

## The biology of equine mesenchymal stem cells: phenotypic characterization, cell surface markers and multilineage differentiation

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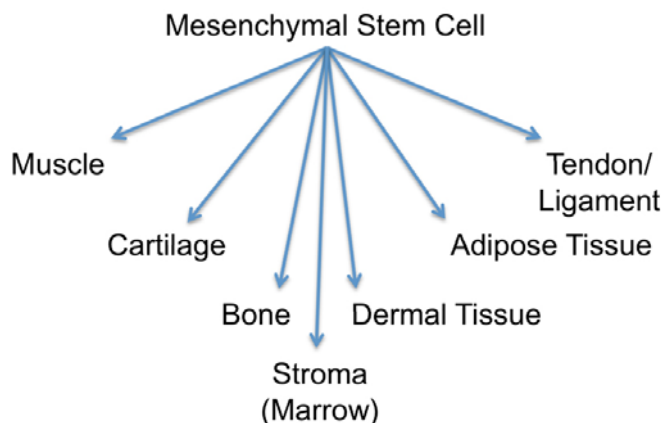
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## 1. ABSTRACT

Mesenchymal stem cells (MSCs) are multipotent stem cells that can give rise to a range of connective tissue cells including osteoblasts, chondrocytes and adipocytes. MSCs have been isolated from humans and a variety of animal species including rodents, dogs, horses and rabbits. There is currently no consensus on how these cells are identified and characterized. This is partly due to the lack of standardized specific cell surface markers for MSCs. The aim of this review is to examine the literature on equine MSCs and establish whether there is a well-defined phenotype for these cells. Equine MSCs have been obtained from four main sources, bone marrow, adipose tissue, umbilical cord (blood and matrix) and peripheral blood. MSCs from these tissue sources have been shown to undergo chondrogenic, adipogenic and osteogenic differentiation. However the markers used to identify these cells vary significantly in the literature. Despite this, CD90 and CD34 seem to be reliable positive and negative markers respectively. Our understanding of the biology of equine MSCs will benefit from better reagents for their phenotypic characterization. The antibodies and molecular probes needed for the reliable identification of equine MSCs are not standardized and this is a high priority for future research.

## 2. INTRODUCTION

Mesenchymal stem cells have been isolated from a variety of species including, humans, rodents, dogs, horses and rabbits (1). They are classed as multipotent cells since they have the potential to give rise to cells from multiple, but still limited number of lineages (2, 3) (see Figure 1). There is an ongoing debate regarding the use of the term 'mesenchymal stem cell' (4, 5). It has been suggested that these cells should be referred to as 'mesenchymal stromal cells', because the word 'stem' may imply the cells have more functions (such as self-renewal and life-long cell division (6)) than they actually possess. However, this is likely to be addressed by the correct use of the terms pluripotent and multipotent. Also, according to the International Society for Cellular Therapy, cells can only be referred to as 'stem' cells if multipotency and long-term survival can be demonstrated *in vivo* (4). One group even argues that the word 'mesenchymal' itself is ambiguous because the noun 'mesenchyme' refers to mesodermal-derived tissues, which includes blood and vascular systems as well as musculoskeletal and connective tissues (7). The uncertainty surrounding the phenotype and origin of mesenchymal stem cells is clear throughout the literature; with some authors using different terms in the title and body of a paper (8) and others using both terms



**Figure 1.** Differentiation potential of human mesenchymal stem cells along the mesengenic lineage. Cell culture has facilitated the characterization of mesenchymal stem cells and the signals and pathways that regulate their development into different cell types.

e.g. ‘mesenchymal stem cells or mesenchymal stromal cells’ in their publications (9-12). Although these can be used interchangeably, the term ‘mesenchymal stem cell’ is the most widely used and accepted. Therefore, the abbreviation ‘MSC’ will be used throughout this review.

Another aspect of MSCs that varies between research papers is their phenotypic characterization. In 2006, the International Society for Cellular Therapy published a Position Statement detailing the criteria that should be used to identify human mesenchymal stromal cells (5). The cells must be plastic adherent, 95% or more should express the cell surface markers, CD73, CD90 and CD105, 2% or less should express CD45, CD34, CD11b, CD19 and HLA-DR and they must be multipotent, capable of undergoing adipogenic, chondrogenic and osteogenic differentiation (5) (see Figure 2). HLA-DR can be expressed by mesenchymal stromal cells but only if they have been stimulated by IFN-gamma (5). A range of papers published between 2006 and 2010, covering a variety of species, were examined to see if these guidelines were followed, particularly in terms of the cell surface molecules expressed (see Table 1). Out of the 23 papers that were reviewed (10 of which focused on human cells), none of the authors tested for CD11b or CD19 expression. Eighteen papers including human, equine and canine focused studies, tested for CD90 expression, 13 looked for CD105 expression and only four research groups checked for CD73 expression (see Table 1). Interestingly, two of these four papers found canine MSCs to be CD73 negative (13, 14). The first of these papers also found canine MSCs to be negative for CD105 expression (13), whereas the second reported that the cells were negative for CD90 expression (14). The functions of the cell surface molecules detailed in these 23 papers are summarized in Table 2. Although the International Society for Cellular Therapy advise testing for eight cell surface molecules (5), our review of a selection of papers published between 2006 and 2010 identified a total of 49 cell surface molecules, indicating that there is currently no consensus as to which cell surface markers should be tested for in any species.

There is one marker, STRO-1, that has been used in a number of studies to isolate a more homogeneous population of human skeletal stem cell progenitors from human bone marrow (15-19). The antibody STRO-1 received its name due to its ability to interact with stromal precursor cells (17). It has since been shown that STRO-1 positive cells can differentiate down the three mesenchymal lineages (15, 18); leading one group to suggest that it could be a potential marker for MSCs (20). STRO-1 binds to a trypsin-resistant cell-surface antigen which is present in only a subpopulation of cells within the bone marrow (6, 15, 16); in one study it was found that this antibody bound to 10 percent of bone marrow mononuclear cells (17). It has recently been shown that STRO-1 positive cells are present in immature bovine cartilage and to a lesser extent mature bovine cartilage (20). However, the majority of studies looking at STRO-1 expression have involved human cells to date. Out of the papers reviewed that were published between 2006 and 2010, only five tested MSCs for STRO-1 expression (see Table 1), all of which were human studies. Antibodies are a key limiting factor in the phenotypic identification of MSCs. For example, two groups researching ovine MSCs admit that they could only test for a limited number of cell surface molecules due to a lack of antibody cross-reactivity with ovine tissue (8, 11). Therefore, there may be a set of markers (including STRO-1) expressed by MSCs in a variety of species but due to the current lack of antigen cross-reactivity across species, consistent MSC cell surface markers have yet to be identified. This may also be why some groups solely rely on differentiation assays to confirm the presence of MSCs (21, 22). Table 3 summarizes the main histological, extracellular matrix (ECM) and phenotypic markers of osteoblasts, chondrocytes and adipocytes differentiated from MSCs.

### 3. PROLIFERATION OF EQUINE MSC RESEARCH

Diseases of the musculoskeletal system such as osteoarthritis (OA) and tendinopathy are the major reasons

**Table 1.** Cell surface markers of MSCs from various species and tissue sources.

Species	Source	Cell surface markers		Reference
		Positive	Negative	
Human	Adipose tissue	CD13, CD29, CD44, CD73 and CD90		(103)
Canine	Bone marrow	CD90 and MHC-I	CD34, CD45 and MHC-II	(78)
Human	Bone marrow Umbilical cord blood Adipose tissue	CD29, CD44, CD73, CD90, CD105, CD106 and HLA-I	CD14, CD34, CD45, CD133, CD144 and HLA-II	(50)
Equine	Bone marrow	CD90, fibronectin, perlecan and collagen type IV	None tested for	(23)
Equine	Umbilical cord matrix	CD54, CD90, CD105, CD146, Oct4, SSEA-4, c-Kit, SSEA-3 and TRA-1-60	CD34, CD45 and CD133	(55)
Canine	Bone marrow	CD90 and CD105	CD34 and CD45	(64)
Equine	Umbilical cord blood	Oct4, TRA-1-60, TRA-1-81 and SSEA-1	None tested for	(100)
Equine	Adipose tissue	Oct4, TRA-1-60 and TRA-1-81	SSEA-1 and SSEA-4	
Ovine	Bone marrow	CD44, vimentin and CD105	CD34 and CD45	(8)
Human	Adipose tissue	CD44, CD90, CD105, CD146, CD166 and STRO-1	CD14, CD31 and CD45	(9)
Human	Bone marrow	CD44, CD90, CD105 and CD166	CD45 and CD117	(104)
Human	Bone marrow	CD29, CD44, CD73, CD90, CD105 and CD146	CD14, CD34 and CD45	(105)
Human	Infrapatellar fat pad	CD13, CD29, CD44, CD90, CD105 and 3G5	CD34, CD56, LNGFR and STRO-1	(106)
Human	Infrapatellar fat pad	CD13, CD29, CD44, CD90 and CD105	CD35, CD56, LNGFR and STRO-1	(107)
Human	Infrapatellar fat pad	CD13, CD29, CD44, CD90 and CD105	CD34, CD56, LNGFR and STRO-1	(108)
Ovine	Bone marrow	CD29, CD44 and CD166	CD31 and CD45	(11)
Human	Bone marrow	CD44 <sup>c</sup> , CD54, CD90 and STRO-1	CD14 and CD45	(12)
Human	Placental chorionic villi	CD49a/VLA-1, CD146, CD106, 3G5, $\alpha$ -SMA and STRO-1	X63 and vWF	(109)
Canine	Adipose tissue	CD29, CD44 and CD90	CD13, CD31, CD34, CD45, CD73, CD105 and CD117	(13)
Canine	Umbilical cord blood	CD29, CD33, CD44, CD105, CD184 and Oct4	CD4, CD8a, CD10, CD14, CD20, CD24, CD31, CD34, CD38, CD41a, CD41/61, CD62p, CD73, CD90, CD133 and HLA-DR	(14)
Equine	Bone marrow	Sox-2, Oct4 and Nanog	CD34	(37)
Equine	Adipose tissue	CD90 and CD44	CD13	(52)
Canine	Adipose tissue	CD44, CD90, CD140a and CD117	CD34 and CD45	(79)
Canine	Adipose tissue	CD90 and CD105	CD34 and CD45	(31)

behind early retirement and euthanasia of race, working and pleasure horses (23). Spontaneous OA frequently occurs in both athletic and older horses (24). The most common causes of OA in younger horses are traumatic injury and concomitant synovitis (25). There are some joints that are more likely to develop OA than others, in the horse it is the metacarpophalangeal joint followed by the carpal joints (25). Equine joints are large, relatively easy to manipulate and the cartilage is similar in thickness and composition to human articular cartilage (4). It is for these reasons that the horse is an established animal model for OA (4). OA is the most common human arthritic disease (26, 27) and is progressive and degenerative (28-31). An alternative name for this disease is degenerative joint disease (28). Age, sex, obesity, genetics, trauma and overuse are all risk factors for OA (26, 27). The main events associated with OA are, the degradation of cartilage, inflammation and the formation of osteophytes (27, 28, 32). OA is a debilitating disease, with acute pain experienced in the early stages. In the later stages of the disease, this pain becomes more chronic and is an indication in human

patients that the joint will need replacing (27). Current treatments for OA are only able to reduce pain and inflammation, not stop the progression of the disease (27). Autologous chondrocyte implantation (ACI) is a relatively successful treatment for small cartilage lesions but it is not suitable for larger areas of damage (30). The situation is similar for the degeneration of the intervertebral disc (IVD), which is a leading cause of lower back pain in humans (33). Patients with IVD degeneration experience pain and limited mobility and like with OA, current treatments only relieve symptoms unless invasive surgery is opted for (33). Both OA and IVD degeneration could potentially benefit from MSC therapy because these cells could be used to generate tissues *in vitro* that can be transplanted into the patient. In addition, a greater understanding of MSC biology may highlight other factors that can be used in the treatment of OA, for example, it has recently been shown that curcumin can protect MSCs from the negative effects of pro-inflammatory cytokines (31). Therefore, curcumin could potentially aid the formation of *in vitro* cartilage and also be an early treatment option for

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**Table 2.** Alternative names and functions of cell surface markers in MSCs (functional descriptions from Genecards - <http://www.genecards.org/>)\*

Cell Surface Antigens/Markers	Alternative Names	Function
CD4	CD4 molecule	Required for MHC class II and T cell receptor interaction.
CD8a	CD8 antigen alpha polypeptide	A cell surface glycoprotein expressed on cytotoxic T cells. It enables these T cells to interact with MHC class I.
CD10	Membrane metallo-endopeptidase	Involved in the destruction of opioid peptides through cleavage of the Glycine-Phenylalanine bond.
CD13	Alanyl (membrane) aminopeptidase	An aminopeptidase with broad specificity. It is involved in the final stages of peptide digestion and can also cleave antigenic peptides which are bound to MHC class II.
CD14	Myeloid cell-specific leucine rich glycoprotein	Involved in the innate immune response to LPS and in the upregulation of other cell surface molecules, including those with an adhesive function.
CD20	Membrane-spanning, 4-domains, subfamily A, member 1	May have a role in the activation and proliferation of B-cells.
CD24	CD24 molecule	Encourages B-cell proliferation in response to antigen and prevents these cells from terminally differentiating into antibody-secreting cells.
CD29	Beta 1 integrin	Integrins are cell-surface adhesion molecules which are made up of alpha and beta subunits. Each integrin has a slightly different function depending on the subunits it contains. There are at least 18 alpha and 8 beta subunits in mammalian cells.
CD31	Platelet/endothelial cell adhesion molecule	A cell adhesion molecule which has a key role in the transendothelial migration of leukocytes during inflammation. It also prevents phagocytosis of viable cells but encourages it if the cells become apoptotic.
CD33	CD33 antigen (gp67)	A reputed adhesion molecule expressed on myelomonocytic-derived cells. It connects cells via sialic acid binding.
CD34	CD34 molecule	Could be involved in early hematopoiesis by aiding stem cell attachment to bone marrow ECM or stromal cells.
CD38	CD38 molecule	Cyclic ADP-ribose synthesis. It also possesses cADPr hydrolase activity and can be a receptor involved in the immune response.
CD41a	Platelet glycoprotein receptor	Is the calcium-dependent part of GPIIb/IIIa, which is expressed on megakaryocytes and platelets. It is possible that CD41a and CD41 are the same molecule (110, 111).
CD41/61	Alpha IIb/beta 3 integrin	These are two CD molecules which are the alpha and beta subunit of an integrin. This particular integrin, alpha IIb/beta 3 is a receptor for a variety of molecules including, fibronectin, plasminogen and thrombospondin. As it can also bind to fibrinogen, it is involved in platelet aggregation.
CD44	Hyaluronate receptor	Hyaluronic acid receptor, therefore has a role in cell migration and tumor growth and progression.
CD45	Protein tyrosine-protein phosphatase, receptor type, C	Protein tyrosine-protein phosphatase, which is essential for the activation of T cells via the antigen receptor.
CD49a	Alpha 1 integrin	This is the alpha subunit of an integrin. Alpha 1Beta 1 integrin is a receptor for collagen and laminin.
CD54	Intercellular adhesion molecule I	Involved in leukocyte trans-endothelial migration through leukocyte adhesion protein (LFA-I) binding.
CD56	Neural adhesion molecule I	A cell adhesion molecule which is involved in a variety of processes including, neuron-neuron adhesion, neurite outgrowth and neurite fasciculation.
CD62p	P selectin	Ca <sup>2+</sup> dependent receptor which binds to carbohydrates on monocytes and neutrophils. It also has a role in leukocyte rolling as well as recognizing the ligand sialyl-Lewis X.
CD73	5'- nucleotidase, ecto	Converts extracellular nucleotides into nucleosides, which are membrane permeable, through hydrolysis.
CD90	Thy-1 cell surface antigen	Putatively involved in synaptogenesis and other developmental processes in the brain.
CD105	Endoglin	The main glycoprotein present in the vascular endothelium; may have an essential role in binding endothelial cells to integrins.
CD106	Vascular cell adhesion molecule I	Aids adherence of leukocytes to endothelial cells and is vital for cell-cell recognition.
CD117	c-Kit	A receptor for stem cell factor (mast cell growth factor) with tyrosine-protein kinase activity.

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CD133	Prominin I	A pentaspan transmembrane glycoprotein frequently found to be expressed on adult stem cells. It is believed to suppress differentiation, therefore helping the cell to maintain its stem cell properties.
CD140a	Platelet-derived growth factor receptor alpha	A receptor for platelet-derived growth factors A and B (PDGFA, PDGFB) which has a tyrosine-protein kinase activity.
CD144	Cadherin 5, type 2	Cadherins are cell adhesion proteins which are dependent on calcium. They connect cells together by adhering to each other in a homophilic way. This particular cadherin is thought to be important in endothelial cell biology.
CD146	Melanoma cell adhesion molecule	Has an adhesive role in the endothelial cell monolayer. It is also a cell surface receptor that initiates tyrosine phosphorylation of FYN and PTK2 and causes a temporary increase in the intracellular calcium concentration.
CD166	Activated leukocyte cell adhesion molecule	Cell adhesion molecule which binds to CD6. It has a role in neurite extension and may be involved in T and B cell binding to activated leukocytes.
CD184	Chemokine (C-X-C motif) receptor 4	Receptor for CXCL12. Important in the vascularization of the gastrointestinal tract.
3G5	3G5 antigen	Could potentially be involved in the regulation of cell shape (112).
alpha-SMA		alpha-Smooth Muscle Actin is a cytoskeletal protein.
HLA-DR		Human Leukocyte Antigen-DR is a specific part of the MHC class II locus. The DR section has one DRA gene and nine DRB genes (A codes for alpha and B for beta) (113).
HLA-II	MHC class II	Human Leukocyte Antigen class I is involved in the presentation of antigens to CD8+ T cells (114, 115).
LNGFR	p75NTR	Low-affinity nerve growth factor receptor binds to a range of molecules including nerve growth factor receptor.
MHC-I	HLA class I	Major Histocompatibility Complex class I is involved in the presentation of antigens to CD4+ T cells (114, 115).
Nanog	Nanog homeobox	A transcriptional regulator involved in maintaining pluripotency of embryonic stem cells. It also has a role in inner cell mass and embryonic stem cell proliferation and self renewal.
Oct4	POU class 5 homeobox	A transcription factor that recognizes the sequence 5'-ATTGTCAT-3'. Is a member of a trimeric complex with Sox-2. This complex regulates the expression of various genes involved in embryonic development. Both Oct4 and Sox-2 are key transcription factors in early embryogenesis.
Sox-2	SRY (sex determining region Y)-box 2	A transcription factor that is part of a trimer with Oct4 (see notes for Oct4).
SSEA-1		Stage Specific Embryonic Antigen-1. Molecular function currently undetermined (116).
SSEA3		Stage Specific Embryonic Antigen-3. Molecular function currently undetermined (116).
SSEA-4		Stage Specific Embryonic Antigen-4. Molecular function currently undetermined (116).
STRO-1		No defined function in the literature.
TRA-1-60	TRA-1-60 antigen	Molecular function remains unclear (117).
TRA-1-81	TRA-1-81 antigen	Molecular function remains unclear (117).
Vimentin	FLJ36605	Class-III intermediate filaments, which form part of the cytoskeleton.
vWF	F8VWF	von Willebrand Factor has multiple functions including acting as a chaperone for coagulation factor VIII and promoting adhesion of platelets to areas of vascular injury.
X63		No defined function in the literature.

\*Some of the functional descriptions were not available on Genecards and were obtained from other resources.

OA (31). In another study, a combination treatment of curcumin and resveratrol were found to protect human articular chondrocytes from the cytotoxic effects of interleukin (IL) 1 beta, which is a major pro-inflammatory cytokine involved in cartilage degradation in OA (34). MSCs are also being studied as potential therapeutic treatments for rheumatoid arthritis (RA) (35). A recent

study has shown that human umbilical cord derived MSCs inhibited the proliferation of fibroblast-like synoviocytes stimulated by tumor necrosis factor alpha (TNF-alpha), which is a major pro-inflammatory cytokine in RA (35). These MSCs were also shown to downregulate the proliferation of T lymphocytes from RA patients (35). In addition, this group demonstrated that umbilical cord

**Table 3.** The main histological, ECM and phenotypic markers of osteoblasts, chondrocytes and adipocytes differentiated from mesenchymal stem cells

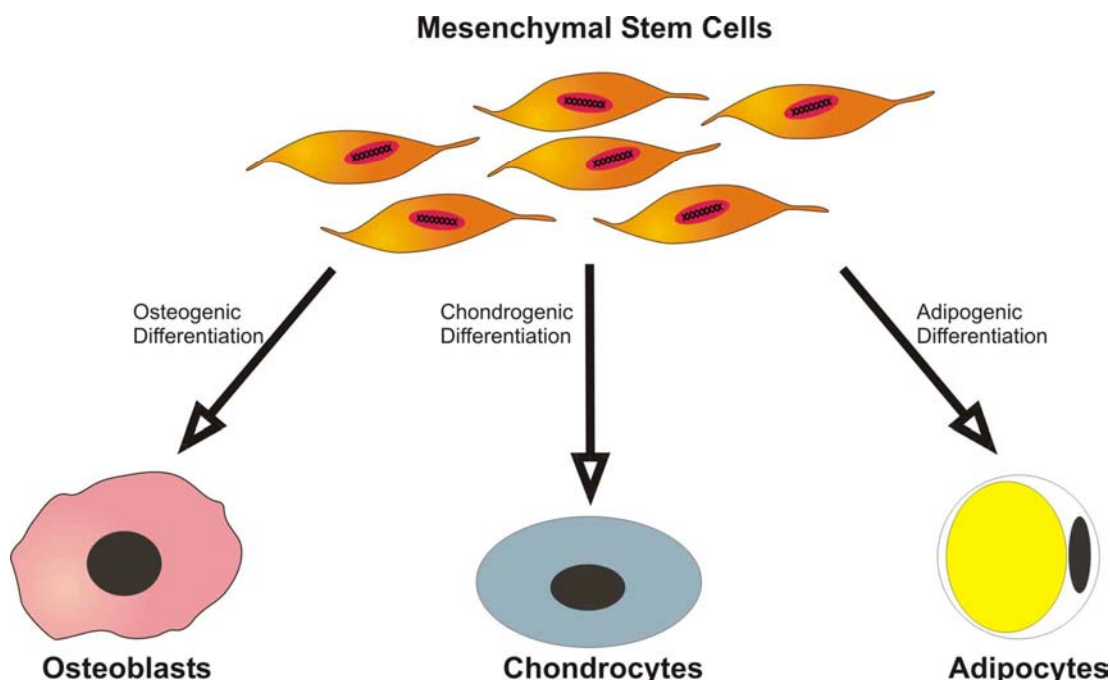
	Osteoblasts	Chondrocytes	Adipocytes
Histological Stains	Alizarin Red (11, 14, 48, 51, 55, 58, 85, 99-101)	Toluidine Blue (13, 14, 23, 54, 55, 66-68, 85)	Oil Red O (11, 13, 23, 48, 51, 55, 58, 64, 68, 85, 101)
	von Kossa (13, 23, 37, 64, 68, 84, 100, 104)	Alcian Blue (11, 47, 64, 65, 83, 84, 99, 101)	
		Safranin O (47, 51, 99)	
		Masson trichrome stain C (48, 101)	
ECM Markers	Type I collagen (11, 14, 64)	Type II collagen (13, 47, 48, 54, 56, 66, 67, 83, 100)	Type I collagen (64)
	Beta 1 integrin (64)	Aggrecan (13, 66)	Beta 1 integrin (64)
Other Markers	Alkaline phosphatase (ALP) (23, 48, 69, 84, 101)	Sox9 (13, 100)	Adiponectin (64)
	Osteonectin (OSTN) (14, 69, 100)		Peroxisome proliferator-activated receptor- gamma (PPAR-gamma) (11, 64)
	Osteocalcin (OCN) (14, 69)		
	Runt-related transcription factor-2 (Runx2) (69, 100) / Core binding factor alpha 1 (11, 64)		Fatty Acid Binding Protein 4 (FABP4) (13)
	Osteopontin (OPN) (13)		Lipoprotein Lipase (LPL) (11, 13)
	Bone Sialoprotein (BSP) (13)		Leptin (11, 13)
	Vitamin D receptor (VDR) (14)		
	Homeobox gene MSX2 (14)		

derived MSCs can induce regulatory T cell expression (35). All these factors combined suggest that these cells have the potential to be a successful therapeutic treatment for RA (35).

In terms of tendinopathy, some equine tendons have a higher incidence of injury than others, with most occurring in the tendons of the forelimb (36). Of these forelimb injuries, the most commonly injured tendon is the superficial digital flexor tendon (SDFT) (36). Tendons take a long time to heal and as scar tissue forms during this healing process, they never function as well as native tendons (37, 38). Horses continually use their tendons to their functional limit and as they get older, their tendons start to degenerate, so it is not surprising that these tissues often fail (3, 37, 38).

Despite the limited understanding of MSCs and their basic cell biology, various groups have already moved on to study their potential for regenerative medicine in small number of clinical cases. Successful regenerative medicine requires cell biologists, tissue engineers and surgeons to collaborate and work together to repair and regenerate damaged or diseased tissues and organs (39, 40). This field is being studied globally and in a variety of animal models, including rats, hens, dogs, pigs and rabbits (41). One review details several studies in the application of canine MSCs for bone regeneration (42). Another research group propose that one-step surgical procedures will eventually be achievable (40). This would involve a tissue engineer isolating MSCs from adipose tissue and seeding them onto a graft whilst the surgeon continues to operate on the patient (40). Once the graft is ready the surgeon can implant it into the patient all in the same operation (40). According to the authors, this whole process would only take a maximum of two and a half hours (40). The support for the use of MSCs in regenerative medicine is strong and they are already being used in equine medicine (38). One study assessed different treatments for collagenase-induced tendonitis in six stallion

Standard bred horses (43). Each treatment, bone marrow MSCs, bone marrow mononuclear cells, fibrin or saline, were directly injected into the tendon lesion (43). The authors used histology to show that both cellular treatments restored the tendon to a state comparable with an uninjured tendon (43). However the authors do acknowledge that only a small sample of horses were studied and that the tendonitis was an experimental model (43). In another study, bone marrow derived MSCs were isolated and implanted into the injured SDFT of an 11 year old pony (44). The results of this study were promising as no lameness was observed after the implantation of the cells (44), although the result may have been completely different in another horse. A more recent study compared the effects of injecting equine embryonic stem cells (ESCs) and equine bone marrow derived MSCs into SDFT lesions (45). Three lesions were created in each tendon in eight thoroughbred geldings (45). Serum was injected into lesion one, MSCs into lesion two and ESCs into lesion three (45). After a period of 90 days, the ESCs were detected in all three lesions, despite the cell numbers remaining relatively constant (45). The authors claim that MSCs were only found in the second lesions after 90 days (45). Also, the survival of MSCs had decreased to only 0.2% during this time (45). There is some debate about the fate of these cells and the authors state that it is impossible to determine this without a reliable tenocyte marker (45). In spite of all this, in the UK, Europe and Australia there is a standard procedure that is followed to treat tendon injuries involving bone marrow derived MSCs (38). Considering the variation in research, it could be argued that already having a treatment program set up for equine tendon lesions using MSCs is premature and potentially dangerous as it can be difficult to maintain the chondrogenic phenotype of MSCs *in vitro* (28). Another problem associated with *in vitro* chondrogenic differentiation of MSCs is premature hypertrophic terminal differentiation (26). This results in cartilage similar to that of the growth plate, not articular cartilage (26).



**Figure 2.** Osteogenic, chondrogenic and adipogenic differentiation of mesenchymal stem cells.

Research has also been carried out to determine the effects of MSC treatment on cartilage lesions in equine femoropatellar joints (46). Transplants consisting of bone marrow derived MSCs and fibrinogen and fibrinogen alone were compared (46). The transplants were observed after 30 days and eight months (46). The lesions treated with MSCs appeared to heal better because more new, homogeneous cartilage-like tissue had filled them compared to the lesions treated with fibrinogen alone (46). Staining for proteoglycans was more intense in the MSC transplants compared to the fibrinogen transplants after 30 days (46). However, the authors do acknowledge that the results for type II collagen content varied between horses (46). Interestingly, this study showed that the differences between treatment groups were no longer present at eight months (46). This implies that MSCs have no significant impact on the treatment of large articular cartilage defects in the long-term (46). The variation between horses in terms of type II collagen content was still present at eight months (46).

Equine MSCs have been obtained from four main sources, bone marrow, adipose tissue, umbilical cord (blood and matrix) and peripheral blood, with bone marrow and adipose tissue being the most frequently used. Some sources are favored over others for a variety of (mainly clinical) reasons. Bone marrow aspiration can be painful and it is quite an invasive procedure (47, 48); there is also the rare but potentially severe risk of pneumopericardium (air in the pericardial space) (49). In addition, some researchers claim that the yield of MSCs in bone marrow decreases with age (50, 51). There is strong support for adipose tissue as an alternative source of MSCs because it can be obtained in large quantities (50, 52, 53) using a less

painful procedure (53). There is also lower donor site morbidity associated with adipose tissue harvest compared to bone marrow aspiration (54). Umbilical cord blood is another potential source of MSCs, which is favorable because the invasive and potentially damaging processes of bone marrow and adipose tissue harvest can be avoided (50, 51, 55). It is with the clinical applications of MSCs in mind that some researchers are comparing these cells isolated from different sources (see section 5.2).

#### 4. EQUINE BONE MARROW DERIVED MSCS

Bone marrow has been aspirated from a variety of sources including the sternum, iliac crest, sternbrae, tuber coxae and tibia. The ages of the animals used in these studies has also varied with the youngest being three days old (23) and the eldest being 15 years old (56). There are two main types of study in this area of research; those that aim to isolate and characterize MSCs and those that intend to identify the factors that affect them.

##### 4.1. Characterization of bone marrow derived MSCs

Fortier *et al* (1998) isolated equine MSCs from bone marrow (from the sternbrae) and showed that these cells can undergo chondrogenic differentiation (57). They used toluidine blue and the spectrophotometric version of the dimethylmethylene blue (DMMB) dye-binding assay to determine the proteoglycan content and showed that the proteoglycan concentration in the media increased from day two to day eight. This suggests that the MSCs had adopted the chondrogenic phenotype and were still producing matrix at eight days (57). The osteogenic and adipogenic capabilities of equine bone marrow derived MSCs has also been studied (58). Osteogenesis and adipogenesis were successfully induced in these cells

through culture with osteogenic induction medium containing, beta-glycerophosphate, dexamethasone and sodium 2-phosphate ascorbate and adipogenic induction medium, consisting of biotin, pantothenate, insulin, dexamethasone, isobutylmethylxanthine, rosiglitazone and rabbit serum. Both types of differentiation became more extensive over time; results from 5 day osteogenic induction and 10 day adipogenic induction are shown (58). In addition, co-culture of equine bone marrow derived MSCs and equine articular chondrocytes has been examined (59). This group found that chondrogenesis was enhanced, in terms of proteoglycan synthesis and expression of collagen type II and Sox9 (chondrogenic specific genes), in the co-cultured pellets compared to pellets containing either MSCs or articular chondrocytes alone (59). Sox proteins are transcription factors and are part of the high mobility group (HMG) superfamily (60, 61). They are involved in a variety of processes including hair follicle, gut and muscle development (60). There are three main Sox proteins involved in chondrogenesis, which are collectively known as the SoxTrio (61). Sox9 is the major regulator of chondrocyte development and Sox5 and Sox6 (the other proteins in the trio) influence chondrogenesis through Sox9 (61). The exact mechanism how they do this is currently not fully understood (61). A similar co-culture study has been performed with canine bone marrow MSCs and canine primary osteoblasts (62). They demonstrated that MSCs, when in high-density culture, require osteogenic induction medium or co-culture with primary osteoblasts to undergo osteogenic differentiation (62). When neither were present the MSCs underwent apoptosis and secondary necrosis (62).

Equine bone marrow derived cells have been shown to express CD90, fibronectin, perlecan and collagen type IV (23). CD90, also called Thy-1, is a glycosylphosphatidylinositol (GPI)-anchored glycoprotein on the external leaflet of the phospholipid bilayer (63). It is expressed on numerous cell types and has varying functions ranging from promoting neurite outgrowth to regulating apoptosis (63) (see Table 2). High levels of beta 1 integrin expression were also observed, which could suggest that MSCs use beta 1 integrins to adhere to the extracellular matrix (23). Beta 1 integrin expression has also been found in canine bone marrow derived MSCs (64). The authors of the equine paper (23) also claim that cryopreservation had no negative impact on the cells; this is demonstrated to a certain extent as the thawed cells were shown to proliferate and differentiate (23). However, the authors' claim would be strengthened if the results presented included data from fresh, non-cryopreserved cells (23).

#### 4.2. Factors affecting bone marrow derived MSC differentiation

Research into the biology of equine bone marrow derived MSCs has focused on the effects of various factors on their differentiation. Such factors include, transforming growth factor beta 1 (TGF beta 1) and insulin-like growth factor 1 (IGF-1) (56), hyaluronic acid and synovial fluid (65), fibroblast growth factor 2 (FGF-2) (66), dexamethasone (67), bone morphogenetic protein-12 (BMP-12) (37, 68) and BMP-2, BMP-7 and BMP-2/7 (69). TGF beta describes a family of cytokines, which are

essential for survival and have a range of functions (70, 71). These molecules have roles in inflammation and repair, embryonic and adult growth and development and immune response regulation (70, 72). There are five isoforms of TGF beta; TGF beta 1 was the first isoform to be purified (72). BMPs are also part of the TGF beta superfamily and were first detected in bone (73). Although they were initially thought to only be involved in ectopic bone formation, it is now known that these proteins have roles in a variety of processes including skeletal repair and regeneration and ligament, tendon and neuron development (73). FGF, also called basic fibroblast growth factor is mainly produced by stromal fibroblasts (74). FGF-2 is involved in wound healing, tissue repair and hematopoiesis (74).

The effect of TGF beta 1 and IGF-1 on chondrogenic differentiation of equine bone marrow derived MSCs has been studied (56). It was found that culturing the cells with TGF beta 1 in monolayer followed by culturing them with IGF-1 in a 3D environment enhanced chondrogenic differentiation (56). IGF-1 alone also induced chondrogenesis but not as much as when the cells were treated with both TGF beta 1 and IGF-1 (56). In addition, the effects of hyaluronic acid and autologous synovial fluid on equine bone marrow derived MSCs have been studied (65). It was hypothesized that hyaluronic acid alone and in combination with TGF beta 1 and autologous synovial fluid would all induce chondrogenic differentiation in these cells (65). Two types of hyaluronic acid were used, Hylartil and Ostenil, both of which induced chondrogenesis in high density micromass culture (65). TGF beta 1 and autologous synovial fluid also stimulated chondrogenic differentiation under the same conditions (65). In fact the most effective induction factor was TGF beta 1, which might be expected as it is expressed in high levels in embryonic cartilage (65).

The effect of FGF-2 on bone marrow derived MSCs cultured in monolayer has also been studied (66). The hypothesis was that treating the cells with FGF-2 in monolayer would enhance their proliferation as well as their ability to undergo chondrogenic differentiation when in 3D culture (66). The limited data provided did show that FGF-2 enhanced chondrogenesis (MSC pellets increased in size following the addition of FGF-2) (66). According to the authors, addition of FGF-2 had no effect on MSC proliferation (66). This research group did another study in 2008, this time looking at the effect of dexamethasone on the chondrogenesis of equine bone marrow derived MSCs (67). They found that  $10^{-7}$ M dexamethasone significantly increased the alkaline phosphatase (ALP) activity and expression, indicating a high level of chondrogenesis (67). This group also hypothesized that addition of FGF-2 and dexamethasone would increase MSC proliferation and chondrogenic differentiation but the endochondral phenotype would not be induced (67). This hypothesis was found to be incorrect as pellets treated with FGF-2 and dexamethasone expressed ALP at significantly higher levels compared to controls which would suggest that these cells are differentiating into hypertrophic chondrocytes rather than articular chondrocytes (67).

More recently, a study comparing three different isolation protocols for equine bone marrow derived MSCs has been carried out (49). The first method, termed the classic protocol, involved isolation by plastic adherence (49). The other two methods were both density gradient techniques, Percoll and Ficoll (49). This group found that the greatest MSC yield was obtained using the classic protocol (49). The Percoll method generated significantly more colony forming units than the classic protocol, whereas the results for the Ficoll method were only slightly lower than the Percoll method (49). Osteogenic and chondrogenic differentiation were unaffected by the isolation procedure (49). The authors had difficulties inducing adipogenesis and did not have enough samples to perform statistical analysis for this lineage (49).

### 4.2.1. BMPs and their effects on bone marrow derived MSCs

It has been shown that equine bone marrow derived MSCs can be induced to undergo tenogenic differentiation when cultured with BMP-12 (37). This group assessed the gene expression of tenomodulin to confirm tenogenic differentiation (37). Tenomodulin is a transmembrane protein that is expressed in a variety of connective tissues including tendons, ligaments and the cornea (75). However it has been suggested that tenomodulin expression is only exclusively detected in tenocytes *in vitro* (75), which implies that tenomodulin could be used as a tenocyte specific marker. Another research group have shown that mice lacking tenomodulin have fewer proliferating tenocytes than mice still expressing tenomodulin (76). Scleraxis has also been recommended as tenocyte marker, but only for *in vivo* studies (75). Tenocytes are still relatively poorly characterized (77) and this may be because their distinguishing morphological features present *in vivo* are often lost during *in vitro* culture (i.e. they become more fibroblastic *in vitro*) (75).

Equine bone marrow derived MSCs have also been shown to express the embryonic stem cell markers Oct4, Sox2 and Nanog and lack expression of CD34 (37). Lack of expression of CD34 and CD45 has also been shown in canine bone marrow derived MSCs (13, 31, 64, 78, 79). Both CD34 and CD45 are markers for hematopoietic stem cells (80). CD45 is a tyrosine phosphatase (81); whereas the precise function of CD34 is still unclear (82) (see Table 2). As well as tenogenic differentiation these cells were also capable of osteogenic differentiation (37). Similarly, it was established that equine bone marrow derived MSCs and superficial digital flexor tenocytes (SDFTNs) are capable of differentiating into tenocytes in the presence of BMP-12 (68). They also studied whether BMP-12 induced osteogenic differentiation and found that it did not (68). BMP-2 however, did stimulate osteogenic differentiation (68). The cells in this study were only observed for six days and the authors acknowledge that doing a longer study would be beneficial (68). It would also be useful to assess BMP-12's apparent inability to induce osteogenesis *in vivo* (68).

A study by Carpenter and co-workers (69) has compared osteogenic differentiation of human and equine bone marrow derived MSCs when cultured with genetically

modified homo- and heterodimers of BMPs, with or without dexamethasone (69). In particular they studied BMP-2 and BMP-7 homodimers and a BMP-2/7 heterodimer (69). It was found that BMP-2 treatment gave the biggest increase in ALP production in both cell types (69). They also discovered that dexamethasone had to be present in the culture medium for the equine cells to differentiate, whereas the human cells still underwent osteogenesis without it (69). This may suggest that MSCs require different environmental signals for differentiation depending on their species.

## 5. EQUINE ADIPOSE TISSUE DERIVED MSCS

Most of the research on equine adipose tissue derived MSCs has involved comparing them with bone marrow derived MSCs (54, 83, 84). There are a couple of studies where the aims were to further characterize the cells (52, 85). In addition, one study assessed the effect of platelet lysate on adipose tissue derived MSCs but no data or figures were provided to support the conclusions drawn (86).

### 5.1. Characterization of adipose tissue derived MSCs

A study aiming to isolate and phenotypically characterize adipose tissue derived MSCs has found that these cells express CD90 and CD44 but lacked expression of CD13 (52). CD44 expression did vary greatly ( $45.23\% \pm 40.01$ ) (52). Canine adipose tissue derived MSCs have been shown to express CD44, CD29 and CD90 but lacked expression of CD13, CD34, CD73, CD105 and CD117 (13). The functions of these CD molecules are summarized in Table 2.

Work by da Silva Meirelles and colleagues (85) has shown that adipose tissue derived MSCs have the ability to undergo chondrogenic and adipogenic differentiation (85). However, the authors did not present any evidence for osteogenic differentiation because cell detachment occurred before the first assay could be completed (85). The main focus of this study was to assess the correlation between MSC frequency and adipose tissue vascularity. The results suggest that there is a relationship between the two factors, however only two horses were used and the authors also acknowledge that the precise location of MSCs *in vivo* remains poorly understood (85). This is not just an equine specific issue; the *in vivo* location of adult stem cell progenitors is poorly understood in all species. There are some groups who believe that there is a close relationship between MSCs and pericytes and even suggest that these cells should be renamed as perivascular stem cells (87). One author has suggested that all MSCs are in fact pericytes (88). Pericytes are present in the microvessels within connective tissues (89) and are CD146 positive and CD34, CD45 and CD56 negative (88, 90). It has been shown that stromal vascular fraction taken from human white adipose tissue contained pericytes that expressed CD44, CD73, CD90 and CD105 (90), which are all cell surface markers associated with MSCs. The same research group also demonstrated that pericytes isolated from human skeletal muscle expressed these markers (90). The authors claim that the same results were seen in

pericytes isolated from a variety of other human tissues including the pancreas, placenta and bone marrow; however the results for these tissues are not included in the paper (90). In addition, cultured pericytes have been shown to undergo chondrogenic, adipogenic and osteogenic differentiation (90), which further supports the idea that MSCs and pericytes are equivalent (89) or that MSCs have developed from pericytes (90).

### 5.2. Comparison of adipose tissue derived MSCs with bone marrow derived MSCs

Chondrogenesis of adipose tissue derived MSCs and bone marrow derived MSCs in agarose and self-assembling peptide hydrogel cultures have been compared (54). Chondrogenesis occurs during embryogenesis and results in the formation cartilage which later undergoes endochondral ossification to form bone (28, 91-95). There are several stages to chondrogenesis, the first of which is the proliferation and condensation of MSCs (28, 91-93, 95). This is followed by the proliferation and eventual terminal differentiation of chondroprogenitors (92). These terminally differentiated chondrocytes secrete extracellular matrix typically associated with hyaline cartilage (93). Different results were seen when the cells were cultured on the different hydrogels (54). TGF beta  $\beta$ 1 enhanced chondrogenesis in the bone marrow derived MSCs on the agarose hydrogel but not in the adipose tissue derived MSCs (54). They also discovered differences in proteoglycan synthesis on the agarose hydrogel (54). Bone marrow derived MSCs synthesize aggrecan and decorin whereas adipose tissue derived MSCs synthesize a wide range of proteoglycans in terms of size and they produce smaller or degraded proteoglycans (54). On the self-assembling peptide hydrogel, TGF beta 1 was found to increase proteoglycan synthesis, total protein content and glycosaminoglycan accumulation in both bone marrow derived MSCs and adipose tissue derived MSCs (54). Aggrecan expression was also observed in both cell types on this hydrogel (54). These results suggest that the type of gel cells are cultured in influences their differentiation (54). Another study comparing chondrogenic differentiation in equine adipose tissue derived MSCs and bone marrow derived MSCs was carried out by Vidal *et al* in 2008 (83). They found that after being incubated with chondrogenic medium and the growth factors TGF beta 3 and BMP-6 for 14 days, bone marrow derived MSCs formed hyaline cartilage whereas the adipose tissue derived MSCs still had a more fibroblast-like morphology (83). This group showed that chondrogenesis was more advanced in the bone marrow derived MSCs; however the authors do acknowledge that the differences seen between the two cell types may be due to the fact that the bone marrow and adipose tissue were taken from different horses (83).

The effects of donor, amount of tissue, source and basic fibroblast growth factor (bFGF) supplementation on equine MSCs were evaluated in one study (84), which compared the proliferation abilities of equine bone marrow derived MSCs and adipose tissue derived MSCs cultured with or without bFGF (84). The presence of bFGF has previously been shown to increase the proliferation of human bone marrow derived MSCs (96-98). Adding bFGF

to adipose tissue derived MSCs caused a change in morphology; they became more spindle-shaped (84). Morphological and histological data is not given for the bone marrow derived MSCs. Adipose tissue derived MSCs had greater (not statistically significant) proliferative ability than bone marrow derived MSCs and the difference between the two cell types increased with the addition of bFGF (84). Addition of bFGF also enhanced osteogenic differentiation in both cell types (84). Based on these results adipose tissue derived MSCs may be more competent than bone marrow derived MSCs in terms of proliferative ability and they are more responsive to bFGF (84).

Equine adipose tissue derived MSCs can differentiate down the osteogenic, chondrogenic and adipogenic pathways. Similar observations have been made with canine adipose tissue derived MSCs with the exception that myogenic differentiation has also been observed and induced in these cells (13).

### 6. EQUINE UMBILICAL CORD BLOOD AND MATRIX DERIVED MSCS

In 2007, Koch *et al* tried to isolate MSCs from equine umbilical cord blood (51). They successfully isolated these cells but only at a rate of 57% (51). During this study, they demonstrated that the cells they isolated could differentiate down the three mesenchymal lineages (51). This low success rate prompted the same group to do further research into equine umbilical cord blood derived MSCs, aiming to improve the protocol for isolating them (99). Three cell separation methods were tested, PrepaCyte-EQ medium (PEQ), Ficoll-Paque PREMIUM medium loaded with undiluted whole blood (FUD) and Ficoll-Paque PREMIUM medium loaded with diluted whole blood (FD) (99). Two types of FBS were also tested, regular and MSC qualified (99). They report that 100% MSC isolation was achieved with the PEQ method, whereas only 60% and 20% was achieved with FUD and FD respectively (99). All umbilical cord blood derived MSCs underwent osteogenesis and adipogenesis regardless of the separation method and FBS used (99). However, only one cell line differentiated down the chondrogenic lineage (99).

Umbilical cord matrix derived MSCs have been found to express Oct4, which is a key transcription factor involved in maintaining the pluripotency of embryonic stem cells (55). Expression of SSEA-4 and c-Kit, which are also markers of embryonic cells, have been reported in the same study (55). Another characteristic of undifferentiated cells is high levels of cytoplasmic ALP and this was seen in the cells observed in this study (55). Umbilical cord matrix derived MSCs were also shown to weakly express SSEA-3 and TRA-1-60 (55). As well as the embryonic stem cell markers, these cells express some cell surface markers usually associated with adult stem cells (including CD54, CD90, CD105 and CD146) (55) but they did not express CD34, CD45 or CD133 (55). The authors also showed that these cells are capable of differentiating into adipocytes, chondrocytes, osteocytes

and neuronal-like cells (55). Neural markers have also been found on canine bone marrow derived MSCs (78). It could be argued however that as these cells expressed several embryonic stem cell markers and had the potential to differentiate into four different cell types, they could possess a more primitive phenotype.

Similarly, another research group also found that umbilical cord blood derived MSCs express Oct4 (more than 90%) (100). They also showed that these MSCs are capable of differentiating into chondrocytes and osteocytes and discovered that umbilical cord blood derived MSCs and adult horse bone marrow derived MSCs could not undergo adipogenic differentiation (100). In addition, it was found that umbilical cord blood derived MSCs have the ability to differentiate into hepatocytes, which are part of the endodermal lineage (100). This could suggest that umbilical cord blood derived MSCs are more plastic than MSCs from other sources in the horse (100). However, like the previous paper, it could be argued that the cells being studied in this research are not technically MSCs but something more primitive. Another study has shown that umbilical cord blood derived MSCs are able to differentiate into adipocytes, although the extent of this differentiation was much less than the osteogenic and chondrogenic differentiation that was observed (101). The authors suggest that this may be because fetal bone marrow lacks adipocytes but such cells are present in adult bone marrow and adipose tissue (101). The main aim of this study was to optimize the isolation and culture of equine umbilical cord blood derived MSCs (101). The authors claim that hypoxic conditions increased the rate of umbilical cord blood derived MSC expansion but the mechanism by which it does this is poorly understood and the result seen in this study may simply be due to a decrease in lymphocyte survival (101). In comparison, the level of chondrogenic differentiation rat bone marrow derived MSCs are capable of can be enhanced by culturing these cells in normoxic conditions for 14 days followed by hypoxic conditions for seven days (102).

### 7. EQUINE PERIPHERAL BLOOD DERIVED MSCS

Out of the four sources of equine MSCs, peripheral blood has been the least researched. Equine peripheral blood derived MSCs have been compared with bone marrow derived MSCs (47). The authors found it difficult to isolate peripheral blood derived MSCs, reporting a success rate of only 36.4% (47). They also found these cells to be very sensitive to trypsinization (47). In addition to this, 50% of cryopreserved peripheral blood derived MSCs were lost after thawing (47). The authors do acknowledge that the cellular degeneration observed may be due to the fact that the cells were cultured for two weeks without any media change (47). In terms of differentiation, the authors demonstrated that equine peripheral blood derived MSCs could differentiate into osteocytes (47). However, only weak adipogenesis and no chondrogenesis were observed (47). The weak adipogenic differentiation could have been caused by sensitivity to the adipogenic induction medium (47). Bone marrow derived MSCs on the other hand were much easier to isolate and culture and

had the ability to differentiate down all three mesenchymal lineages (47). From these results, the authors of this paper draw the conclusion that equine bone marrow derived MSCs are the best option for tissue engineering (47).

In the previous study mentioned, the culture conditions used were based on a method for culturing human MSCs (47). This prompted another research group to complete a study aiming to optimize the culture conditions for equine peripheral blood derived MSCs, which would hopefully help to clarify the differentiation abilities of these cells (48). Unlike the first study, these investigators changed the culture medium for the first time after 10 days and they achieved an isolation success rate of 66.7%, which was much higher than in the earlier paper (48). The authors showed that equine peripheral blood derived MSCs do have the ability to undergo adipogenic differentiation; however their adipogenic induction medium contained 5% rabbit serum (48). Rabbit serum has a high concentration of free fatty acids (linoleic and palmitic fatty acids) and it is these which are thought to induce adipogenesis (48). Both osteogenic and chondrogenic differentiation were also observed in these cells (48). In fact, TGF beta 3 was found to enhance chondrogenesis in both peripheral blood derived MSCs and bone marrow derived MSCs and was more effective at doing so than TGF beta 1 (48). Therefore, like the MSCs from equine bone marrow, adipose tissue and umbilical cord (blood and matrix), peripheral blood derived MSCs do have the potential to differentiate down the three mesenchymal lineages but the culture conditions have to be optimized (48).

### 8. SUMMARY AND CONCLUSIONS

There is an ongoing debate regarding the nomenclature of MSCs, which is partly due to the fact that there is currently no consensus for identifying and characterizing these cells. In terms of cell surface markers, a total of 49 cell surface markers (see Table 1) were tested for in the studies reviewed in this paper. The aim of this review was to look at the literature on equine MSCs and determine whether these cells have a well-defined phenotype.

MSCs are increasingly being studied due to their potential applications in regenerative medicine. OA and IVD degeneration are both debilitating diseases where current treatments only alleviate symptoms unless invasive surgery is opted for (27, 33). Horses can also develop OA and because their joints are large and their articular cartilage is similar in composition to human articular cartilage, they are a good animal model for OA (4). Despite the lack of understanding of basic MSC biology, in terms of cell surface markers, *in vivo* location and *in vivo* survival after transplantation, these cells are already being used to treat equine tendon injuries (38).

Equine MSCs have been isolated from four different sources, bone marrow, adipose tissue, umbilical cord (blood and matrix) and peripheral blood. Bone marrow and adipose tissue derived MSCs have been shown

to be able to undergo adipogenesis and chondrogenesis (57). Osteogenesis has also been shown with both cell sources although some difficulties, such as cell detachment during culture, were found with the adipose tissue derived MSCs (85). Umbilical cord blood and matrix derived MSCs can also differentiate down the three mesenchymal lineages, although the results for chondrogenesis and adipogenesis have varied (99). Additionally, umbilical cord matrix derived MSCs have been shown to differentiate into neuronal-like cells (55) and umbilical cord blood derived MSCs into hepatocytes (100). Therefore it could be argued that this clearly demonstrates the pluripotency of these cells and the word 'stem' in 'mesenchymal stem cell' is perfectly justified. However, as hepatocytes are part of the endodermal lineage it could also be argued that the adjective 'mesenchymal' is no longer appropriate. Peripheral blood derived MSCs are the least studied out of the four. One paper confirms that these cells can differentiate down all three mesenchymal lineages but the culture conditions have to be modified for the cell source (48).

This review also highlights the diversity of equine MSC research, as a great variety of factors have been tested in terms of their effect on MSCs, including numerous growth factors and bone morphogenetic proteins. However, there is one growth factor (TGF beta 1) that has been assessed on three of the cell types mentioned in this review (bone marrow derived MSCs (56), adipose tissue derived MSCs (54) and peripheral blood derived MSCs (48)). All of these papers show that TGF beta 1 enhances chondrogenesis, although one group found that TGF beta 3 was more effective at inducing chondrogenesis in both bone marrow derived MSCs and peripheral blood derived MSCs (48).

The field of equine MSCs is still in its infancy and is an exciting area of research where much still needs to be done. A major priority is to identify reliable cell surface markers for these cells. This could potentially make MSC isolation more straight forward for both researchers and those involved in generating MSC preparations for application in regenerative medicine, therefore decreasing the bench to bedside time. Equine MSCs may express the same repertoire of markers recommended by the International Society for Cellular Therapy for human MSCs, but due to lack of antigen cross-reactivity across different species, the same guidelines are currently not applied to equine MSCs.

In conclusion, many research groups have shown that equine MSCs can differentiate into osteocytes, chondrocytes and adipocytes regardless of their tissue of origin and CD90 and CD34 are emerging as potential positive and negative cell surface markers respectively for these cells. We now need to move forward with a much more detailed investigation of the functional phenotype of these cells. Much research into the basic biology of these cells still needs to be done, with the most pressing concern being the standardization of the isolation and phenotypic characterization of these cells.

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**Abbreviations:** MSC: mesenchymal stem cell; ECM: extracellular matrix; OA: osteoarthritis; RA: rheumatoid arthritis; IVD: intervertebral disc; ESC: embryonic stem cell; TGF: transforming growth factor; IGF: insulin-like growth factor; FGF: fibroblast growth factor; BMP: bone morphogenetic protein; ALP: alkaline phosphatase; bFGF: basic fibroblast growth factor

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