RA, OA, and back pain are important causes of disability-adjusted-life years in both the developed and developing world (Brooks, 2006). Back and knee pain are common in the community and are likely to increase with the aging population (Brooks, 2006). Until recently OA was viewed as a "degenerative" or "wear-and-tear" disease and held little interest for most clinicians. It is now accepted that the age-related degeneration of articular cartilage as part of the clinical syndrome of OA is one of the most common causes of pain and disability in middle-aged and older people (Buckwalter and Mankin, 1998; Mollano et al., 2002; Aigner et al., 2004, 2007).

In particular, joint and back pain are among the major causes of disability and morbidity within the adult population. OA is the most common form of joint disease, with the majority of the population over 65 years of age demonstrating radiographic evidence of OA in at least one joint. Likewise, around two-thirds of the adult population suffer from lower back pain (LBP) at some point in their lifetime.

The current inadequacy of treatments for these conditions, combined with their increasing prevalence exacerbates the burden on healthcare systems. Therefore, researchers and clinicians are striving for novel, innovative treatment options and the emergence of the fields of tissue engineering and regenerative medicine offer hope that long-term tissue repair may be possible. However, one of the current limiting factors for treatment of cartilage and intervertebral disc (IVD) diseases is a source of cells. While a range of cell sources have been proposed, adult mesenchymal stem cells (MSCs) offer the greatest potential for clinical application. This review will focus on the requirements for articular cartilage and IVD regeneration and the factors which are essential for lineage-specific differentiation of MSCs which leads to formation of the correct, functional matrix with similar or improved properties to the original tissue.

Bone and cartilage defects are common features of joint diseases, such as RA and OA (Noel et al., 2002). They have significant social and economic impact on the aging population. Despite progress in orthopedic surgery, bone and cartilage repair is a major challenge as large defects will not spontaneously heal (Noel et al., 2002). Regenerative medicine is an emerging field that seeks to repair or replace injured tissues and organs through natural or bioengineered means. Recent research on stromal MSCs has provided a new and exciting opportunity for bone and cartilage tissue engineering. We have learned a great deal about the isolation, cultivation, and characterization of MSCs in recent years. A huge amount of research effort is focused on their differentiation. MSCs have generated a great deal of public, scientific, and media interest because of their potential use in regenerative medicine and tissue engineering. In this review we critically evaluate the strengths and limitations of regenerative medicine strategies and tissue engineering approaches that employ MSCs for articular cartilage and IVD regeneration and repair.

Identification and Application of Adult Stem Cells

Until recently, scientists primarily worked with two types of stem cells from animals and humans: embryonic stem cells and non-embryonic "somatic" or "adult" stem cells. Embryonic stem cells are found in the blastocyst whereas adult stem cells are found in adult tissues. Adult stem cells maintain the normal turnover of organs with a high intrinsic regenerative capacity. These include blood, skin, and intestinal epithelium. Adult stem cells can be found in children, adolescents as well as adults and are generally unipotent or multipotent. Pluripotent adult stem cells are very rare and are generally found in small numbers. However, they can be found in a number of tissues including umbilical cord blood. The best studied adult stem cells are multipotent and are generally referred to by their tissue origin (i.e., hematopoietic stem cells that differentiate into erythrocytes, white blood cells, platelets, etc.) and bone marrow stromal cells (also known as MSCs), which have the capacity to differentiate into connective tissue cells. The hierarchy of stem cells is illustrated in Figure 1 and the differentiation pathways involved in the specialization of MSCs into a selected number of musculoskeletal lineages is depicted in Figure 2.

MSCs possess the capacity to differentiate into cells of connective tissue lineages (Pittenger et al., 1999; see Fig. 3). These include bone (Noel et al., 2002; Arinzeh, 2005; Hong et al., 2006), fat (Barry and Murphy, 2004; Helder et al., 2007), cartilage (Noel et al., 2002; Barry and Murphy, 2004; Caplan, 2007), IVD (Trubiani et al., 2005, 2006; Richardson et al., 2007), ligament (Trubiani et al., 2005, 2006; Sonoyama et al., 2006), and muscle (Barry and Murphy, 2004). Evidence suggests that these cells are also capable of differentiation along myogenic and neurogenic lineages, although these pathways are not normally utilized to prove multipotentiality of isolated MSCs.

Adult MSCs were originally isolated from bone marrow in 1999 by Pittinger and co-workers, who demonstrated their multilineage differentiation potential. Subsequent studies have identified the presence of stem cells in a number of adult tissues, including adipose, muscle, dermis, periosteum, synovial membrane, synovial fluid, and articular cartilage. Thus far, MSC-like progenitor cells have been isolated from bone marrow (Grigoriadis et al., 1988), periosteum, trabecular bone, adipose tissue, synovium, skeletal muscle, and deciduous teeth (Barry and Murphy, 2004; Sonoyama et al., 2006). While there are no definitive markers of MSCs a range of cell surface markers are routinely used. These include immunopositivity for STRO-1, CD73, CD105, CD106, CD145, and CD166, combined with negative immunoreactivity for CD11b, CD31, CD34, CD45, and CD117 (Fig. 4). These markers can also be used to identify a more homogeneous population of cells than previous methods

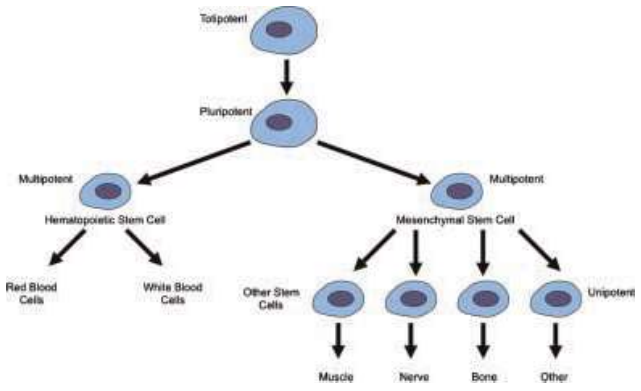

Fig. 1. The structural hierarchy of stem cells. The potency of stem cells specifies the differentiation potential of stem cells. Totipotent stem cells are produced from the fusion of an egg and sperm cell. Cells produced by the first few divisions of the fertilized egg are also totipotent. These cells can differentiate into embryonic and extraembryonic cell types. Pluripotent stem cells are the descendants of totipotent cells and can differentiate into cells derived from any of the three germ layers. Multipotent stem cells can produce only cells of a closely related family of cells (e.g., hematopoietic stem cells differentiate into red blood cells, white blood cells, platelets, etc. and other stem cells which include mesenchymal stem cells). Unipotent cells only have the capacity to produce one cell type, but have the property of self-renewal which distinguishes them from non-stem cells (e.g., muscle stem cells). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

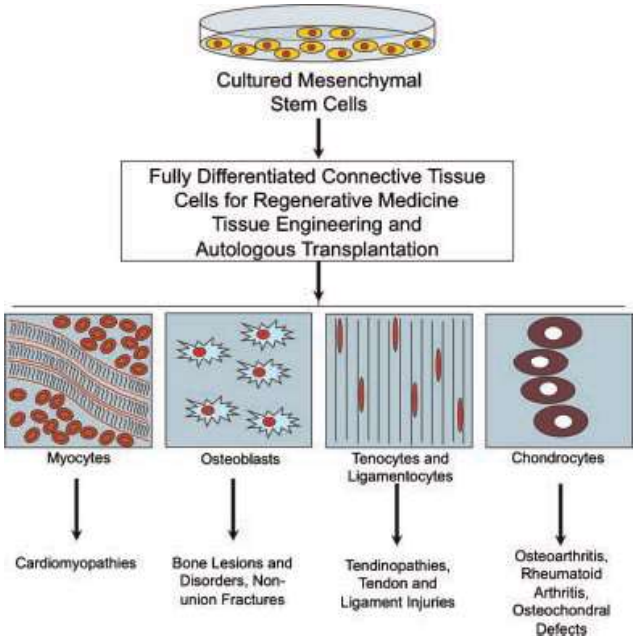

Fig. 2. Differentiation potential of cultured mesenchymal stem cells. Mesenchymal stem cells have the capacity to differentiate into connective tissue and musculoskeletal cells for tissue engineering, autologous implantation/transplantation and regenerative medicine. This process involves commitment, lineage progression, differentiation and maturation. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

utilizing either density-gradient centrifugation, or even simple plastic adherence. The general heterogeneity of bone marrow cell populations can lead to variable results; however, MSCs are generally regarded to be capable of differentiation along the chondrogenic, osteogenic, and adipogenic pathways. Work from our laboratory, along with that of other groups, also suggests that MSCs are capable of differentiation to nucleus pulposus (NP) cells of the IVD, chondrocytes, and osteoblasts (Csaki et al., 2007, 2009; Richardson et al., 2007; Mobasheri et al., 2009). However, since no definitive markers of NP cells are in existence, a range of chondrocyte markers, with which they share a large phenotypic similarity, are routinely used.

Musculoskeletal Tissue Regeneration

Connective tissues, such as bone, cartilage, tendon, and the IVD all suffer from both traumatic and age-related degenerative injuries. While bone repairs relatively well, the avascular and hypocellular nature of cartilage and in particular the IVD means these tissues suffer from a very limited self-repair capacity. During OA and IVD degeneration (one of the main causes of LBP) there is an imbalance between synthesis and degradation of the extracellular matrix which leads to an overall loss of tissue. This tissue destruction over time leads to pain and reduced mobility.

While the increased repair capacity of bone prevents this, a number of metabolic and degenerative disorders, such as osteoporosis, osteonecrosis, and Paget's disease, can cause bone loss or abnormal bone turnover. Traumatic injury and loss of bone following surgery for other diseases such as cancer can also lead to bone lesions which are too large for the body's self-regeneration capacity to cope with. Current clinical interventions for large bone defects, such as bone grafting, suffer from problems with donor site morbidity and limited

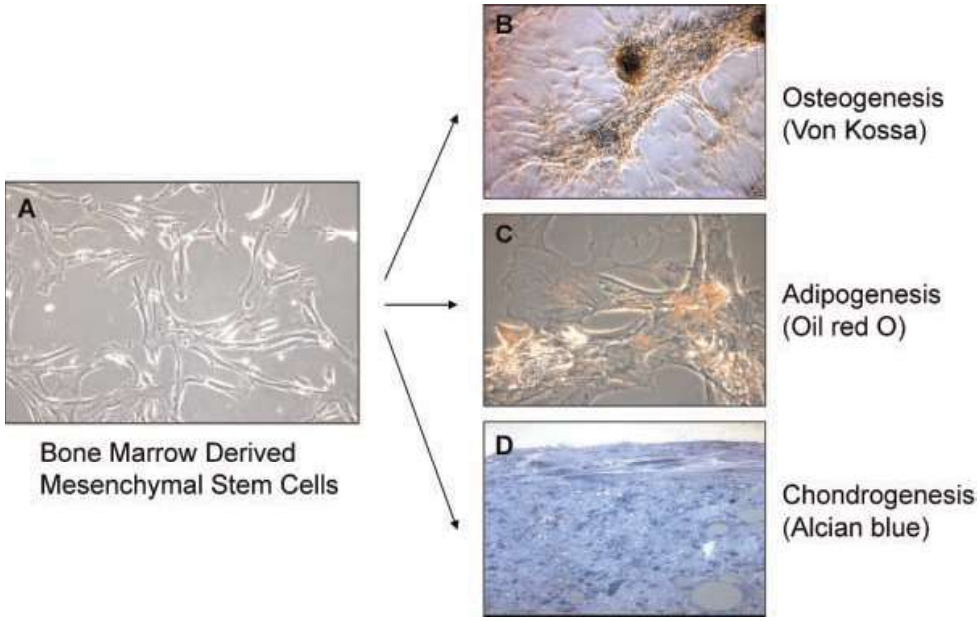

Fig. 3. Light microscopic demonstration of osteoid, adipose, and cartilage tissue formation with von Kossa, Oil Red O, and Alcian blue staining. MSCs incubated with the osteogenic induction medium from a fibroblastic appearance (A) to a more cuboidal appearance; cells were surrounded with an abundant matrix and formed mineralized nodules (B). After 3 weeks in culture, stimulated cells were stained positive with von Kossa stain for mineral deposition in their newly formed extracellular matrix. C: In monolayer cultures treated with the adipogenic induction medium an abundance of vacuoles were observed within the cells. Oil Red O staining for lipids revealed the presence of neutral lipids. D: After 21 days high-density cultures treated with chondrogenic induction medium (i.e., TGF-β1 and dexamethasone) were intensely stained with Alcian blue revealing a high content of cartilage-specific proteoglycans. Magnification: A, B (10×); C and D (40×). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

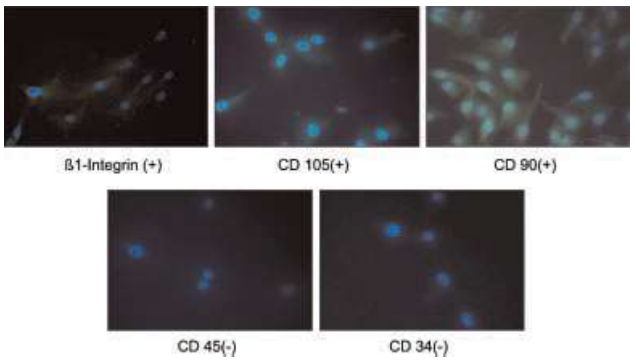

Fig. 4. Immunofluorescence images showing expression of canine MSC markers. Positive expression: β-1 integrin, CD-105, and CD-90. Negative expression: CD-45 and CD-34. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

material for large-scale autologous grafts and infection or immune rejection with allogeneic grafts.

These limitations in current clinical treatments for bone defects and disorders have led to the development of tissue engineering strategies using both synthetic and natural scaffold implants. Materials such as tricalcium phosphates (Solchaga et al., 1999; Guo et al., 2004a,b; Shao et al., 2006; Jiang et al., 2007), calcium carbonates (Kreklau et al., 1999), hydroxyapatite (Reddi, 2000; Yoshikawa and Myoui, 2005; Chajra et al., 2008), and Bioglass (Wilda and Gough, 2006; Helen and Gough, 2008) have all been utilized alongside growth factors to promote new growth, or act as supports for implanted cells to regenerate new tissue. These materials all aim to mimic the specialized microenvironment or bone and cartilage and are growing in their clinical application.

Articular cartilage and the IVD share similarities in cellular phenotype and extracellular matrix composition. Consequently similar approaches have been adopted for tissue engineering cartilage and IVD (Richardson et al., 2007; Kalson et al., 2008). As with bone repair, there are major limitations to current clinical treatments for OA and IVD degeneration. Scaffold-free autologous chondrocyte implantation (ACI) is currently used clinically to treat small cartilage lesions, such as those caused by traumatic injury. For this treatment a biopsy is taken from a non-load-bearing region of cartilage, the chondrocytes enzymatically extracted, expanded in monolayer culture, then reimplanted into the lesion and covered with a periosteal flap (Brittberg et al., 1994). The therapy has been used to treat over 12,000 patients worldwide and offers cartilage repair and reduced pain equivalent to existing surgical cartilage repair therapies (Peterson et al., 2002). However, while this treatment appears to work well for small cartilage lesions it has limited practical application for larger osteoarthritic lesions.

Cell implantation into degenerate IVDs has been demonstrated in animal models to both inhibit degeneration and regenerate tissue (Ganey et al., 2003). A small-scale study in human patients comparing standard discectomy for disc prolapse with discectomy plus autologous disc cell implantation demonstrated improvements in pain reduction, preservation of disc height and prevention of adjacent disc segment degeneration in patients who underwent the cell implantation therapy (Meisel et al., 2006, 2007). Clinical follow-up data is limited and there is no biochemical or biomechanical data, but this study demonstrates the potential for cell-based therapies for IVD tissue regeneration. However, studies suggest that isolation of cells from degenerate IVD could accelerate degeneration, while isolation of cells from non-degenerate levels may induce degenerative changes and needle puncture is a common method of inducting degeneration in animal models of disease (Kim et al., 2005; Masuda et al., 2006; Korecki et al., 2008; Zhang et al., 2009). Additionally, as IVD degeneration, like OA in cartilage, affects the phenotype of resident cells the identification of a suitable cell source for novel tissue engineering and regenerative medicine strategies is one of the key determinants of its success.

Comparison of Cartilage and Intervertebral Disc Cell and Matrix Phenotypes

During embryogenesis articular cartilage forms through the condensation of MSCs, which then undergo differentiation to chondrocytes. The avascular, aneural, and alymphatic tissue formed during this process is rich in collagen and proteoglycans (PGs) and serves to protect the ends of bones and articulate movement. Around 40–50% of total cartilage ECM is collagen and while 90% of total collagen is type II, other collagens are present, including types VI, IX, and XI. In deeper, hypertrophic regions of cartilage type X collagen is expressed. PGs represent around 25% of cartilage ECM, of which over 50% is aggrecan, with decorin, biglycan, and fibromodulin among the other predominating PGs present. The cellular component of the tissue is relatively small, with rounded chondrocytes embedded within the matrix at a density of around 15,000 cells/mm^3 (Stockwell, 1971). The chondrocytes interact with their ECM through a specialized pericellular matrix rich in type VI collagen. In addition to facilitating cell–matrix interactions this PCM is also thought to protect the cells from compressive mechanical loading forces experienced within the cartilage during normal joint movement. Homeostatic matrix turnover is coordinated by the chondrocytes through tightly regulated anabolic and catabolic processes and involves a diverse array of growth factors, cytokines, and matrix degrading enzymes.

The IVD is notochordal in origin and comprises a central gelatinous NP, surrounded by a more fibrous annulus fibrosus (AF), with cartilaginous end-plates connecting the adjacent vertebral bodies (Trout et al., 1982; Humzah and Soames, 1988; Buckwalter, 1995). During developing the NP contains a population of notochordal cells, which die out usually by the age of 10 and are replaced by NP cells (Trout et al., 1982), although the exact source of these NP cells is still unclear. Like articular cartilage, the NP is rich in type II collagen and aggrecan (Sive et al., 2002). However, unlike articular cartilage the NP has around 27 times more PG than collagen (Mwale et al., 2004). The PGs confer a much higher osmotic potential to the tissue, resulting in an increased hydration and producing a more gelatinous tissues than cartilage. The NP cells share a common morphology and phenotype with articular chondrocytes (Sive et al., 2002), although their density within the tissue is much lower at around 5,000 cells/mm^3 (Maroudas et al., 1975). Like chondrocytes the NP cells are embedded within a PCM rich in type VI collagen, again due to the compressive mechanical loading forces experienced within the spine (Wu et al., 1987; Poole et al., 1988; Roberts et al., 1991a,b; Guilak et al., 2006). While there are similarities in phenotype between chondrocytes and NP cells the differences in the ECM of the two tissues clearly demonstrates that differences must exist and studies have recently identified molecular markers which can be used to identify NP cells specifically. However, clear evidence of the origin and defined phenotype of human NP cells is still lacking and may hamper subsequent MSC-based tissue regeneration strategies.

Cell and Matrix Changes During OA and IVD Degeneration

During OA and IVD degeneration loss of the tightly controlled matrix turnover mechanisms leads to matrix degradation. Both

OA chondrocytes and degenerate NP cells express higher levels of catabolic cytokines such as IL-1 and TNF-α compared to their normal phenotypes (Le Maitre et al., 2005, 2007b; Daheshia and Yao, 2008). The increase in these catabolic cytokines is associated with increases in a range of matrix metalloproteinase (MMP) and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) enzymes which degrade the ECM (Cawston et al., 1999; Flannery et al., 1999; Seguin et al., 2006; Le Maitre et al., 2007a; Millward-Sadler et al., 2009; Pockert et al., 2009). The catabolic cytokines have also been shown to decrease matrix production, particularly aggrecan, and increase the secretion of pain mediators such as NGF and Substance P (Rannou et al., 2000; Le Maitre et al., 2005; Purmessur et al., 2008; Sutton et al., 2009). There is also evidence of cell apoptosis and senescence, which further affect cell function and limit self-repair capacity (Martin and Buckwalter, 2001, 2002; Mobasheri, 2002; Goggs et al., 2003; Zhao et al., 2006; Le Maitre et al., 2007a; Shakibaei et al., 2008). In articular cartilage OA there is a breakdown of the smooth articular surface, with lesions radiating down into the cartilage, eventually causing full thickness erosion of the cartilage and exposing underlying bone. This erosion causes pain and limited mobility in the joint and current clinical treatment involves invasive surgical intervention, often necessitating total joint replacement.

In IVD degeneration the loss of matrix from the NP leads to dehydration and eventually loss of disc height. This loss of height removes tension from the collagen fibers of the AF and destabilizes the whole motion segment. Disc innervation causes pain and changes in anatomy of the spine leads to both pressure on the nerve root and limited mobility. While prosthetic disc replacements are available, such as Charite (Blumenthal et al., 2005) or Prodisc (Bertagnoli et al., 2005), the current standard treatments for end-stage degeneration are microdiscectomy and spinal fusion, both of which limit mobility and change the biomechanics of the spine leading to degenerative changes at adjacent disc levels (Levin et al., 2007).

In both cases there is clear need for novel cell-based tissue engineering strategies. However, the changes in cellular physiology preclude the use of autologous OA chondrocytes, or degenerate NP cells. Therefore, there is an increasing focus on the potential use of MSCs for regeneration of the tissues.

Molecular and Physiological Determinants of Chondrogenic Differentiation

Since Pittenger et al. (1999) demonstrated the chondrogenic potential of MSCs during pellet culture numerous methods have been described which allow MSC chondrogenesis (Tew et al., 2008a). In particular gels such as alginate (Ma et al., 2003) and agarose (Fukumoto et al., 2003) have been utilized and more recently a range of tissue engineering biomaterials have been described which allow or promote chondrogenesis. Similarly we have demonstrated differentiation of MSCs to NP-like cells within chitosan glycerophosphate hydrogels (Richardson et al., 2008a) and poly-L-lactic acid scaffolds (Richardson et al., 2006a), both of which have potential for clinical application for the regeneration of cartilaginous tissues.

However, a number of basic principles underpin the majority of this research. Firstly, MSC chondrogenesis requires a three-dimensional environment and secondly differentiation requires the addition of exogenous anabolic factors. In particular the addition of TGF-β, as part of a serum-free differentiating medium, is one of the most widely used growth factors (Johnstone et al., 1998; Pittenger et al., 1999) and in addition to promoting chondrogenesis has also been shown to inhibit osteogenic and adipogenic differentiation (Zhou et al., 2004; Jian et al., 2006). IGF-1 and members of the BMP family of growth factors, particularly BMP-7, have also been shown to induce chondrogenesis of MSCs and expansion of monolayer MSCs in medium containing FGF-2 is also thought to promote chondrogenesis following transfer to a 3D culture environment (Solchaga et al., 2005; Chiou et al., 2006; Knippenberg et al., 2006; Longobardi et al., 2006). However, one of the main problems with in vitro differentiation methods is the complexity of the signaling pathways involved in chondrogenesis, compared to the simplicity of culture systems. Pellet cultures mimic the mesenchymal condensation which occurs during embryogenesis and numerous studies have demonstrated the importance of cell–cell contact for MSC differentiation to either chondrocytes or NP cells (Richardson et al., 2006b). Likewise anabolic growth factors are known to stimulate differentiation and matrix formation and operate through a number of pathways, predominantly the MAPKinase and Smad pathways (Murakami et al., 2000; Jian et al., 2006; Longobardi et al., 2006).

Successful chondrogenesis is routinely assessed by the induction of SOX-9, which subsequently drives the production of type II collagen, and the increased expression of the PG aggrecan (Tsuchiya et al., 2003; Tew et al., 2008b). The similarities in the phenotype of articular chondrocytes and NP cells of the IVD (Sive et al., 2002) mean, in the absence of validated and highly specific NP marker genes, these markers are also routinely used to identify NP-like cells. However, MSC differentiation in standard in vitro culture systems appears to be unstable and routinely results in expression of hypertrophic markers such as type X collagen and alkaline phosphatase (Johnstone et al., 1998; Yoo et al., 1998). The possibility that chondrogenic differentiation may lead to hypertrophy is problematic for clinical application given that healthy surface and mid zone chondrocytes and NP cells do not express either type X collagen or alkaline phosphatase (Boos et al., 1997; Nerlich et al., 1997; Gan et al., 2003). This was evidenced by Pelttari et al. (2006) who compared MSCs and chondrocytes in pellet cultures. Their work demonstrated that, following implantation into SCID mice, the MSCs showed high levels of type X collagen and alkaline phosphatase expression which promoted vascular invasion and calcification, while chondrocytes produced a cartilaginous matrix. For cartilage or IVD regeneration it is therefore essential to ensure a stable differentiated phenotype and current in vitro culture systems may be too simple to allow this.

However, stimulation with multiple growth factors may improve differentiation or inhibit terminal differentiation. For example, combination of TGF-β3 with BMP-2 gave improved chondrogenic differentiation of MSCs compared to either growth factor alone, or the combination of TGF-β3 with either BMP-4 or BMP-6 (Sekiya et al., 2005). Addition of PTHrP to TGF-β3 stimulated MSCs in poly-glycolic acid scaffolds has also been shown to inhibit the expression of type X collagen and suppress terminal differentiation of these cells (Kim et al., 2008). Hypoxia and TGF-β3 drive MSC differentiation towards a phenotype consistent with that of the NP and these cells could be used to repopulate the damaged or degenerate IVD (Risbud et al., 2004).

Physiological Microenvironmental Challenges for MSC-Based Tissue Regeneration Strategies: A Case of Nurture Versus Nature?

In order to understand more clearly the interplay between different growth factors and produce cells with a correct and stable phenotype, more complex, biologically relevant test systems are required. However, in order to develop these test systems it is important to understand fully the microenvironmental niche into which cells will eventually be implanted.

Both articular cartilage and the NP of the human IVD are avascular and aneural tissues, which leads to a unique environment where cells rely on diffusion of nutrients, such as glucose, and oxygen from capillaries in adjacent bony tissue (Humzah and Soames, 1988). This diffusion problem is thought to be one of the major reasons for the molecular diversity of glucose transporters in articular chondrocytes and IVD cells (Mobasheri et al., 2002a,b, 2005a, 2008; Richardson et al., 2003, 2008b; Phillips et al., 2005). The lack of vasculature results in low oxygen concentrations and consequently cells in both tissues are exposed to an environment containing only around 2% oxygen. The hypoxic conditions in the extracellular matrix of cartilage and IVD also have profound effects on glucose transport and metabolism (Mobasheri et al., 2008; Richardson et al., 2008b; Peansukmanee et al., 2009). However, chondrocytes, NP and AC cells have shown molecular adaptations to this limited oxygen environment and use anaerobic glycolysis, mediated through the actions of the highly conserved HIF-1α transcription factor, to generate ATP (Holm et al., 1981; Bibby and Urban, 2004; Bibby et al., 2005; Mobasheri et al., 2005b, 2008). HIF-1α has key functions in controlling energy generation, cell survival, and matrix synthesis by articular and growth-plate chondrocytes (Pfander and Gelse, 2007). However, there appear to be differences in the adaptations of NP cells and chondrocytes, with NP cells demonstrating stable expression of HIF-1α under both normoxic and hypoxic conditions, while chondrocytes lose expression of HIF-1α in normoxia (Risbud et al., 2006; Agrawal et al., 2007; Pfander and Gelse, 2007). While HIF-1α is known to regulate glycolysis, in NP cells the stabilized form of HIF-1α has also been shown to regulate aggrecan expression (Agrawal et al., 2007).

The avascular nature of the tissues also limits the diffusion of nutrients to and waste products away from cells, which in the case of NP cells can be as far as 8 mm from the nearest blood vessel (Brodin, 1955a,b). The consumption of glucose during anaerobic glycolysis decreases the glucose concentration within the tissues, resulting in a nutrient gradient towards the core of the tissues. This metabolic signature is similar to the metabolism of cancer cells (Airley and Mobasheri, 2007), producing lactic acid (Mobasheri et al., 2006, 2008), which can build up in addition to protons generated by chondrocytes in the extracellular matrix of cartilage and IVD tissue, producing an acidic environment with a pH as low as 5.7 (Diamant et al., 1968; Mobasheri et al., 1998), although a pH of around 7.0–7.2 is more common in normal NP tissues (Wuertz et al., 2009a).

The high PG content of the tissues, particularly the NP and the deep zone of articular cartilage, confers a high fixed negative charge, which is equilibrated through the distribution of cations (Na^+, K^+, Ca^{2+}, and H^+) within the extracellular matrix (Mobasheri et al., 1997, 1998; Mobasheri, 1998, 1999). The high abundance of protons reduces the pH of the extracellular matrix to around 0.5 pH units lower than surrounding tissues, even without the effect of lactic acid (Urban, 2002). In addition, PGs also confer a high osmotic potential which acts to draw in water and give a high swelling pressure to the extracellular matrix, which maintains hydration of cartilage and IVD. This swelling pressure allows the tissues to withstand the high compressive loads experienced within the articulating joints and IVD, which are known to be as high as 2.3 MPa in the normal IVD (Wilke et al., 1999) and 6 MPa in normal articular cartilage within the knee joint (Herberhold et al., 1999).

During IVD degeneration and OA the nutrient supply to the tissues is compromised, making these already harsh conditions even worse. This is thought to be caused, in part, by calcification of the end-plates within the IVD, which blocks the flow of oxygen, nutrients, and lactic acid, to and from blood vessels in the vertebral bodies. As a result hypoxia has been shown to reach 1%, while pH levels drop to around 6.5. Both of these features have been shown to be detrimental to NP cell survival and matrix production (Ohshima and Urban, 1992; Bibby and Urban, 2004; Bibby et al., 2005). Therefore, in order to ensure that MSCs will survive and function properly following implantation into either degenerate NP tissue or osteoarthritic cartilage model in vitro systems are required which take into consideration microenvironmental features such as pH, oxygen concentration, mechanical load, osmolarity, glucose concentration, and high catabolic cytokine levels.

MSC Pre-Conditioning to Enhance Cell Survival and Function

One proposed method to ensure MSC survival and function following implantation into the degenerate IVD or osteoarthritic cartilage is to pre-condition, or pre-differentiate the cells. A recent study by Wuertz et al. (2008) demonstrated that when cultured in an IVD-like environment containing low glucose, low pH, and high osmolarity, MSCs demonstrated a lower proliferation rate and lower expression of matrix genes compared to standard conditions, highlighting the importance of the microenvironmental niche on MSC biology. In particular, low pH has been shown to inhibit expression of aggrecan by MSCs (Wuertz et al., 2009a), while the presence of catabolic cytokines IL-1 and TNFα, which are increased in both IVD degeneration and OA, inhibited chondrogenesis of MSCs (Wehling et al., 2009). Conversely, hypoxia has been shown to promote MSC chondrogenesis via the up-regulation of HIF-1α and this increased chondrogenesis was abolished following siRNA knock-down of HIF-1α (Kanichai et al., 2008). The importance of the HIF gene HIF-2α in hypoxia-enhanced chondrogenesis was also demonstrated in stem cells isolated from intrapatellar fat pad of OA patients (Khan et al., 2007). Studies investigating the differentiation of MSCs to NP-like cells have also demonstrated that hypoxia, combined with exogenous addition of TGF-β in 3D alginate hydrogel cultures, elevated expression of matrix genes collagen types II and XI and aggrecan, as well as the transcription factor SOX-9 through increases in both phosphorylated ERK and p38 (Risbud et al., 2004). The authors suggest that this combination of factors may produce cells which can be used to repopulate the degenerate NP. Similarly, studies by the same authors showed that adaptation of the NP cells to their hyperosmotic milieu is dependent on activation of the ERK and p38-MAPK pathways acting through TonEBP, a transcription factor that permits adaptation to osmotic stress and regulates aggrecan gene expression in addition to other target genes (Tsai et al., 2007). Combination of TGF-β stimulation with application of cyclical mechanical load also promotes chondrogenesis of MSCs compared to stimulation with TGF-β or cyclical mechanical loading alone (Li et al., 2009). Interestingly this study also demonstrated that mechanical loading stimulated the expression of TGF-β1 and TGF-β3 by MSCs seeded in fibrin-biodegradable polyurethane scaffolds, suggesting that load may be a key mediator in driving MSC chondrogenesis and production of a stable chondrocyte or NP-like phenotype. While there are no studies investigating the effect of load on MSCs to NP-like cells, load is known to be important in regulating matrix gene expression by NP cells (Le Maitre et al., 2008; Wuertz et al., 2009b) and we have recently demonstrated that a combination of hypoxia and load can accelerate MSC differentiation to NP-like cells during culture in type I collagen gels in media containing TGF-β.

Development of Novel Ex Vivo Model Systems for Assessing MSC Differentiation and Function

While mimicking the microenvironmental niche of articular cartilage and the IVD in vitro demonstrates which features

promote MSC differentiation and which factors are inhibitory, these systems are not truly representative of the tissue into which the cells will be implanted.

Co-culture systems have been utilized to investigate the interaction of chondrocytes or NP cells with MSCs and demonstrated that direct cell–cell contact in monolayer (Richardson et al., 2006b), or co-culture in 3D culture without cell–cell contact can induce differentiation. A recent study by Kim et al. (2009) has also demonstrated that when MSCs and NP cells, each cultured in separate alginate beads, were exposed to mechanical stimulation expression of SOX-9 and aggrecan was increased compared to co-cultures without load. However, while these systems highlight the fact that NP cells and chondrocytes stimulate MSC differentiation, to date this appears to be the only study to have incorporated microenvironmental conditions into their study. Novel high-density pellet culture systems, such as those shown in Figures 5 and 6, stage MSCs and co-cultures of chondrocytes, osteoblasts, and MSCs at the air–liquid interface using a steel bridge, with nutrients being supplied via diffusion through a nitrocellulose filter membrane (Haisch et al., 2006; Csaki et al., 2007, 2009; Mobasheri et al., 2009). These studies have been used to demonstrate MSC differentiation in a three-dimensional environment which more closely mimics the environment of articular cartilage in vivo. Modeling IVD tissue is however more complex, since the NP is enclosed within the fibrous AF circumferentially and by the end-plates and vertebrae to the inferior and superior aspects. We have previously developed an NP explant model in which the tissue is constrained circumferentially and have utilized this system to demonstrate that human MSCs injected into explanted bovine caudal discs undergo spontaneous differentiation to NP-like cells, increasing expression of SOX-9, type II collagen, and aggrecan, but importantly not type X collagen (Le Maitre et al., 2009a) after 4 weeks. To allow culture of these explants we have also developed a bioreactor capable of mimicking the microenvironmental conditions found within the normal and degenerate human IVD. This system allows modification of media pH, glucose concentration, osmolarity, and mechanical load, all of which have been shown to be important in maintaining high cell viability and directing MSCs to a stable differentiated phenotype. We have demonstrated using this bioreactor that if explants are exposed to a daily regime of mechanical loading comparable to that experienced in vivo, then cell viability and matrix retention are improved compared to unloaded controls (Le Maitre et al., 2009b). This novel bioreactor-explant model system will therefore allow us to test novel cell-based tissue regeneration strategies under controllable microenvironmental conditions. We hope it will also be possible to combine this style of bioreactor with whole-disc organ culture models currently under development. These models allow natural constraint of the NP and offer an opportunity to test regenerative medicine therapies in the most relevant biological systems without the need for unsuitable animal models. This aspect is particularly important for IVD research, where current animal models do not represent the cell, humoral or biochemical microenvironment of the human lumbar spine. In particular many animal discs contain a population of notochordal cells, which are not present in adult humans; the discs are smaller than human discs and hence the oxygen and nutrition supplies are not comparable; the NP tissue is less fibrous than that of adult human tissue; and importantly the biomechanics of animal spines is different to that of humans given the quadrapedal nature. Additionally, the synovial joints and IVD of most animals do not undergo spontaneous degeneration and current methods for inducing degeneration are not representative of the human condition (the suitability of animal models was recently reviewed in detail by Alini et al., 2008).

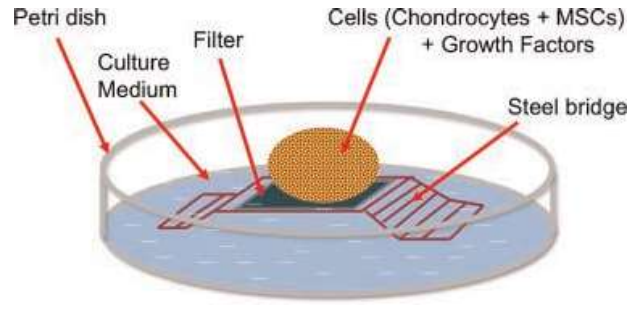

Fig. 5. Schematic of the air–liquid interface system for high-density (3D) cultures. A nitrocellulose filter is placed on a steel-net-bridge and cells are cultured on the filter. Cell culture medium reaches the filter–medium interface, nurturing cells through diffusion, thus, mimicking an environment similar to that found in vivo. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

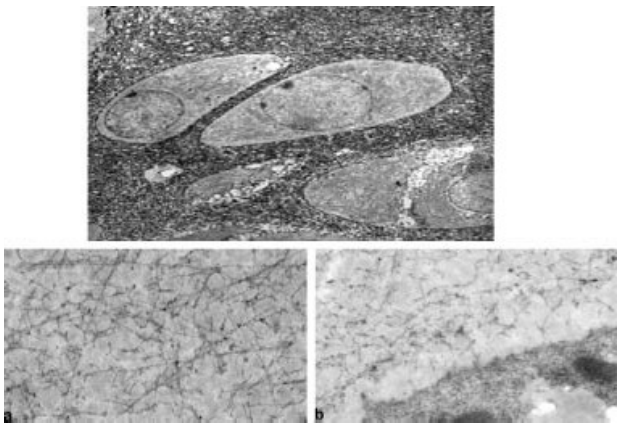

Fig. 6. Electron microscopic demonstration of chondrogenesis induced in high-density 3D cultures of canine MSCs. After 14 days, nodules with typical round or oval chondrocytes with small processes embedded in a network of extracellular matrix fibrils could be observed. Transmission immunoelectron microscopy of MSC cultures induced with chondrogenic induction medium in high-density culture. Chondrogenic-induced cultures were labeled by immunogold using anti-collagen type II (a) or anti-cartilage-specific proteoglycan antibodies (b).

Summary and Concluding Remarks

In this review we have focused on the potential of MSCs as an alternative to cells derived from patient tissues for tissue engineering and therapeutic applications such as ACL. There are still many technical challenges associated with isolating, expanding, differentiating, and pre-conditioning MSCs for subsequent implantation into the spine and degenerate joints. The physiological microenvironment of the degenerate joint or spine is likely to be hypoxic, acidic, deprived of nutrients, and exposed to higher than normal concentrations of pro-inflammatory cytokines and reactive oxygen species. Furthermore, MSCs may be exposed to abnormal physical loads. Future regenerative medicine strategies will need to address these issues.

The molecular determinants of MSC differentiation into chondrogenic and osteogenic lineages continue to be elucidated using existing 2D models. However, new research using 3D models is more likely to shed light on fundamental processes such as paracrine-mediated differentiation, and mechanisms responsible for the recruitment of endogenous progenitor populations in response to chemical, biological, and physical cues. Furthermore, three-dimensional and high-density culture models have huge potential as "3Rs" models for replacing and reducing the use of animals in OA and IVD research. There are numerous animal models for OA and other types of arthritis. However, none of them have a proven track record of predictability for the equivalent disease in humans and the majority of them are plagued with problems (Pritzker, 1994). Three-dimensional culture conditions, hypoxia and the use of specially supplemented culture media will ultimately need to be assessed in pre-clinical animal models that closely mimic the human disease. However, 3D culture models utilizing tissue engineered articular cartilage and IVD should reduce the numbers of experimental animals used before moving onto larger and more expensive pre-clinical animal models.

Acknowledgments

The authors thank their collaborators Professor Anthony J. Freemont (Manchester) and Professor Pablo Martín-Vasallo (Tenerife). This study was made possible by grants from the National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs) (grant number: Mobasheri.A.28102007), the Biotechnology and Biological Sciences Research Council (BBSRC) (grants BBSRC/S/M/2006/13141 and BB/G018030/1), the WALTHAM Centre for Pet Nutrition, the Engineering and Physical Sciences Research Council (EPSRC), The Ingenuity Programme (University of Nottingham Business School), and The Wellcome Trust (grant number: CVRT VS 0901). Stephen M. Richardson is a Research Councils UK (RCUK).

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