

Review Article

Mesenchymal stem cell therapy in equine musculoskeletal disease: science, fact or clinical fiction?

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Summary

The goal in the therapeutic use of mesenchymal stem cells (MSCs) in musculoskeletal disease is to harness the regenerative nature of these cells focussing on their potential to grow new tissues and organs to replace damaged or diseased tissue. Laboratory isolation of MSCs is now well established and has recently been demonstrated for equine MSCs. Stem cell science has attracted considerable interest in both the scientific and clinical communities because of its potential to regenerate tissues. Much has been investigated and learnt but it must be appreciated that in spite of this, the field is still relatively young and both communities must prepare themselves for considerable time and effort to develop the technology into a highly efficient treatment. However, the promise of functional tissue engineering to replace old parts with new fully justifies the interest. At present, it is important to balance the understanding of our current limitations with a desire to progress the technology.

Introduction

Mesenchymal stem cells (MSCs) have the potential to revolutionise the treatment of equine orthopaedic disease because of their ability to self-renew and differentiate into various tissue lineages under specified conditions (Lee and Hui 2006). The use of haemopoietic stem cells for the clinical treatment of leukaemia (e.g. bone marrow transplants) is already well established whereas the use of mesenchymal stem cells is much more in its infancy. The goal in the therapeutic use of MSCs in musculoskeletal disease is to harness the regenerative nature of these cells focussing on the potential to grow new tissues and organs to replace damaged or diseased tissue. In addition to *in vitro* experiments and *in vivo* studies in animals, studies on human patients seem to be showing promise that stem cell therapy can be beneficial in the treatment of osteogenesis imperfecta and myocardial infarction (Horwitz *et al.* 2002; Wollert *et al.* 2004). Currently, degenerative diseases under investigation for treatment with stem cells in man are extensive, but include diseases such as

osteoarthritis, diabetes mellitus, Parkinson's disease, ischaemic heart disease and retinal degeneration (Hows 2005).

This review article describes the isolation and characterisation of MSCs and focusses on the evidence that MSCs can potentially enhance repair of 3 orthopaedic tissues: tendon, articular cartilage and bone.

Definition of a stem cell

The term stem cell was first coined in the 19th Century by Edmund Beecher Wilson who described the term as a synonym for a mitotically quiescent primordial germ cell (Wilson 1896). Stem cells have also been described as the natural units of embryonic generation or adult regeneration of a variety of tissues (Weissman 2000). In the 1960s, it was first identified that bone marrow derived cells were capable of differentiating into cells (osteoblasts) of mesenchymal origin (Friedenstein and Petrokova 1966). The ability of these bone marrow derived MSCs to differentiate into cells of different lineages (osteoblasts, chondrocytes and adipocytes) is now widely established in many laboratories (Johnstone *et al.* 1998; Pittenger *et al.* 1999; Barry *et al.* 2001; Jiang *et al.* 2002) and has been recently demonstrated for equine bone marrow derived MSCs (Koerner *et al.* 2006; Vidal *et al.* 2006). Mature bone marrow derived mesenchymal stem cells (BM-MSCs) are found post natally in the nonhaemopoietic fraction of the bone marrow stroma which comprises a heterogeneous population of cells including reticular cells, adipocytes, osteogenic cells, smooth muscle cells, endothelial cells and macrophages (Lee and Hui 2006).

Stem cell terminology

Stem cells have 2 basic characteristics: they are able continually to self-renew; and they are capable of differentiating into multiple specialised cell types (Baksh *et al.* 2004; Ryan *et al.* 2005). Truly **totipotent** stem cells are able to create an entire organism, a property only of the early embryo (Fortier 2005; Lee and Hui 2006). **Pluripotent** stem cells harvested from the embryonic blastocyst are capable of differentiating into cells of the 3

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mammalian germ lines (mesoderm, endoderm and ectoderm) under specific signalling conditions (Conley *et al.* 2004). **Multipotent** is the term commonly applied to mature stem cells capable of differentiating into a limited number of different lineages (Lee and Hui 2006). Multipotent stem cells capable of differentiating into more specialised cell types (for example osteocytes, chondrocytes and adipocytes) can be aspirated from nonhaemopoietic fraction of bone marrow (Pittenger *et al.* 1999). Stem cell **plasticity** refers to the ability of mature stem cells to acquire mature phenotypes that are different from their tissue of origin (Grove *et al.* 2004).

Transdifferentiation refers to the ability of a stem cell committed to one specific cell line to switch to another cell type of a different lineage sometimes across embryonic germ layers (Song *et al.* 2006). For example, a mesenchymal stem cell derived from the bone marrow, that is stimulated to become an osteoblast with the relevant stimuli, may be able to revert to different phenotype, such as, an adipocyte following addition of stimulatory factors that are appropriate for adipogenic differentiation (Song and Tuan 2004). To date, the exact pathways involved in transdifferentiation are unclear (Song *et al.* 2006).

Stem cell niches are specialised microenvironments that safeguard against excessive stem cell production and progression to cancer but also allow controlled replication for progenitor production and tissue repair when necessary. The balance between quiescence and activity is the important characteristic of a functional stem cell niche (Moore and Lemischka 2006)

'**Stemness**' has been described as stem cell potency - the cell's breadth of competence for differentiation (Shostak 2006). Bone marrow MSCs harvested from mature mice have been described as **multipotent adult progenitor cells** (MAPCs) by virtue of their ability to differentiate across cell lines representing the 3 embryonic germ layers (Jiang *et al.* 2002). The breadth of differentiation demonstrated by Jiang *et al.* (2002) showed that bone marrow derived cells could develop into tissues of all the 3 germ lines (haemopoietic cells, epithelium of the liver, lung and gut; in addition to cells of mesenchymal origin). Our current understandings of the molecular mechanisms that maintain self-renewal and control differentiation are not at a point where 'tissues could be made on demand'. However, advances in identifying the molecular signature of embryonic stem cells (ESCs) has highlighted pathways, such as those involving the transcription factors Wnt and Smad, involved in the maintenance of 'stemness' (Tabibzadeh and Hemmati-Brivanlou 2006).

Techniques used to assess 'stemness'

Differentiation experiments

Mesenchymal stem cells can be encouraged to progress down particular differentiation routes by the addition of specific factors under defined culture conditions. The multilineage differentiation ability of MSCs has been extensively studied *in vitro* and most authors consider that to demonstrate the presence of a stem cell, it should be possible to differentiate it into at least 3 different cell types. Some debate centres around whether this property is solely an *in vitro* phenomenon and does not relate to the situation *in vivo*. The demonstration of 'Koch's postulates' for stem cells implanted *in vivo* has been rarely demonstrated (i.e. the ability to implant stem cells which will differentiate and form new target tissues and from which stem cells with tri-lineage potential can be recovered).

Furthermore, due to the multitude of elaborate isolation protocols and the huge variety of tissues from which MSCs can be sourced, differentiation of MSCs is difficult to compare between studies. To complicate matters further factors such as donor age and disease status can affect the differentiation abilities of MSCs (Majors *et al.* 1997). More recent studies describe the phenomenon of clonal heterogeneity (Pittenger *et al.* 1999; Muraglia *et al.* 2000; Okamoto *et al.* 2002; Baksh *et al.* 2004). If a single cell is isolated *in vitro* and expanded, so that all daughter cells are derived from this single cell, the phenotype, gene expression and protein expression should be similar. However, it has been shown that cells isolated in this manner do not have similar properties; Pittenger *et al.* (1999) and Muraglia *et al.* (2000) reporting only one third of clones having tri-lineage (osteochondro/adipo) differentiation potential.

Quantification of differentiation of stem cells down different lineage pathways relies upon the identification and objective measurement of specific genes or markers associated with each particular cell lineage. For gene expression, osteogenesis is routinely measured by quantification of Runx2, a gene that regulates osteogenesis; chondrogenesis can be identified by SOX9 expression; and adipogenesis can be proven by the expression of PPAR γ . In addition, histology is often used to identify various types of differentiation: oil red O staining for fat, increase in alkaline phosphatase and calcium deposition for bone (Pittenger *et al.* 1999); and safranin O for proteoglycans in cartilage (Chang *et al.* 2006). Tendon specific 'markers' have not yet been identified although scleraxis, a gene identified during embryonic tenogenesis, was proposed to possess such a property (Schweitzer *et al.* 2001; Wang *et al.* 2005). However, it remains uncertain whether the expression of this gene is tendon specific. An equine homologue of this gene has not yet been identified and therefore tenogenesis is currently best demonstrated by the histological appearance of longitudinally aligned collagen fibres and the presence of a 'bank' of proteins that distinguishes tendon from other musculoskeletal tissues (e.g. the presence of collagen I and III and COMP, and the absence of collagen II).

Cell surface antigens

Mesenchymal stem cells carry cell surface proteins that can be recognised by specific antibodies and therefore can be used to characterise the cells. A unique 'mesenchymal stem cell marker' has not yet been identified in contrast to the hallmark antigen CD34 used for positive immunoselection of HSCs (Tuan *et al.* 2003) There is general agreement that MSCs lack the typical haematopoietic antigens; CD45, CD34 and CD14 (Pittenger *et al.* 1999; Koc *et al.* 2000; Jones *et al.* 2002; Baksh *et al.* 2004) while possessing other cell surface antigens such as CD44, CD106 (Pittenger *et al.* 1999), CD166 (SB-10), CD105 (SH-2), CD73 (SH-3, SH-4) (Barry and Murphy 2004).

These antigens can then be used to sort subpopulations of cells using the powerful technique of fluorescence activated cell sorting (FACS). Unfortunately these MSC surface markers share many common features with endothelial, epithelial and muscle cells (Minguell *et al.* 2001). Most of these markers have not been validated as equine cell surface markers to date and there is evidence that some have been shown not to cross-react with the horse (L.A. Fortier, F.P. Barry, personal communications). For stem cell biology to progress in the horse, it is imperative that such putative markers are developed for equine use.

Gene expression

In addition to cell surface antigens, cells can be defined by the genes expressed. The gene profile of human MSCs can now be evaluated using techniques such as gene microarray (Pazianos *et al.* 2003), quantitative assessment of gene expressions using real-time PCR and serial analysis of gene expression (SAGE) (Li and Akashi 2003; Pazianos *et al.* 2003), and the function of particular genes identified using small interfering RNA (siRNA) (Song *et al.* 2006). With the imminent sequencing of the equine genome it is likely that these techniques will become more available for use in equine research.

Protein expression

The cytokine expression profile for MSCs has been extensively described (for review see Minguell *et al.* 2001) demonstrating that MSCs produce several growth factors, interleukins and other cytokines and also their receptors suggesting that MSCs can interact with their microenvironment.

Types of stem cells capable of forming musculoskeletal tissues

1) Embryonic stem cells

Embryonic stem cells (ESCs) are the *in vitro* cultured pluripotent cells obtained from the inner cell mass of the embryonic blastocyst (Bongso *et al.* 1994; Laslett *et al.* 2003; Conley *et al.* 2004; Lee and Hui 2006). The human embryonic blastocyst is formed approximately 5 days following *in vitro* fertilisation and comprises 2 tissue types: the inner cell mass, which develops into all tissues of the embryo; and its extra embryonic trophoblast, which contributes structures of the placenta (Kimber 2000). It is during this period that pluripotent ESCs can be derived through immunodissection or physical micro dissection (Laslett *et al.* 2003; Pera *et al.* 2003; Conley *et al.* 2004). The cells of the 5 days blastocyst *in vivo* are able to proliferate and self-renew for a transient period before they become restricted to more specific and committed cell precursors. ESCs have been cultivated from several species including the mouse, rat, man (Thomson *et al.* 1998) and, recently, the horse (Li *et al.* 2006; Saito *et al.* 2006). Following their isolation, these cells are transferred to coculture with murine or bovine embryonic fibroblasts, also known as ‘feeder cells’, which are necessary to ensure the ESCs retain their pluripotent ability. The mechanisms and specific factors needed to maintain pluripotency remain unknown (Conley *et al.* 2004; Gruen and Grabel 2006).

A successful test of pluripotency of a stable ESC line is its ability to differentiate into ectoderm, mesoderm and endoderm when injected into animals (Thomson *et al.* 1998). This is most frequently demonstrated by the propensity to form teratomas when injected into mice (Thomson *et al.* 1998).

Mesenchymal stem cells

Mesenchymal stem cells were isolated originally from the nonhaemopoietic bone marrow stroma and have the potential to differentiate into tissues of mesenchymal origin including chondrocytes, osteoblasts, adipocytes, fibroblasts and marrow stroma (Tuan *et al.* 2003). The ability of MSCs to differentiate into many types of musculoskeletal tissue holds great possibilities in

the repair and regeneration of cartilage, bone and tendon. However, the mesenchymal stem cell fraction of the bone marrow constitutes only 0.001–0.01% of the nucleated cells found in the bone marrow (Pittenger *et al.* 1999) and, in many cases, the number needed are much greater than can be harvested directly from these clinical aspirates. Techniques for expanding these cells in culture are being developed although, as with ESCs the problem with control of differentiation still exists.

Mesenchymal stem cells can be recovered from a wide range of tissues from different ages (see Table 1). In the fetus, MSCs have been isolated from a range of tissues including spleen, lung, pancreas and kidneys (In ’t Anker *et al.* 2004). Circulating MSCs have also been found in first trimester fetal blood, liver and bone marrow (Kern *et al.* 2006; Lee *et al.* 2006). A growing body of literature suggests that mid trimester MSCs may have better growth and plasticity properties compared to adult MSCs (Gotherstrom *et al.* 2005; Guillot *et al.* 2006).

Neonatal MSCs can be sourced from umbilical cord blood and Wharton’s jelly (umbilical cord perivascular tissue). It has been reported that umbilical cord blood contains a more primitive population of multipotent MSCs capable of differentiating into cells of the 3 germ layers (Lee *et al.* 2004). However, MSCs from cord blood have been shown to have a reduced adipogenic differentiation (Bieback *et al.* 2004; Chang *et al.* 2006). Other studies suggest umbilical cord blood MSCs retain their multipotentiality but only have a limited lifespan (Karahuseyinoglu *et al.* 2006).

TABLE 1: Sources and differentiation potential of adult and neonatal MSCs

Source tissue	Differentiation potential	References
Mature		
Bone marrow	Osteo, adipo, chondro Mesoderm, ectoderm, endoderm (haematopoietic, liver, lung, gut)	(Pittenger <i>et al.</i> 1999) (Jiang <i>et al.</i> 2002)
Equine bone marrow	Osteo, adipo, chondro Osteo, adipo	(Yoshimura <i>et al.</i> 2006) (Vidal <i>et al.</i> 2006)
Trabecular bone	Osteo, adipo, chondro Osteo, adipo, chondro	(Koerner <i>et al.</i> 2006) (Noth <i>et al.</i> 2002) (Sottile <i>et al.</i> 2002) (Tuli <i>et al.</i> 2003)
Periosteum	Osteo, chondro Osteo, chondro, myo Osteo, adipo, chondro	(De Bari <i>et al.</i> 2001) (De Bari <i>et al.</i> 2006) (Yoshimura <i>et al.</i> 2006)
Cartilage	Osteo, adipo, chondro Osteo, chondro, tendon, perimysium	(Barbero <i>et al.</i> 2003) (Dowthwaite <i>et al.</i> 2004)
Tendon	Osteo, adipo	(Salingcamboriboon <i>et al.</i> 2003)
Synovial membrane	Osteo, adipo, chondro, myo Osteo, adipo, chondro	(De Bari <i>et al.</i> 2001) (Yoshimura <i>et al.</i> 2006)
Synovial fluid	Osteo, adipo, chondro	(Jones <i>et al.</i> 2004)
Muscle	Osteo, adipo, myo Osteo, adipo, myo	(Asakura <i>et al.</i> 2001) (Wada <i>et al.</i> 2002) (Williams <i>et al.</i> 1999)
Fat	Osteo, adipo, chondro Osteo, adipo, chondro, myo	(Im <i>et al.</i> 2005) (Zuk <i>et al.</i> 2001)
Blood	Osteo, adipo, fibro	(Zvaifler <i>et al.</i> 2000)
Equine blood	Osteo, adipo	(Koerner <i>et al.</i> 2006)
Dermis	Osteo, adipo, chondro, myo	(Young <i>et al.</i> 2001)
Neonatal		
Umbilical cord blood	Osteo, adipo, chondro	(Chang <i>et al.</i> 2006) (Romanov <i>et al.</i> 2003)
Wharton’s jelly	Osteo	(Sarugaser <i>et al.</i> 2005)
Placenta	Osteo, adipo	(In ’t Anker <i>et al.</i> 2004)

In adults, MSCs have been sourced predominantly from bone marrow (Pittenger *et al.* 1999) although, more recently, MSCs have been harvested from a wide variety of other tissues (Table 1). The presence of MSCs in mature tissues other than the bone marrow stroma may suggest that these cells are further down the lineage commitment pathway with a more limited potential for differentiation (Tuan *et al.* 2003).

Other proposed properties of MSCs

Immunotolerance

There is increasing evidence that allogenic MSCs are able to avoid rejection by 3 broad mechanisms: MSCs are hypoimmunogenic, often lacking MHC-II; can prevent the normal T cell response through disruption of natural killer cells and CD8+ and CD4+ cells; and are able to induce a suppressive microenvironment through production of prostaglandins and interleukin-10 (Ryan *et al.* 2005). These findings support the use of allogenic MSCs in regenerative medicine.

Trophism/paracrine action

The trophic effects of MSCs refer to the local effects that do not bring about exogenous stem cell differentiation and include suppression of the local immune system, inhibition of fibrosis and apoptosis, enhancement of angiogenesis and stimulation of mitosis of intrinsic tissue stem cells (Caplan and Dennis 2006). The trophic effects have been alluded to in a goat model of meniscectomy where meniscal regeneration was associated with the presence of some prelabelled MSCs, but too few to account for the degree of tissue regeneration (Murphy *et al.* 2003).

Homing ability

Chemokines are molecules that attract cells to move to specific locations and may be involved in the homing of MSCs to certain tissue sites. The chemokine receptor and ligand profile of MSCs is beginning to unfold (Sordi *et al.* 2005; Honczarenko *et al.* 2006) and may explain the homing and migratory capacity of endogenous and exogenous MSCs.

Mesenchymal stem cells are the body's own systemically derived healing cells. However, in most tissues the numbers of resident and those derived from the bone marrow via the circulation are thought to be insufficient (with possible exceptions of organs with a highly regenerative capacity such as liver, muscle and bone). The relative contribution of the endogenous bone marrow has been debated. Some studies suggested that endogenous MSCs could be attracted into particular scaffolds (such as small intestinal submucosa; Zantop *et al.* 2006). However, more recent studies, using chimaeric GFP in rats (Izuta *et al.* 2005; Kajikawa 2007), suggest that much of the bone marrow-derived cells immediately after injury are white blood cells concerned with the inflammatory response and repair relies more on resident (and therefore limited) multipotential cells. This supports the exogenous administration of MSCs.

Isolation and culture of MSCs

In most cases, the isolation of MSCs relies on the ability of the MSC to adhere to tissue culture plastic and form colonies of cells

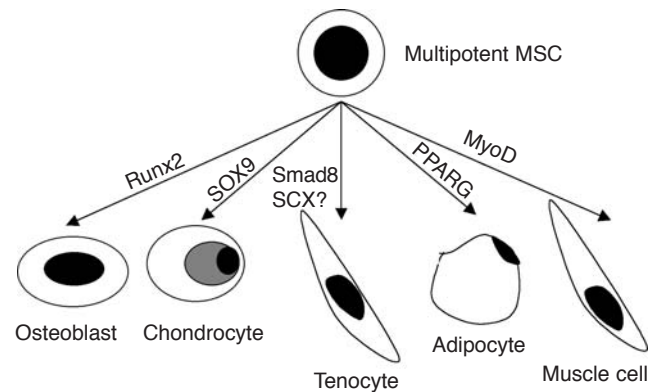


Fig 1: Schematic representation of the multiple differentiation pathways of multipotent mesenchymal stem cells. Runx2 is the principal protein that mediates the transcriptional control of osteogenesis, SOX9 is the equivalent for chondrogenesis and PPAR γ is the equivalent for adipogenesis. MyoD is a regulatory factor that controls myogenesis. Smad8 and scleraxis are transcription factors thought to be important in tenogenesis.

when plated at low cell densities (the number being defined the number of colonies formed (the CFU-f assay). This adherent property is used to separate MSCs from haemopoietic cells removed with the media when the culture media is changed, usually 2 days after initial plating. Frequently the whole bone marrow aspirate is subjected to fractionation on a density gradient solution such as Percoll. Bone marrow aspirates in recent equine studies have yielded $6.4 \pm 3.4 \times 10^9/l$ nucleated cells in bone marrow aspirate (Smith *et al.* 2003; Vidal *et al.* 2006) which is comparative to human bone marrow aspirates (Pittenger *et al.* 1999). Cells are then plated onto Petri dishes or tissues culture plates and grown in standard culture media (Pittenger *et al.* 1999) where they have a fibroblastic morphology (Barry and Murphy 2004). Once the cells have grown to confluence trypsin digestion allows collection of the cells for further cultivation. Equine bone marrow aspirates have yielded $1-2 \times 10^5$ adherent cells after 3 days culture (Fortier 2005) from 10 ml of sternal bone marrow aspirate (Smith *et al.* 2003).

Although it is now generally accepted that the number of MSCs obtained from the bone marrow reduces with age (subjects over ~ 30 years), site and disease status (Majors *et al.* 1997) there are still huge variations in the yield of stem cells from different individuals and even in aspirates obtained from the same individual and the same site (Phinney *et al.* 1999). Furthermore, the morphology and other cellular properties of even clonally isolated cells can change (Okamoto *et al.* 2002) as bone marrow MSCs are expanded in culture (Sekiya *et al.* 2002). Small rapidly dividing cells with high multilineage potential become larger slower growing cells with limited differentiation ability (Digirolamo *et al.* 1999) as they are expanded through several passages.

Mesenchymal stem cell therapy for cartilage, bone and tendon

The use of MSCs to regenerate diseased tissue relies on the successful extrapolation of *in vitro* differentiation to *in vivo* engraftment and long-term survival. Tissue engineering is thought to require at least 3 separate components; a scaffold in which the cells can survive, an appropriate environment (humorally and mechanically) and a cell source (Tuan 2006).

Cartilage

Joint disease in the form of osteoarthritis is a common cause of equine lameness (Riggs 2006). The limited potential articular cartilage has for regeneration has been recognised for over 2 centuries (McIlwraith 2002). Full thickness cartilage defects have been shown to heal with fibrous tissue that may or may not undergo a degree of metaplasia to become fibrocartilage with inferior biomechanical properties compared to articular hyaline cartilage (Vachon *et al.* 1991). Current concepts for the mechanism of cartilage repair include a combination of limited intrinsic repair orchestrated by the resident chondrocytes, extrinsic repair from mesenchymal elements from the subchondral bone, and finally 'matrix flow' where lips of cartilage are created at the periphery of a defect that then migrate towards the centre of the defect (McIlwraith 2002).

In vitro chondrogenic differentiation: Mesenchymal stem cells are encouraged to pursue a chondrogenic differentiation pathway in high density pellet cultures using serum free media containing transforming growth factor β 3 (TGF β 3) (Barry *et al.* 2001). Certain transcription factors and growth factors have been shown to increase the size and weight of such pellets in culture (Fischer *et al.* 2002; Sekiya *et al.* 2002).

In vivo cartilage regeneration: The technique of 'microfracture' has been employed in equine surgery to release 'bone marrow cells' and growth factors to promote cartilage repair. Although microfracture appears to improve clinical outcome and volume of repair tissue the aggrecan content is not comparable to normal cartilage (Frisbie *et al.* 2003). Cell based therapies have been advocated in equine joint resurfacing (Litzke *et al.* 2004). In the human field, research is pursuing the use of MSCs for cartilage repair (Caplan *et al.* 1997; Barry *et al.* 2001; Caterson *et al.* 2001; Wakitani *et al.* 2002). In a caprine model of meniscectomy to induce osteoarthritis, the intra-articular injection of MSCs suspended in hyaluronan reduced degeneration of articular cartilage and subchondral bone sclerosis 6 weeks post injection compared to control animals. This was thought to arise primarily from the regeneration of a meniscal-like tissue rather than the formation of new cartilage (Murphy *et al.* 2003).

Successful outcome of cell-based cartilage tissue engineering depends ultimately on the proper differentiation of stem cells into chondrocytes and the assembly of the appropriate cartilaginous matrix to achieve the load-bearing capabilities of the natural articular cartilage (Chen *et al.* 2006). Although MSCs have been shown to progress down a chondrogenic pathway *in vitro*, recent applications of MSCs in a mouse model was associated with premature hypertrophy. The authors stating that cultured MSCs assume an unnatural pathway of differentiation to chondrocyte cells (Pelttari *et al.* 2006). Mesenchymal stem cells have been shown to progress down a chondrogenic pathway in the horse (Worster *et al.* 2000, 2001; Vidal *et al.* 2006) however, surgical challenges of implant retention in the horse may continue to hamper the use of such cell based therapy where immediate weight bearing is important.

Bone

It has been suggested that, with the exception of bone fractures, most diseases of the musculoskeletal tissue do not stimulate regeneration

of the original tissue (McIlwraith 2002). In contrast to tendon and cartilage repair, bone repair parallels the embryological formation of bone (Kraus and Kirker-Head 2006) allowing regeneration of a tissue with similar biochemical and biomechanical properties. Bone regeneration occurs through osteogenesis, osteoinduction, osteoconduction and osteopromotion (Gamradt and Lieberman 2004). Repair of many fractures occurs by secondary bone union and formation of a fracture callus with the appropriate internal fixation or coaptation. Circumstances that require additional interventions include delayed-unions, arthrodesis or situations where there has been substantial loss of bone (Kraus and Kirker-Head 2006).

In vitro osteogenic differentiation: Dexamethasone, β glycerophosphate and Vitamin D3 are needed to promote osteogenesis (Bellows *et al.* 1990; Chung *et al.* 1992; Liu *et al.* 1999). Dexamethasone has been shown to stimulate MSC proliferation and support osteogenic differentiation (Bellows *et al.* 1990). Organic phosphates are thought to play a role in mineralisation and modulation of osteoblast activity (Chung *et al.* 1992) additionally free phosphates have been shown to induce osteogenesis regulatory genes such as Runx-2 (Fujita *et al.* 2001) and osteogenic markers; osteopontin (Beck *et al.* 2000). Vitamin D3 is thought to increase alkaline phosphatase activity and promote osteocalcin production (Liu *et al.* 1999).

In vivo bone regeneration: Cancellous bone grafts have been used as an adjunct to treat open fracture repairs in the horse for many years (McIlwraith 1998). They are believed to supply a cell source for osteogenesis, an osteoconductive matrix and osteoinductive growth factors (Gamradt and Lieberman 2004). Autologous cancellous bone grafting could be described as a crude stem cell procedure. More recently, bone marrow MSCs have been loaded into 3 mm ceramic carriers and implanted into rat femurs (Bruder *et al.* 1998). Both immunohistochemistry and mechanical properties were superior in the fracture repairs that received the ceramics loaded with MSCs. A similar experiment has been carried out on sheep tibiae with encouraging results (Kon *et al.* 2000). Implantation of either autologous or allogenic MSCs have been shown to promote bone formation in a canine model of atrophic nonunion (Arinzeh *et al.* 2003).

Use of allogenic MSCs did not induce any demonstrable immunologic reaction supporting the use of cryopreserved MSCs thereby avoiding any delay from culturing a patient's own bone marrow aspirate (Kraus and Kirker-Head 2006). Shen *et al.* (2002) demonstrated that systemically administered MSCs transduced to produce IGF-1 were able to localise preferentially to the fracture site and accelerate healing in a mouse model. Brodke *et al.* (2006) compared selectively retained MSCs to a conventional autograft in a canine model of atrophic nonunion and demonstrated comparable regenerative capacity (Kraus and Kirker-Head 2006). Clinical use of osteogenic MSCs in the horse could, in the future, enhance arthrodesis techniques and treatment of osseous cyst-like lesions as well as in the management of selected fractures.

Tendon

Superficial digital flexor tendonitis continues to be one of the most common orthopaedic injuries in the performance horse with a reported incidence of 8–43% in racing Thoroughbreds. Many different therapies are currently used for the treatment of tendonitis suggesting that no current therapy is particularly/convincingly

efficacious. Because of the poor functionality of scar tissue natural repair, new treatments should aim, ideally, at regenerating a tissue as close to tendon as possible.

In vitro tenogenic differentiation: In comparison with adipogenic, osteogenic and chondrogenic differentiation little is currently known about the signalling pathways involved in tenogenesis, and studies investigating tenogenesis have been limited by the lack of unique markers for tendon matrix. However, both MSCs and tendon-derived cells can be induced to form an extracellular matrix that closely resembles tendon tissue *in vitro* (R.K.W. Smith, unpublished data). Recent literature suggests that tensional mechanical load of cells is necessary for the formation of optimally organised tendon and ligament matrix (Hsieh *et al.* 2000; Wall and Banes 2005).

In vivo tendon regeneration: There are a number of experimental animal models which have demonstrated efficacy of MSCs implantation over controls, usually using laceration models in rabbits or rats (Young *et al.* 1998; Awad *et al.* 2003). In a recent study using a window model in the rat patellar tendon, MSCs were better than controls or a differentiated cell line (from a different species) in both mechanical functionality and matrix quality (Hankemeier *et al.* 2005). The use of autologous MSCs has been described in the horse (Smith *et al.* 2003) which utilises an autogenous adherent nucleated cell population recovered from sternal bone marrow aspirates and expanded in the laboratory until approximately 10×10^6 cells are available for implantation into the damaged tendon under ultrasound guidance. The technique is being used currently in equine practice and, while clinical experience suggests a positive effect on re-injury rates compared to conventional management (Smith and Webbon 2005), there are no published controlled studies proving efficacy to date.

Future challenges

Optimising the technology

Current techniques in use clinically are simple and likely to require more sophisticated methodologies to optimise efficacy. This is also hampered by relative ignorance of the scientific mechanism of action and clinical benefit. Despite a huge amount of exciting and encouraging research in the stem cell field there is still much to be learned about the signals necessary to: 1) enable a stem cell to remain a stem cell capable of self-renewal and multilineage differentiation; 2) initiate differentiation down one particular lineage; 3) indicate to a stem cell to home and engraft to a specific tissue site - MSCs that have been passaged many times *in vitro* appear to lose their homing ability; and 4) elaborate, or induce the formation of, a functional extracellular matrix, either pre- or post implantation.

Determining efficacy

While laboratory animal models do provide some information, they do not fully resemble many clinical orthopaedic injuries, such as equine over-strain tendon disease. Therefore, the best and, probably, only legitimate model of this disease is the use of naturally occurring disease in well designed controlled studies within the equine industry. These studies are difficult to do on clinical cases, not only because randomised trials are complex to

perform without inducing bias in the case populations within a privately funded medical service, but also because outcome measures have limited objectivity, are difficult to define and often influenced by other, uncontrolled factors. Clinical data therefore provide only limited confidence of efficacy and the use of expensive, randomised, placebo controlled studies of disease cohorts with tissue analysis are required.

Allogenic MSCs

The use of allogenic MSCs introduces the concept of 'off-the-shelf' therapy that has added advantage of immediate availability. Additionally the use of allogenic cells could eliminate factors such as genetic predisposition and diminished potency of MSCs due to natural ageing (Leung *et al.* 2006).

Transfection of stem cells

The ability to transfect stem cells *ex vivo* offers exciting possibilities in utilising the stem cell as a 'delivery vehicle' for certain important mediators of tissue regeneration. This has already been performed successfully for muscle regeneration (Mourkioti and Rosenthal 2005). However, transferring this to clinical practice is challenging. At present, both virally and nonvirally transfected MSCs are capable of expressing the candidate genes for only approximately 6 months (for review see Reiser *et al.* 2005).

Genetic modification of stem cells to express bone morphogenetic proteins and enhance bone repair is currently at the level of *in vitro* (Zachos *et al.* 2006) and experimental animal research (Gamradt and Lieberman 2004). Optimisation of both vector and delivery method are necessary before these tools can become available in the clinical setting.

Conclusions

Stem cell science has attracted considerable interest in both the scientific and clinical communities because of its potential to regenerate tissues. Much has been investigated and learnt but it must be appreciated that, despite this, the field is still relatively young and both communities must prepare themselves for considerable time and effort to develop the technology into a highly efficient treatment. However, the promise of functional tissue engineering to replace old parts with new fully justifies the interest. At present, it is important to balance the understanding of our current limitations with a desire to progress the technology.

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