UNIVERSITY OF LIVERPOOL

Tendon and ligament stem cells and their niche

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy

By

Katie Joanna Lee

May 2017

Abstract

Tendon and ligament are prone to age- and injury-related degeneration. Tendon and ligament are unable to heal effectively after injury and current treatment strategies have variable success rates. The identification of stem cell populations in tendon and ligament holds potential for new therapeutic strategies to treat tendon and ligament injuries. The stem cell niche has been shown to be vital for survival and function of a number of stem cell populations, including tendon. Modulation of the stem cell niche can induce changes in stem cell phenotype and function and holds therapeutic potential.

The aim of this study was to characterise stem cell populations in equine superficial digital flexor tendon (SDFT) and canine cranial cruciate ligament (CCL), as well as characterise the stem cell niche of these cell populations. Tendon- and ligament-derived stem cells (TDSCs and LDSCs respectively) and tenocytes and ligamentocytes (tendon and ligament fibroblasts respectively) were isolated from tendon and ligament tissue and assessed for clonogenicity, proliferation, stem cell and tenogenic marker expression and multipotency. The extracellular matrix niche of the different ligament cell populations was also investigated using metabolic labelling and proteomic analysis.

Cell populations isolated from equine SDFT demonstrated many properties of stem cells however differentiation potential was restricted. There were very few differences in stemness and niche composition between TDSCs and tenocytes suggesting a heterogeneous mixture of cells within equine SDFT at different stages of differentiation. LDSCs isolated from canine CCL demonstrated all of the hallmarks of a stem cell and differed in phenotype and gene expression to ligamentocytes which showed reduced stemness. The proteomic composition of the LDSC and ligamentocyte niche was similar with differential expression and turnover of some extracellular matrix proteins between cell types. These results indicate the presence of two cell populations and niches within canine CCL. Stem cell populations present in equine tendon and canine ligament may hold therapeutic potential and modulation of the stem cell niche in these tissues may provide a less invasive alternative treatment strategy than current therapies.

Table of contents

Index of figures

Index of tables

List of abbreviations

AB	Alcian blue
ACL	Anterior cruciate ligament
ACLDSC	Anterior cruciate ligament derived stem cell
AGG	Aggrecan
ALC	Average local confidence
ALP	Alkaline phosphatase
AR	Alizarin red
BAPN	β-aminopropionitrile
BCA	Bicinchoninic acid
BCIP/NBT	5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium
BCP	1-Bromo-3-chloropropane
BMSC	Bone marrow stromal cell
BMP	Bone morphogenetic protein
BSA	Bovine serum albumin
CCL	Cranial cruciate ligament
CD	Cluster of differentiation
CID	Collision induced dissociation
CMFDA	5-chloromethylfluorescein diacetate
COMP	Cartilage oligomeric matrix protein
CTGF	Connective tissue growth factor
Da	Dalton
DAB	3,3'-Diaminobenzidine
DCN	Decorin
DDA	Data dependent acquisition
DMEM	Dulbecco's modified eagles medium
DMMB	Dimethyl-methylene blue
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate

DTS	Decellularised tendon slice
DTT	Dithiothreitol
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGR-1	Early growth response protein-1
ESI	Electrospray ionisation
FABP4	Fatty acid binding protein 4
FACS	Fluorescently activated cell sorting
FBS	Foetal bovine serum
fdESCs	Foetal-derived embryonic stem cell
FGF	Fibroblast growth factor
FITC	Fluorescein isothiocyanate
FT-MS	Fourier transform ion cyclotron
FWHM	Full width at half maximum
GAG	Glycosaminoglycan
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HCD	Higher energy collisional dissociation
HCl	Hydrochloride
HRP	Horseradish peroxidase
HSC	Haematopoietic stem cell
IAA	Iodoacetamide
IBMX	3-isobutyl-1-methylxanthine
IBSP	Integrin binding sialoprotein
IFM	Interfascicular matrix
IGF	Insulin-like growth factor
ISCT	International Society for Cellular Therapy
ITS	Insulin, Transferrin, Selenium
LC	Liquid chromatography
LCM	Laser capture microdissection
LDSC	Ligament-derived stem cell
LDSC-F	Ligament-derived stem cell – fibronectin adhesion method

LDSC-L	Ligament-derived stem cell – low density plating method
MALDI	Matrix-assisted laser desorption/ionisation
MCL	Medial collateral ligament
MCLSC	Medial collateral ligament stem cell
MKX	Mohawk
M-MLV RT	Moloney Murine Leukemia Virus Reverse Transcriptase
MS	Mass spectrometry
MSC	Mesenchymal stem/stromal cell
NCM	Non-collagenous matrix
OA	Osteoarthritis
OC	Osteocalcin
OCT	Optimum cutting temperature
OCT4	Octamer-binding transcription factor 4
OMD	Osteomodulin
OPG	Osteoprotegerin
OPN	Osteopontin
ORO	Oil red O
PBS	Phosphate buffered saline
PDL	Periodontal ligament
PDLSC	Periodontal ligament stem cell
PDT	Population doubling time
PEN	Polyethylene naphthalate
PG	Proteoglycan
PGE_2	Prostaglandin E_2
PPARγ	Peroxisome proliferator-activated receptor gamma
PPM	Parts per million
PRP	Platelet-rich plasma
PTM	Post-translational modifications
qPCR	Quantitative polymerase chain reaction
RIA	Relative isotope abundance
RT	Retention time

RUNX2	Runt-related transcription factor 2
Sca-1	Stem cell antigen 1
SCID	Severe combined immunodeficiency
SCX	Scleraxis
SDFT	Superficial digital flexor tendon
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
SILAC	Stable isotope labelling using amino acids in cell culture
SLRP	Small leucine-rich proteoglycan
SO	Safranin O
SOX	Sex determining region Y box
SSEA	Stage-specific embryonic antigen
TBS	Tris buffered saline
TDSC	Tendon-derived stem cell
TDSC-F	Tendon-derived stem cell – fibronectin adhesion method
TDSC-L	Tendon-derived stem cell – low density plating method
TE	Tris- ethylenediaminetetraacetic acid
TFA	Trifluoroacetic acid
TGF	Transforming growth factor
THBS	Thrombospondin
TNC	Tenascin C
TNMD	Tenomodulin
TOF	Time-of-flight
VEGF	Vascular endothelial growth factor

Acknowledgements

This PhD project would not have been possible without the generous funding of the Marjorie Forrest Bequest and the University of Liverpool. I would also like to acknowledge the University of Liverpool's Technology Directorate for co-funding the proteomics work.

Firstly, I would like to thank my supervisory team. I wish to express my gratitude to my primary supervisor, Dr Elizabeth Canty-Laird, for her unwavering support, guidance and patience as well as her profound expertise. I would also like to thank my secondary supervisors, Professors Eithne Comerford and Peter Clegg, for their continued support throughout my project and their invaluable knowledge and contribution.

I wish to thank all of my colleagues, past and present, in the Department of Musculoskeletal Biology for creating such an enjoyable working environment, as well as their continued support, patience and assistance throughout the course of my PhD. Particularly I would like to thank Dr Kate Williamson for her invaluable help and patience, as well as Jade Gumbs, Luke Tregilgas, Kirsty Johnson, James Anderson, Megan Barrow and Drs Ben McDermott, Danae Zamboulis, Alan Mueller, Othman Ali and Sumaya Allaith.

I would also like to extend my thanks to Drs Deborah Simpson and Philip Brownridge and Professor Rob Beynon of the University of Liverpool's Centre for Proteome Research for their assistance in planning, processing and analysing my proteomics work. I also would like to extend my gratitude to Drs Mandy Peffers and Yalda Ashraf-Kharaz for their help with proteomics data analysis.

Finally I would like to thank my family and friends, particularly my parents, for their continued support and encouragement throughout my PhD studies, without which this would not have been possible.

Chapter 1

General Introduction

1.1 Introduction

Tendon and ligament are prone to injury and degeneration, and this is most often seen in occupational and sporting environments (Cimino *et al*., 2010; Maffulli *et al*., 2003). The healing process for tendon and ligament is poorly understood, however it is well documented that tendon and ligament tissue is unable to heal effectively resulting in painful and debilitating scar tissue, unable to perform its normal physiological function (Frank, 2004; Maffulli *et al*., 2003).

The current treatment for damaged or degenerated tendon varies depending on the severity and location of the tendinopathy (Goldin and Malanga, 2013; Lempainen *et al*., 2015; Mayor, 2012; Schwartz *et al*., 2015). Physiotherapy is often prescribed for less severe injuries such as tendinitis, as well as other physical therapies including ultrasound and cryotherapy, however these strategies are largely ineffective and/or lack any real clinical evidence as to their efficacy (Mayor, 2012). Pharmacotherapies, such as anti-inflammatories, are used as an alternative or in combination with physiotherapy with limited effect (Mayor, 2012; Schwartz *et al*., 2015). For more severe tendinopathies peritendinous steroid injections can be given or surgical intervention is performed, with these procedures being invasive with poor success rates (Coleman *et al*., 2000; Mayor, 2012). Therefore, an alternative approach for the management and treatment of tendinopathies is currently being sought. One such alternative is stem cell therapies which hold great potential particularly since the identification of a stem cell population within tendon tissue (Bi *et al*., 2007).

The current treatment strategies are also limited for ligament injuries; as with tendinopathies, rest, immobilisation and physiotherapy are often prescribed (Temponi *et al*., 2015). Where conservative therapies have failed, surgery is often performed, however there appears to be little difference in success outcome between surgical and conservative treatment options (Dawson *et al*., 2016). Most cases requiring surgery for anterior cruciate ligament (ACL) injuries, one of the most commonly injured ligaments (Gianotti *et al*., 2009; Woo *et al*., 2000), undergo reconstruction of the ACL using a section of the patients' hamstring or patellar tendon, or less commonly, cadaveric tissue (Murawski *et al*., 2014). There are

variable success rates associated with ACL reconstruction, dependent upon the patients' lifestyle, age and health (Dawson *et al*., 2016; Murawski *et al*., 2014), however recent advances in the field of stem cell research may provide a treatment option with improved success rates. For example, the injection of mesenchymal stromal cells (MSCs) alone (Kanaya *et al*., 2007) or with the use of a biosynthetic scaffold (Figueroa *et al*., 2014) to treat ACL rupture shows promising results at the preclinical research stage. It is clear that stem cell therapies hold potential for treatment of ligament injuries.

1.2 Function and structure of tendon and ligament

1.2.1 Function

Tendons are dense, regularly arranged, connective tissues which transfer forces from muscle to bone, and tendons vary in composition and location dependent upon their function (Sharma and Maffulli, 2006; Thorpe *et al*., 2014). The main function of tendon is to transmit loads from muscle to bone as well as to resist tension, however tendon is also compliant to enable easier physiological and mechanical movement (Shepherd and Screen, 2013). These conflicting functions are achieved through stiff collagen fibres, preventing excessive strain and loss of contractile energy during movement, and a viscous matrix which allows for sliding of fibres within tendon and an increased range of movement (Shepherd and Screen, 2013). Tendon also plays a role in proprioception and maintaining posture (Nourissat *et al*., 2015) as well as acting as an energy store (Thorpe *et al*., 2014). These final two functions of tendon are dependent on the type of tendon. Positional tendons such as the human digital flexor tendon and equine common digital extensor tendon are stiff and help to position limbs, whereas energy-storing tendons such as the human Achilles tendon and equine superficial digital flexor tendon act as a spring to aid motion (Thorpe *et al*., 2014).

Ligaments provide a similar function to tendon, however there are notable differences between the two tissue types. Ligaments are also dense, regularly arranged connective tissues, however they connect bone to bone in the case of

skeletal ligaments. Ligaments can also vary in composition, positioning and location which has an impact on their function, for example, skeletal ligaments span joints and insert into bones (known as insertion points), providing multiple functions such as stabilisation of the joint, as well as allowing movement during locomotion (Frank, 2004; Tozer and Duprez, 2005). Similar to tendon, ligaments are compliant due to their viscoelastic properties which aid joint mobility, as well as being able to adapt to repetitive strain or loads by "creeping" which involves deformation of the ligament. In addition, skeletal ligaments are involved in proprioception of joints, allowing coordinated joint movement (Birch *et al*., 2013; Frank, 2004).

1.2.2 Structure

Tendon is surrounded by a sheath known as the paratenon which is composed of loose connective tissue and is quite distinct from the tendon itself (Benjamin and Ralphs, 1998). Immediately surrounding the tendon is the epitenon which is a layer of connective tissue continuous with the interfascicular matrix (IFM), also known as the endotenon (Benjamin and Ralphs, 1998). The paratenon and epitenon contain blood vessels and nerves (Franchi *et al*., 2007), whereas the tendon body varies in the extent of vasculature and innervation between species, tendon types and location in the tendon. For example, the human Achilles tendon is well vascularised at the insertion and origin points, however the mid-region is largely avascular (Theobald *et al*., 2005). Murine flexor tendons show poor vascularisation (Wong *et al*., 2006) whereas the equine superficial digital flexor tendon has a strong vascular network (Kraus-Hansen *et al*., 1992).

Tendon is largely acellular and is mainly composed of extracellular matrix (ECM) in the form of collagen fibrils which are held together by ECM components such as decorin and aggrecan (Kannus, 2000; Thorpe *et al*., 2013). Collagen type I is predominant, accounting for 95% of the total collagen within tendon, with the remainder being made up of types II, III, V, XI, XII and XIV (Franchi *et al*., 2007; Thorpe *et al*., 2013). Collagen type I is a heterotrimer composed of two alpha 1 chains (encoded by the *COL1A1* gene) and one alpha 2 chain (encoded by the *COL1A2* gene) (Nourissat *et al*., 2015). Collectively the collagen fibrils form collagen

fibres and fascicles (Fig.1.1), which are inter-related and the movement or sliding of fascicles and fibres aids tendon movement and function. In addition, a helical formation of some fascicles provides elasticity to aid extension and recoil of tendon (Franchi *et al*., 2007; Sharma and Maffulli, 2006; Thorpe *et al*., 2014).

Between each fascicle is the IFM which acts to maintain the structure of the tendon. This matrix contains many different glycoproteins including proteoglycans (PGs) which are the predominant class of glycoprotein found in tendon. PGs consist of varying numbers of glycosaminoglycan (GAG) chains which branch from a core protein; sulphation of these chains is common (Yoon and Halper, 2005). There are various different GAGs which alter the structure and function of PGs, these include dermatan sulphate, chondroitin sulphate, heparin sulphate, keratin sulphate and hyaluronan (Kannus, 2000). PGs enable resistance to high tensile and compressive forces which are present during mobilisation. In addition, they are involved in collagen fibrillogenesis, aiding collagen fibril formation and maturation (Franchi *et al*., 2007). There are two types of PG in tendon, small leucine-rich PGs (SLRPs) and large aggregating PGs. SLRPs are the most common PGs in tendon, and these include decorin (accounting for approximately 80% of total PG content), biglycan, fibromodulin and lumican (Thorpe *et al*., 2013). Aggrecan and versican are larger PGs and are only found in small quantities in tendon, approximately 10% (Yoon and Halper, 2005). Other proteins found within tendon include tenomodulin, tenascin-C, COMP, lubricin (Thorpe *et al*., 2013), thrombospondin, fibronectin and undulin (Kannus, 2000). These non-collagenous proteins found within the IFM and between fascicles, often termed the non-collagenous matrix (NCM), are found in a greater abundance in areas under compression, such as the enthesis. This is possibly due to the increased water content associated with PGs which hydrates the tendon and resists compression (Kjaer, 2004; Thorpe *et al*., 2013). There are also variations in the NCM composition between hierarchical levels of tendon, with the NCM increasing in complexity as the subunits increase in size (Thorpe *et al*., 2013).

The IFM also contains elastic fibres, blood vessels and cells (Franchi *et al*., 2007). Tenocytes are tendon-specific fibroblasts and traditionally were thought to be the only cell type present in tendon, however it is now thought that tenocytes account for approximately 95% of the cellular content of tendon, with progenitor

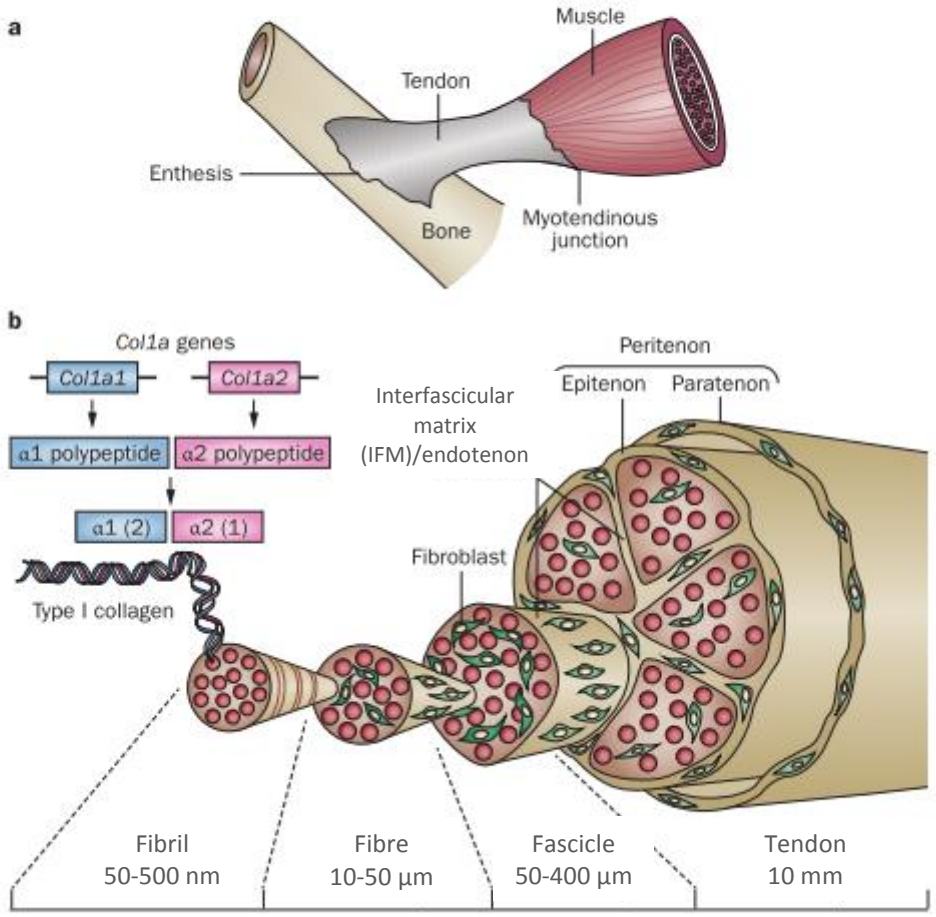


Figure 1.1. Tendon structure. The role of tendon in connecting muscle and bone (a). The hierarchical structure of tendon (b). Adapted from (Nourissat *et al*, 2015).

cells, endothelial cells and chondrocytes comprising the remaining 5% (Franchi *et al*., 2007). Tenocytes are located between collagen fibrils and in the IFM and they are responsible for the production of the ECM as well as the repair and maintenance of tendon tissue (Franchi *et al*., 2007; Kannus, 2000).

Ligament structure is similar to that of tendon, therefore only a brief description is provided, including any differences in nomenclature. The outer layer of ligament tissue, known as the epiligament, is normally very vascular and highly innervated, with nerves running parallel to blood vessels, however the ligament itself has a limited blood supply and innervation. The epiligament is continuous with the interfascicular matrix (IFM), also known as the endoligament (Frank, 2004).

Ligaments are composed of dense bands made up of collagenous fibres. These fibres are inter-related and can tighten or loosen independently of one another suggesting a complex structure (Benjamin and Ralphs, 1998). As described previously for tendon tissue these fibres form fibrils and fascicles. The collagen fibres are connected by IFM, which is composed of a variety of proteins, blood vessels, elastic fibres and cells (Birch *et al*., 2013; Clark and Sidles, 1990). The predominant cell type within ligament are fibroblasts known as ligamentocytes, which are present within the IFM as well as between fascicles, and are responsible for the production of ECM (Frank, 2004; Lo *et al*., 2002).

Ligaments are predominantly composed of water, approximately two thirds, with the remaining third comprising ECM mainly in the form of collagen, proteoglycans and other proteins. Collagen type I accounts for 85% of the total collagen within ligament, with the remainder made up of types III, V, VI, XI and XIV (Frank, 2004). The majority of water found within ligament is associated with PGs and GAGs, which play a similar role in ligament as they do in tendon (Frank, 2004; Kjaer, 2004). In addition, the complement of PGs and GAGs seen in ligament is similar to that of tendon (Birch *et al*., 2013; Frank, 2004), although differences between the two tissue types have recently been demonstrated (Kharaz *et al*., 2016).

1.3 Mesenchymal stem/stromal cells (MSCs)

Mesenchymal stem cells (MSCs) are a population of tissue-specific cells which have the ability to form skeletal tissues. MSCs have been shown to self-renew, express pluripotency markers and differentiate into a number of different cell types, including adipogenic, osteogenic and chondrogenic lineages *in vitro* (Baddoo *et al*., 2003; Bianco *et al*., 2013; Caplan, 1991; Colter *et al*., 2001; Dominici *et al*., 2006). However, more recently these properties of MSCs have been called into question, as well as the classification of these cells as stem cells. For example, other cells besides stem cells demonstrate clonogenicity, such as fibroblasts (Williamson *et al*., 2015). The expression of some pluripotency markers has been shown to be restricted to embryonic stem cells, not somatic cells (Lengner *et al*., 2008); studies demonstrating expression of Oct4 in MSCs may be identifying non-functional pseudogenes or non-specific staining of Oct4 (Lengner *et al*., 2008). Traditional MSC markers are also found on other cell types such as fibroblasts (Cadby *et al*., 2014; Lupatov *et al*., 2015; Williamson *et al*., 2015). In addition, despite the ability of MSCs to differentiate down multiple lineages *in vitro*, this ability seems to be restricted *in vivo* (Caplan, 2017). Despite this, many studies have shown the efficacy of MSCs as a cell transplantation therapy for a number of conditions (Caplan and Correa, 2011). Traditionally the mechanism of action was thought to be differentiation of the cells into tissue-specific cells to aid repair, however it is now thought that the beneficial effects of these cells is actually due to the release of paracrine factors which stimulate local tissue repair (Caplan and Correa, 2011; de Windt *et al*., 2017). For the reasons described above, there is now a call for MSCs to be renamed “mesenchymal stromal cells” or “medicinal signalling cells”, rather than “mesenchymal stem cells” (Caplan, 2017). Despite the reduced stemness of these cells they still hold potential for the treatment of a range of conditions. The majority of research into MSCs has isolated cells from bone marrow, however MSC-like sub-populations have been isolated from a number of tissues, including tendon and ligament, which may hold therapeutic potential.

1.4 Tendon-derived stem cells

1.4.1 Tendon-derived stem cell characteristics

Bi and others (2007) discovered a population of cells within tendon that possessed stem cell-like properties including self-renewal, clonogenicity and the ability to differentiate into a variety of cell types (Bi *et al*., 2007). Since this publication a considerable amount of research has been conducted (Lee *et al*., 2012; Lovati *et al*., 2011; Mienaltowski *et al*., 2013; Rui *et al*., 2010; Rui *et al*., 2013b; Zhang and Wang, 2010b; Zhang and Wang, 2013a) in an attempt to characterise tendon-derived stem cells (TDSCs) as they may hold potential for future treatment of tendinopathies.

1.4.1.1 Stem cell markers

Many markers have been identified for TDSCs in a range of species (Table.1.1), however the majority of these markers are present in other cell types. TDSCs have been shown to possess many markers which are also expressed by MSCs indicating that TDSCs are a form of MSC. The majority of identified MSC markers are surface cluster of differentiation (CD) antigens which are expressed by a number of different cell types. There are many CD antigens, and the presence or absence of particular markers is indicative of MSCs (De Schauwer *et al*., 2011; Dominici *et al*., 2006). In order to distinguish between TDSCs and MSCs it is possible to use tendon specific markers however such markers are usually expressed by tenocytes also, making isolation of pure TDSC cultures difficult, therefore a panel of MSC and tenogenic markers needs to be used.

TDSCs have been found to express the pluripotency markers Oct-4, SSEA-4 and nucleostemin (Zhang and Wang, 2010b; Zhang and Wang, 2015) indicating that they possess stem cell-like characteristics and these markers can be used to distinguish TDSCs from tenocytes. However, Oct4 has been shown to be expressed in embryonic stem cells only, not in somatic stem cell populations (Lengner *et al*., 2008). The apparent expression of Oct4 in TDSCs and MSCs is most likely due to the

Species	Oct-4	Nanog	SSEA-4	Nucleostemin	Nestin	Sca-1	CD90	CD105	CD73	CD44	CD146	CD29	TNC	SCX	TNMD	CD34	CD45
Human	+[t]	+[t]	+[t]	+[t]		NA	+[g]	+[g,j]	+[g]	+[d,g,j]	+[d,g]	+[j]	+[g,t]	+[g]	+[d,g]	-[d,g,j]	-[d,g,j]
Mouse	+[s]	+[a]		+[q]	+[a]	+[a,c,d,i]	+[a,d,i]	+[a]	+[a]	+[a,c,d,i]	+[a]	+[c]	+[d]	+[a,d,i,c]	+[a,i,s]	-[d]	-[d]
Rat	+[m]	+[m]	+[b]	+[b,m]		NA	+[b,f,k,l]		+[l]	+[b,f,k,l,m]	+[m]	+[f]	+[e,k]	+[b,f,l,m]	+[b,k,l,m]	-[b,k,l]	-[f,l]
Rabbit	+[n,r]		+[n,r]	+[n,r]		NA							+[n]	+[n]	+[n]		
Cow						NA				+[p]			+[p]				
Horse	+[o]	+[o]	+[h]			NA	+[o]	+[h,o]	+[o]	+[h,o]		+[h]	+[o]	+[o]		-[h, o]	

Table 1.1. The expression of stem cell and tenogenic markers in TDSCs in various species. **+** = positive expression, **-** = no expression, **NA** = not applicable in this species, **blank** = expression unknown. Information gathered from: [a]Alberton *et al*., 2014; [b]Chen *et al*., 2015; [c]Asai *et al*., 2014; [d]Bi *et al*., 2007; [e]Chen *et al*., 2012a; [f]Chen *et al*., 2016; [g]Lee *et al*., 2012; [h]Lovati *et al*., 2011; [i]Mienaltowski *et al*., 2013; [j]Nagura *et al*., 2016; [k]Rui *et al*., 2010; [l]Rui *et al*., 2013a; [m]Tan *et al*., 2013; [n]Tao *et al*., 2015; [o]Williamson *et al*., 2015; [p]Yang *et al*., 2016; [q]Zhang and Wang, 2010a; [r]Zhang and Wang, 2010b; [s]Zhang and Wang, 2013a; [t]Zhang and Wang, 2013b.

presence of non-functional Oct4 pseudogenes in these cell types or non-specific staining (Lengner *et al*., 2008), indicating that Oct4 is not a useful marker for TDSCs.

TDSCs have also been shown to express MSC markers, as TDSCs derived from human patellar tendon were subjected to flow cytometry in order to analyse surface antigens on the cells and were found to express CD29, CD73, CD44, CD90 and CD105. These TDSCs were negative for CD34 and CD45, markers which are absent on MSCs, but present on haematopoietic stem cells (HSCs) (Lee *et al*., 2012; Nagura *et al*., 2016). Bi and others (2007) attempted to identify markers to distinguish between TDSCs and MSCs, they found that murine TDSCs expressed stem cell antigen-1, CD90.2 and CD44 (Bi *et al*., 2007). The cells were negative for CD34, CD117, CD45 and CD18. Similar results were found for human TDSCs with the cells expressing CD90 and CD44 but not CD18 (Bi *et al*., 2007). Bi and others (2007) found no expression of CD90.2 in MSCs but high expression in TDSCs, however the use of CD90.2 as a marker to distinguish between TDSCs and MSCs is not possible as CD90.2 expression has since been demonstrated in murine MSCs (Li *et al*., 2014). A more promising marker is the MSC marker CD18, with Bi and others (2007) demonstrating no expression of CD18 in murine and human TDSCs (Bi *et al*., 2007), which is consistent with a more recent study (Mienaltowski *et al*., 2013). Therefore it is clear that many of the markers present on TDSCs are also present on MSCs with few exceptions.

Other studies have investigated tenogenic markers as a means of isolating TDSCs (Bi *et al*., 2007; Rui *et al*., 2010). Several studies have demonstrated expression of tenascin C in TDSCs which is a component of tendon ECM (Lee *et al*., 2012; Rui *et al*., 2010). Similarly, TDSCs have been shown to express scleraxis, a transcription factor specific to tendon tissue and tenomodulin, a component of tendon ECM (Bi *et al*., 2007; Mienaltowski *et al*., 2013). Tenogenic markers may be useful candidates to distinguish between TDSCs and MSCs as expression of scleraxis and tenomodulin has been shown to be increased in TDSCs when compared with MSCs (Bi *et al*., 2007). It is therefore necessary to use a range of markers to identify TDSCs as at present there is not one single marker which can be used for their identification.

1.4.1.2 Stem cell culture

TDSCs have been cultured successfully *in vitro* for several species including human, mouse (Bi *et al*., 2007), rat (Rui *et al*., 2010), rabbit (Zhang and Wang, 2010a) and cow (Yang *et al*., 2016). Initially the tendon tissue must be digested which is routinely performed using collagenase type I or II overnight. The cells are then counted and seeded at low seeding densities for selection of clonal cell populations (Asai *et al*., 2014; Lee *et al*., 2012; Lui and Wong, 2013; Mienaltowski *et al*., 2014; Ni *et al*., 2012; Rui *et al*., 2010). TDSCs adhere to the culture flask and produce distinct colonies after several days (Rui *et al*., 2013a). There are various methods for isolation of TDSCs; individual colonies can be scraped off or detached by local application of trypsin (Zhang and Wang, 2010a) or by trypsinising the whole culture vessel (Rui *et al*., 2013a). However, the latter method of complete vessel trypsinisation does not distinguish between TDSC colonies and tenocyte colonies. TDSCs are then transferred to a culture vessel and allowed to proliferate.

Certain conditions are conducive to proliferation with hypoxia causing an increase in proliferation when compared with a normoxic environment (Zhang and Wang, 2013b). In addition, TDSCs obtained from younger animals have increased proliferation rates when compared with aged tendon (Zhang and Wang, 2015; Zhou *et al*., 2010). Tendon derived from younger animals also possesses higher numbers of TDSCs than older tissue, indicating that younger tendon tissue may have greater regenerative potential (Zhou *et al*., 2010). The passage number has also been shown to have a significant effect on stem cell characteristics with one study finding that cell proliferation rates increased with passage number as well as clonogenicity (Tan *et al*., 2012a). However other studies have found that cell proliferation decreases with passage number (Zhang and Wang, 2010b) suggesting that the discrepancy may be due to species and methodological variations as the two studies used differing methods. However, Tan and others (2012a) did note an increase in β-galactosidase activity in higher passages (Tan *et al*., 2012a) which is indicative of cell senescence and consistent with the study by Zhang and others (2010b), although it should be noted that Zhang and others (2010b) did not provide the data or figures relating to this experiment. In addition, mechanical factors can also affect proliferation, with cyclic tensile strain causing an increase in proliferation of rat

TDSCs at moderate frequencies and amplitudes such as 4%, but a decrease at extreme strain, such as 8% (Xu *et al*., 2015).

1.4.1.3 Stem cell differentiation capacity

TDSCs have been found to differentiate into tenocytes *in vitro* (Guo *et al*., 2016; Zhang and Wang, 2010a) as well as adipocytes, osteocytes and chondrocytes (Bi *et al*., 2007; Rui *et al*., 2010). TDSCs have also been shown to differentiate into tenocytes, chondrocytes and osteocytes *in vivo* in a rabbit model (Zhang and Wang, 2010b).

The culture conditions of the TDSCs determine their differentiation capacity as certain factors are conducive to tenogenic or non-tenogenic differentiation. For example transforming growth factor-β1 (TGF-β1) promoted tenogenic differentiation of rat TDSCs (Guo *et al*., 2016). Similarly, platelet-rich plasma (PRP) promoted differentiation of TDSCs into tenocytes when added in cell culture, as well as increasing TDSC proliferation (Zhang and Wang, 2010a). In contrast, PRP has been found to inhibit non-tenogenic differentiation with the addition of PRP to TDSCs in culture preventing adipogenic, osteogenic and chondrogenic differentiation (Chen *et al*., 2012b). Similarly hypoxia has also been found to exhibit an inhibitory effect on adipogenic, osteogenic and chondrogenic differentiation of human TDSCs (Lee *et al*., 2012). However a more recent study has found the opposite with hypoxia producing an increase in non-tenogenic differentiation of human TDSCs (Zhang and Wang, 2013b). This discrepancy may be due to the different oxygen tensions used with Lee and others (2012) using 2% oxygen (Lee *et al*., 2012) and Zhang and others (2013b) using 5% (Zhang and Wang, 2013b), or the induction periods, as Lee and others (2012) induced differentiation for 14 days, whereas Zhang and others (2013b) induced differentiation for 21 days. The additional seven days incubation may have been sufficient to induce non-tenogenic differentiation. In addition, the glucocorticoid drug dexamethasone, which is often included in non-tenogenic differentiation induction media, has been shown to inhibit tenogenic differentiation of rat TDSCs *in vitro*, with a reduction in type I collagen and tenomodulin gene expression seen with prolonged exposure. The inhibitory effect of dexamethasone on tenogenic differentiation is due to inhibition

of the scleraxis gene highlighting the key role this gene plays in the normal function of TDSCs (Chen *et al*., 2015c).

The location of the TDSCs within the tendon does not have an impact on their differentiation capacity as TDSCs isolated from two sites within the murine Achilles tendon (tendon body and peritenon) were both found to exhibit multipotency (Mienaltowski *et al*., 2013). Similarly, there were no differences in multipotency between tenocytes isolated from the peritenon and tendon body (Cadby *et al*., 2014). However the type and location of the tendon may have an impact on the function of TDSCs in certain species such as humans. TDSCs isolated from the human patellar tendon (Lee *et al*., 2012) demonstrated differentiation down osteogenic, adipogenic and chondrogenic lineages, whereas TDSCs isolated from human semitendinosus and gracilis tendons only showed osteogenic and chondrogenic differentiation (Stanco *et al*., 2014). This would suggest that these cell populations are not true stem cells but a form of tendon progenitor cell. However it should be noted that the chondrogenic induction media used differed between the two studies mentioned above. Lee and others (2012) also added L-proline and bone morphogenetic protein 2 (BMP-2) to their induction media which may account for their observation of chondrogenic differentiation of TDSCs when compared with the study conducted by Stanco and others (2014). The difference in multipotency seen between TDSCs isolated from these two tendon types may therefore be due to the culture conditions and the artificial nature of differentiation assays, rather than inherent differences in the cell populations. Similarly, TDSCs isolated from equine superficial digital flexor tendon (SDFT) also showed osteogenic and adipogenic differentiation, but limited chondrogenic differentiation (Lovati *et al*., 2011), highlighting the variability in differentiation potential of TDSCs isolated from certain tendon types. In addition, TDSCs from rat Achilles (Chen *et al*., 2012b) and flexor tendons (Chen *et al*., 2015c) were able to differentiate down osteogenic, adipogenic and chondrogenic lineages. However tendon cells isolated from the rat patellar tendon did not show differentiation towards any of the three lineages, except when CD146+ cells were enriched from the population, with these cells exhibiting differentiation down all three lineages (Lee *et al*., 2015a). The CD146+ cell population was located in the perivascular region, however the study did not

analyse expression of haematopoietic markers, so it is unknown whether these cells are true TDSCs or whether they are contaminated with HSCs. In contrast, in some species there appears to be little difference between TDSCs isolated from different tendon types. For example, in mice, TDSCs isolated from the tail (Alberton *et al*., 2014) and Achilles tendon (Asai *et al*., 2014) demonstrated differentiation down all three lineages, and in rabbits, TDSCs isolated from the patellar and Achilles tendon also demonstrated differentiation down all three lineages (Zhang and Wang, 2010b).

Certain growth factors promote non-tenogenic differentiation, such as BMP-2 which promotes adipogenic, osteogenic and chondrogenic differentiation of TDSCs (Rui *et al*., 2013b). However other growth factors such as early growth response-1 (EGR1) and fibroblast growth factor-2 (FGF-2) can promote tenogenic differentiation of TDSCs. In lapine patellar tendons EGR-1 induced tenogenic differentiation and promoted tendon repair, whilst inhibiting non-tenogenic differentiation (Tao *et al*., 2015). FGF-2 also promoted tenogenic differentiation of TDSCs, with an increase in tenomodulin and scleraxis seen in rat TDSCs subjected to FGF-2 in cell culture. In addition, FGF-2 promoted tendon repair with an increase in cells expressing stem cell markers as well as increases in tenomodulin and scleraxis expression seen in injury sites when compared with healing tendon in the absence of FGF-2 (Tokunaga *et al*., 2015).

Mechanical stretching of TDSCs also affects their differentiation capacity, as 4% stretching has been shown to increase tenogenic differentiation, whereas 8% stretching increased both tenogenic and non-tenogenic differentiation when compared with controls (Zhang and Wang, 2010d). Another study also found that 4% and 8% stretching of TDSCs seeded on collagen scaffolds caused an increase in tenogenic differentiation (Xu *et al*., 2015).

The passage number also affects the multipotency of TDSCs with those at a lower passage being able to differentiate into tenocytes, adipocytes, osteocytes and chondrocytes, whereas cells at higher passage numbers tended to differentiate into osteocytes only, and lost the ability to differentiate into tenocytes, adipocytes and chondrocytes (Tan *et al*., 2012a).

1.4.2 The role of TDSCs in failed healing of tendon

As TDSCs have been shown to differentiate into tenocytes, and tenocytes are responsible for maintaining and repairing tendon, then it is reasonable to hypothesise that TDSCs play a crucial role in tendon maintenance and repair, which may be the case for acute injury (Kajikawa *et al*., 2007; Lui, 2013; Rui *et al*., 2011a). However evidence has shown that TDSCs can contribute to poor healing in chronic tendinopathy due to their erroneous differentiation (Rui *et al*., 2011a) (Fig.1.2). Chronic tendinopathy is characterised by increased cellularity, vascularity, proteoglycan deposition, ECM degradation, ossification, lipid deposition, and the presence of chondrogenic and osteogenic cell types (Omachi *et al*., 2014; Rui *et al*., 2013a). Rui and others (2013a) have recently shown that TDSCs differentiate into chondrocytes and osteocytes after collagenase-induced injury in a rat model of chronic tendinopathy (Rui *et al*., 2013a). TDSCs taken from the patellar tendon of injured rats had an increased proliferation rate, increased numbers of cells which differentiated into osteocytes and an increase in calcium nodule formation and ALP activity. Similarly, there was an increase in the number of TDSCs which differentiated into chondrocytes with an increase in collagen type II and Sox9 gene expression in the injured rat tendons compared with the healthy controls. Finally there was a decrease in tenogenic markers in the injured rat tendons compared with the controls (Rui *et al*., 2013a).

Previously, Bi and others (2007) produced an Fmod and Bgn double knockout mouse to investigate the role of these proteins in the TDSC niche (Bi *et al*., 2007). Fmod (fibromodulin) and Bgn (biglycan) are ECM proteins found in tendon tissue. They found that in the knockout mouse there was significant ossification, indicating differentiation of TDSCs into osteocytes as well as the expression of chondrogenic markers, indicating the presence of chondrocytes. They also found an increase in the activity of the BMP/Smad pathway which may be a cause of the increase in non-tenogenic differentiation of TDSCs. Bi and others (2007) hypothesised that tendinopathy causes changes in the ECM which in turn causes an imbalance in growth factors and cytokines within the niche. This imbalance may

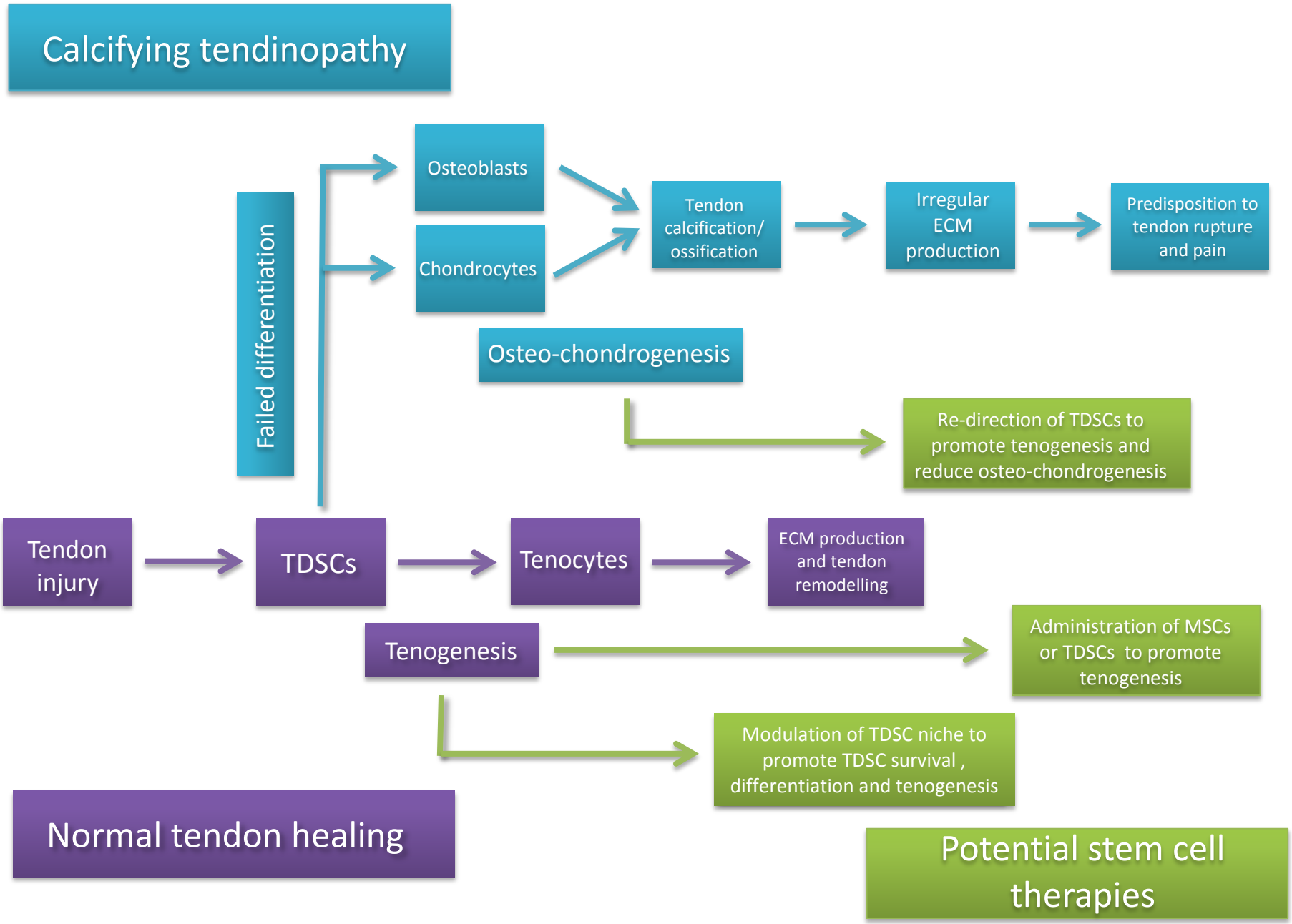


Figure 1.2. The possible route by which TDSCs can lead to failed healing and calcification and ossification of tendon, and treatment strategies by which this process can be attenuated. Adapted from (Rui *et al*., 2011a).

change the fate of TDSCs from a tenogenic lineage to an osteogenic and chondrogenic lineage and therefore cause ossification of the tendon and lead to further damage and pain (Bi *et al*., 2007). There also seems to be changes in the phenotype of TDSCs after tendon injury (Runesson *et al*., 2015). After a tendon injury was induced in rat Achilles tendons, different populations of TDSCs were noted throughout the length of the tendon. TDSCs expressing high levels of nucleostemin were dispersed throughout the length of the tissue, however at the site of injury and also in the connective tissue surrounding the tendon there were distinct populations of cells with different marker expression, principally increased OCT4 expression (Runesson *et al*., 2015). It is unknown whether these OCT4+ cells contribute towards tendon healing after injury or whether they in fact contribute towards poor tendon healing, however it is possible that these cells are derived from the peritenon. In addition, areas of increased proteoglycan deposition with resident chondrocyte-like cells were also found in the injury and mid-proximal regions with high levels of nucleostemin+ cells. It is unknown why these areas have developed, but it is possible that such proteoglycan-rich areas lead to inferior tendon regeneration (Runesson *et al*., 2015).

There is currently no evidence to confirm why the fate of TDSCs is altered during chronic tendinopathy however there are many possible causes in addition to changes to ECM components. Excessive mechanical loading causes non-tenogenic differentiation of TDSCs as discussed previously, and overloading of tendon, often due to exercise or sport, is a common cause of tendinopathy (Zhang and Wang, 2010d). Certain biological mediators have been implicated in tendinopathy, including BMPs and prostaglandin E_2 (PGE_2) (Liu *et al*., 2014a; Zhang and Wang, 2010c). PGE_2 is an inflammatory mediator and its expression is increased in tendon during and after prolonged mechanical loading. After an intensive treadmill run TDSCs isolated from murine Achilles and patellar tendons had increased levels of PGE_2 and differentiated into adipocytes and chondrocytes *in vitro* upon PGE_2 stimulation (Zhang and Wang, 2010c). A recent study has identified the signalling pathway linking PGE_2 and adipogenic differentiation of TDSCs; PGE_2 activates BMP-2 and IGF-1 signalling leading to PPARγ2 activation and adipogenic differentiation (Liu *et al*., 2014a). Tenocytes may also play a role in chronic tendinopathy as when

tendon is damaged the native cells release inflammatory mediators and other growth factors (Wang *et al*., 2003) which have a detrimental effect on the tissue and disturb the balance of cytokines within the TDSC niche. This may then result in erroneous differentiation and further chronic damage. Another possible cause of the altered fate of TDSCs during tendinopathy may be due to treatments, for example the use of glucocorticoids is common for chronic tendinopathies. One such glucocorticoid, dexamethasone, has been shown to inhibit tenogenic differentiation of TDSCs (Chen *et al*., 2015c) and thereby prevent the ability of TDSCs to aid tendon healing, exacerbating the problem by inducing osteogenic and chondrogenic differentiation of TDSCs.

It is clear that, although TDSCs may play a key role in tendon repair for acute injury, they have a detrimental effect on the capacity of tendon tissue to repair itself after chronic injury or mechanical overloading (Bi *et al*., 2007; Rui *et al*., 2013a; Zhang and Wang, 2010d).

1.4.3 TDSCs and ageing

As tendon ages there is a loss of regenerative capability, demonstrated by foetal tendon which showed healing properties after injury as opposed to scar tissue formation in adult tendon (Barboni *et al*., 2012). Barboni and others (2012) isolated ovine amniotic epithelial cells and indirectly co-cultured them using transwell chambers with fresh tendon explants from both foetal and adult calcaneal tendon. The cells co-cultured with foetal tendon were able to differentiate down the tenogenic lineage and formed tendon-like structures. These cells also expressed stem cell markers whereas cells co-cultured with adult tendon did not display this expression. These findings indicate that in foetal tendon inducive tenogenic soluble factors may play a vital role in promoting tenogenesis, which was not observed in adult tendon (Barboni *et al*., 2012). In addition to this Russo and others (2014) observed distinct differences between foetal and adult ovine tendon. During development they observed a decrease in cellularity and vascularity as well as morphological changes to cells and nuclei, with adult tendon displaying elongated, spindle-shaped cells and nuclei and foetal tendon showing heterogeneous cell and

nuclei morphology. Cells expressing stem cell markers were seen in foetal tendon but not in adult tendon, and the proliferative capacity of cells was reduced with age (Russo *et al*., 2014).

Such changes with ageing have also been observed in other species, with aged human (Kohler *et al*., 2013; Ruzzini *et al*., 2013) and rat (Zhou *et al*., 2010) TDSCs also showing decreased cellularity and self-renewal potential as well as a reduced ability to differentiate into tendon cells. However their ability to differentiate down other cell lineages was retained. Aged rat TDSCs have also been shown to change in size and morphology compared with young TDSCs, with aged cells being large and flat and younger cells smaller and elongated. In addition, aged TDSCs also showed increased stiffness and viscosity (Wu *et al*., 2015a) contributing to overall stiffness of the tendon which is commonly seen with age. The ability of young tendon to rescue the phenotype of aged tendon has also been demonstrated in rats. In one study media was collected from young rat patellar TDSCs grown in culture under hypoxic conditions and added to aged TDSCs for ten days. The aged TDSCs grown in the conditioned media exhibited increased proliferation, reduced senescence and increased expression of tenogenic genes when compared with aged TDSCs grown in regular media (Jiang *et al*., 2014). Again, these findings suggest that young tendon produces soluble factors which promote tenogenesis which are not present in aged tendon.

Rat TDSCs from older patellar tendon show earlier entry into senescence, which is possibly caused by a decrease in microRNA-135a (miR-135a). miR-135a binds to ROCK-1 and normally inhibits entry into senescence in young tendon, a decrease in miR-135a in aged tendon may be the cause of early entry into senescence and reduced stem cell proliferation, self-renewal and tenogenic differentiation (Chen *et al*., 2015b). Another study has shown that miR-140-5p may also play a role in tendon ageing (Chen *et al*., 2015a). Pin1, a peptidyl-prolyl isomerase, has been found to regulate senescence in human TDSCs with increased levels delaying entry into senescence. Aged TDSCs were found to express decreased levels of Pin1 and therefore entry into senescence occurred more rapidly. In addition, miR-140-5p was found to regulate the expression of Pin1 and therefore control TDSC senescence (Chen *et al*., 2015a). Alternatively, a loss of tenomodulin, a

marker for the tenogenic linage, may be the cause of TDSC senescence in older tendon with a tenomodulin mouse knock-out model showing reduced cellular self-renewal and early entry into senescence (Alberton *et al*., 2014).

It is clear that tendon ageing has a large impact on function and the ability of tendon to heal after injury or degeneration (Alberton *et al*., 2014; Barboni *et al*., 2012; Russo *et al*., 2014). However a recent study demonstrated the ability of moderate exercise to rescue aged tendon with a reversal of age-related TDSC characteristics (Zhang and Wang, 2015). TDSCs cultured *in vitro* from aged mice showed reduced proliferation and stem cell marker expression and showed non-tenogenic differentiation. After 4% stretching of the TDSCs, stem cell marker expression was increased and the expression of non-tenogenic markers remained the same, however after 8% stretching stem cell marker expression was decreased and non-tenogenic marker expression was increased (Zhang and Wang, 2015). The study also described an *in vivo* experiment that used treadmill running to assess the effects of exercise on TDSC characteristics (Zhang and Wang, 2015). Moderate treadmill running caused an increase in TDSC proliferation as well as stem cell marker expression. In addition, there was a decrease in the osteogenic, adipogenic and chondrogenic differentiation of TDSCs. The study indicated that moderate exercise may be able to prevent or reverse the age-related degeneration of tendon, however intense exercise can have detrimental effects (Zhang and Wang, 2015).

The aforementioned studies have all investigated the effects of ageing on TDSCs, with a comparison of tendon isolated from young and older adults. Chen and others (2016) investigated the changes in TDSCs during early postnatal development comparing seven day old rats with 56 day old rats. They found many differences between the two groups with colony forming ability, stem cell marker expression, proliferation and multipotency all decreasing with age. Adipogenic differentiation was the only parameter maintained across both groups (Chen *et al*., 2016). In combination with the studies outlined previously, the study conducted by Chen and others (2016) suggests that the stem cell properties of TDSCs increase during early postnatal development and then progressively decline during tendon ageing.

1.4.4 The use of TDSCs in tendon repair

TDSCs have been used successfully in tissue engineering for the repair of tendon and other tissues such as ligament in a number of different species. When compared with BMSCs, TDSCs exhibit many advantages including increased clonogenicity, proliferation, differentiation potential and stem cell marker expression (Tan *et al*., 2012b), making TDSCs an ideal cell source for tendon tissue engineering strategies.

The application of TDSCs to an injury site usually requires the use of a synthetic scaffold or construct to allow integration of the cells. Ni and others (2012) used fibrin glue constructs to introduce TDSCs into patellar tendon defects in rats. The application of TDSCs promoted earlier and more effective tendon repair than controls, measured by increased collagen production and improved cell and collagen fibre alignment, as well as increased ultimate stress and Young's modulus (Ni *et al*., 2012). Other studies have used decellularised tendon matrices to enhance TDSC-mediated tendon repair. The use of decellularised matrices prepared from rabbit patellar tendon and seeded with autologous TDSCs led to the formation of tendon-like tissues and increased collagen production, which was not seen in controls. These matrices also increased the proliferation and maintained the stemness of TDSCs, however no mechanical testing was performed to analyse the strength of the newly formed tissue (Zhang *et al*., 2011a). Similarly, decellularised matrices were also prepared from rat tendon, and along with TDSCs were implanted into rat patellar defects. There was formation of tendon-like tissue and an increase in collagen fibre diameter which was not witnessed when TDSCs alone were implanted (Yin *et al*., 2013). These findings were mirrored in another study, in which rat TDSCs were seeded on to canine decellularised tendon slices (DTSs) (Ning *et al*., 2015). The combination of DTSs and TDSCs produced aligned tendon tissue, with the TDSCs showing an increase in tenogenic markers, compared with TDSCs alone (Ning *et al*., 2015). Alternatively, matrices derived from fibroblast ECM can also be used, with positive results seen in a rat patellar tendon defect model (Jiang *et al*., 2013). In the study by Jiang and others (2013), fibroblasts were stimulated to produce ECM and subsequently decellularised before the addition of TDSCs and

implantation into the injury site. Tendon-like structures were observed with increased collagen production and mechanical strength when compared with controls (Jiang *et al*., 2013). Bioimprinting of bovine tendon tissue, mimicking the natural tendon tissue microenvironment, provided a scaffold for a successful tissue engineering approach using MSCs. This involved using tendon tissue sections as a template for imprinting using polydimethylsiloxane to produce a synthetic tendon mimic. MSCs were then seeded on to the synthetic scaffolds and the cells underwent tenogenesis and expressed tenomodulin, suggestive of a new approach for tendon tissue engineering (Tong *et al*., 2012).

Certain factors, such as cellular and matrix alignment, are conducive to successful tissue engineering in tendon. Human TDSCs grown on aligned poly (L-lactic acid) nanofibres highly expressed tendon-specific genes, whereas cells grown on randomly-orientated nanofibres expressed only low levels of these genes (Yin *et al*., 2010). In addition, cells grown on randomly-orientated nanofibres differentiated into osteoblasts, whereas aligned nanofibres prevented this process. The use of aligned nanofibres also induced tendon tissue formation whereas randomly-orientated fibres did not, however the mechanical integrity of the tissue was not tested (Yin *et al*., 2010). The study by Yin and others (2010) emphasises the importance of the structural environment on TDSC survival and function.

Mechanical stimulation also has a positive impact on the ability of TDSCs to form tendon tissue. Rabbit TDSCs subjected to a mechanical stretch of 4% at 0.5 Hz expressed tendon-specific ECM proteins and when seeded on to poly(L-lactide-co-ε-caprolactone)/collagen scaffolds followed by implantation into rabbit patellar tendon injuries caused tendon regeneration (Xu *et al*., 2014).

Successful tendon engineering has also been achieved without the use of a scaffold (Lui *et al*., 2016; Ni *et al*., 2013). Rat TDSCs were used to form a scaffold-free tendon tissue with the use of connective tissue growth factor (CTGF) and ascorbic acid. When implanted into nude mice they formed neo-tendon and also assisted with tendon repair in a rabbit patellar tendon defect model (Ni *et al*., 2013). These results have been mirrored in a rat patellar tendon injury model (Lui *et al*., 2016) indicating that scaffolds may not be necessary for successful tissue engineering in tendon. Further to this, the combined use of TDSCs and platelet-rich

plasma (PRP) without the use of a scaffold showed positive tendon healing in a collagenase-induced rat injury model when compared with an injured control, however the use of PRP and TDSCs produced tendon tissue mechanically inferior to healthy tendon (Chen *et al*., 2014).

TDSCs have been used for repair of non-tendon tissue including anterior cruciate ligament (ACL) (Lui *et al*., 2014). A cell sheet was produced from rat TDSCs, CTGF and ascorbic acid, which was wrapped around rat patellar tendon grafts before insertion during ACL reconstructive surgery. Rats which received the cell sheet and graft had a higher tunnel bone mineral density, better cell alignment, graft osteointegration and intra-articular graft integrity, and higher collagen birefringence. In addition, the ultimate load and stiffness of the joint were higher when compared with graft only rats (Lui *et al*., 2014). The study suggested that the use of TDSCs for tissue engineering may have wide applications, with the potential to repair and regenerate a number of tissues besides tendon.

1.4.5 Equine TDSCs

The majority of current research into TDSCs has been conducted in rodents and small animals, with a small amount in humans, however there is very little data available regarding TDSCs in larger animals. Large animal models can be invaluable as they are able to provide a comparative representation of disease and injury in humans, including musculoskeletal disorders (Innes and Clegg, 2010).

Horses provide a useful model for tendon research as equine tendon has many similarities with that of humans. For example, the equine superficial digital flexor tendon (SDFT) (Fig.1.3) is functionally and clinically equivalent to the human Achilles tendon, with both tendons being energy-storing and aiding with locomotion (Lichtwark and Wilson, 2005; Lui *et al*., 2011; Patterson-Kane and Rich, 2014; Wilson *et al*., 2001). In addition, tendon injury and degeneration manifest in a similar manner in both humans and horses, with matrix alterations, increased cellularity and increased metalloproteinases present in both species (Birch *et al*., 1998; Clegg *et al*., 2007; Innes and Clegg, 2010; Ireland *et al*., 2001; Jones *et al*., 2006; Riley *et al*., 1994).

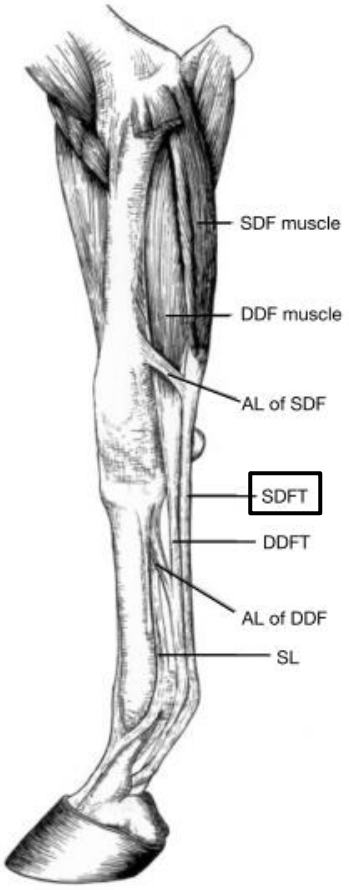


Figure 1.3. Schematic diagram of an equine forelimb showing the location of the superficial digital flexor tendon (SDFT). AL, accessory ligament; DDF, deep digital flexor; SDF, superficial digital flexor. Adapted from (Wilson *et al*., 2001).

The study of equine tendon is important, not only to inform on tendon function and tendinopathy in humans, but also because equine tendon injuries are extremely common, particularly within the horse racing industry. One study found that 15% of racehorses will suffer a tendon or ligament injury during one racing season (Kasashima *et al*., 2004). SDFT injury is common in athletic horses, resulting in increased lameness and reduced performance. One study identified SDFT injuries as accounting for 98% of all tendon and ligament injuries in the horse (Ely *et al*., 2009). SDF tendinopathies are also more prevalent with age, indicating that this tendon is prone to both injury and age related degeneration (Kasashima *et al*., 2004).

As with humans, the treatment options are limited and rarely successful for equine tendon injuries, therefore alternative therapies, including stem cell research, are being investigated. The majority of current research on equine tendon repair is focused on MSCs and their potential use in tissue engineering approaches. For example, Lovati *et al* (2012) isolated bone marrow derived MSCs and indirectly co-cultured the cells with native equine tendon tissue. The cells expressed tenogenic markers, including tenomodulin and tenascin-C, indicating tenogenic differentiation of the MSCs (Lovati *et al*., 2012). In addition, research has also focused on the use of foetal-derived embryonic stem cells (fdESCs), with the cells being introduced into collagenase-induced tendon injuries (Watts *et al*., 2011). The study revealed the tendon lesions to be reduced and the overall tendon size to be increased after fdESC treatment. Tissue architecture was also improved with more aligned tendon fibres (Watts *et al*., 2011). However to date, there is little research available on the characterisation of equine TDSCs. Lovati and others (2011) isolated and characterised TDSCs from equine SDFT and found that TDSCs were able to form colonies and proliferated for an extensive period *in vitro.* Reverse transcription-polymerase chain reaction (RT-PCR) showed expression of MSC markers by TDSCs and tri-lineage differentiation assays resulted in osteogenic and adipogenic differentiation but not chondrogenic (Lovati *et al*., 2011). Similarly, Durgam and others (2016a) isolated TDSCs from equine lateral digital extensor tendon which demonstrated similar properties: MSC marker expression and tri-lineage differentiation, although clonogenicity was not assessed (Durgam *et al*., 2016a).

Durgam and others (2016b) have also introduced TDSC populations into a collagenase-induced injury model in equine SDFT with limited beneficial effects. Collagen and ECM gene expression was similar between TDSC and saline controls, as was the elastic modulus. However yield and maximal stresses were significantly higher in the TDSC group compared with controls and collagen alignment was significantly improved in the TDSC group (Durgam *et al*., 2016b). The progenitor cell populations identified within equine tendon in these studies highlight a potential cell population for use in equine tendon tissue engineering strategies.

1.5 Ligament-derived stem cells

1.5.1 Ligament-derived stem cell characteristics

In 2004 Seo and others identified a population of cells within periodontal ligament which expressed certain stem cell markers and were able to differentiate down a number of different cell lineages (Seo *et al*., 2004). Following this discovery, a large amount of research has been conducted into periodontal ligament stem cells (PDLSCs); both into the characterisation of these cells and their use in tissue engineering strategies. Therefore, the majority of published research on stem cells in ligament has focussed on PDLSCs. The promising results seen in these cells may also be applicable for other ligaments in other areas of the body and in recent years research has turned to the anterior cruciate ligament (ACL) and the potential for ligament-derived stem cells (LDSCs) to provide therapies for other types of ligament injury.

1.5.1.1 Stem cell markers

Many of the markers used to identify LDSCs (Table 1.2) are found in other cell types including MSCs and TDSCs. For example, stem cells derived from human periodontal ligament have been found to express Stro-1, CD146 (Seo *et al*., 2004), CD44, CD105 (Gay *et al*., 2007), CD29 (Kim *et al*., 2012), Oct-3/4, SSEA-4, Sox2, CD13, CD73, CD90 (Eleuterio *et al*., 2013), Nanog (Navabazam *et al*., 2013) and also scleraxis, a tendon specific marker (Fujii *et al*., 2008). However a study focussing on

Species	Oct-4	Nanog	SOX2	SSEA-4	Nucleostemin	Nestin	STRO-1	CD90	CD105	CD73	CD44	CD146	CD166	CD29	CD13	SCX	TNC	TNMD	CD34	CD45
Human	+[c,l]	+[a]	+[a,c]	+[c, l]	+[l]	+[e]	-[j,l]	+[a,c,e,j,l]	+[a,c,e,j]	+[a,c,e,j]	+[a,c,g,j,l]	+[cg]	+[c]	+[c]	+[c]	+[d]	+[b]	+[j]	-[a,c]	-[a,c]
Dog							+[h]	+[h]	+[k]		+[h]	+[k]				+[k]			-[k]	
Rabbit								+[f]			+[f]								-[f]	
Horse	+[i]							+[i]	+[i]	-[i]										-[i]

Table 1.2. The expression of stem cell and tenogenic markers in LDSCs in various species. **+** = positive expression, **-** = no expression, **blank** = expression unknown. Information gathered from: [a]Cheng *et al*., 2009; [b]Cheng *et al*., 2010; [c]Eleuterio *et al*., 2013; [d]Fujii *et al*., 2008; [e]Hakki *et al*., 2014; [f]Jiang *et al*., 2015; [g]Kowalski *et al*., 2015; [h]Lee *et al*., 2014; [i]Shikh Alsook *et al*., 2015; [j]Steinert *et al*., 2011; [k]Wang *et al*., 2012; [l]Zhang *et al*., 2011.

the use of PDLSCs from a single human patient has shown expression of CD44, CD105, CD90 and Nestin, although there was no expression of CD146 or Stro-1 (Prateeptongkum *et al*., 2015) indicating that there may be significant individual variation in stem cell marker expression. This individual variation may be a feature of age as one study has demonstrated a significant decline in the expression of Oct-4, Nanog, Sox-2 and CD146 with age (Wu *et al*., 2015b). LDSCs isolated from non-dental ligaments appear to express similar markers to PDLSCs, with LDSCs isolated from human interspinous ligaments expressing CD29, CD44, CD73, CD90 and CD105, but not CD34 and CD45 (Kristjánsson *et al*., 2016). Stem cells derived from human ACL tissue (ACLDSCs) have been shown to express the MSC markers CD13,CD29, CD49c, CD73, CD90, CD97, CD105, CD146, CD166, SSEA-4, STRO-1 and HLA A, B, C, as well as the pluripotency markers Oct4, Nanog and Sox2, and negative expression of CD34 and CD45 (Cheng *et al*., 2009; Steinert *et al*., 2011; Zhang *et al*., 2011b). Stem cells derived from ruptured ACL remnants and healthy tissue show a similar marker expression profile (Cheng *et al*., 2009; Zhang *et al*., 2011b). Stem cells derived from rabbit medial collateral ligament (MCL) have been shown to express CD44 and CD90, however not CD34, CD106 or CD11b (Jiang *et al*., 2015). The cells isolated in the above studies have all been negative for haematopoietic and endothelial markers, however one group has identified a cell population within the ACL which is positive for CD34 (Matsumoto *et al*., 2012; Uefuji *et al*., 2014), suggesting an alternative vascular source for stem cells located within the ACL. However the CD34+ cells isolated in these studies are located in ruptured regions so these cells may be a feature of ligament injury. In addition, the cells lost expression of CD34 after two weeks expansion, with a phenotype similar to that reported for ACLDSCs in other studies (Matsumoto *et al*., 2012). It is possible that CD34+ cells infiltrate ligament tissue during times of injury and undergo ligamentogenic differentiation to aid tissue repair. Alternatively, it is possible that CD34+ cells are an entirely separate cell population to LDSCs resident in ligament tissue and their phenotype changes during prolonged periods in culture, which is a common phenomenon for *in vitro* experiments. An additional explanation may be that all LDSCs initially express CD34, however this expression is lost by the time cell phenotype is normally analysed. Other studies investigating ACLDSCs from ruptured

ACL tissue found negative expression of CD34 for cells at passage three (Fu *et al*., 2015; Nohmi *et al*., 2012), it is possible that these cells had already lost expression of CD34 by this passage, or that they never expressed this marker at all. Further investigation into the effects of passaging on LDSC phenotype is warranted.

1.5.1.2 Stem cell culture

As the majority of research on LDSCs has focussed on cells extracted from periodontal ligament, most of the published studies have used human tissue, due to the ease of dental tissue access (Fujii *et al*., 2008; Hakki *et al*., 2014; Seo *et al*., 2004). The majority of studies investigating ACLDSCs have also used human tissue (Cheng *et al*., 2009; Steinert *et al*., 2011). However, there are a number of other studies which have isolated and cultured dental and non-dental LDSCs from other species, including dogs (Chang *et al*., 2009; Wang *et al*., 2012), pigs (Liu *et al*., 2008), horses (Shikh Alsook *et al*., 2015) and rabbits (Jiang *et al*., 2015).

As with TDSCs the isolation of LDSCs involves tissue extraction, digestion in collagenase and low-density seeding of cells (Seo *et al*., 2004; Zhang *et al*., 2011b). After several days in culture the LDSCs start to form colonies (Gay *et al*., 2007; Seo *et al*., 2004) and can be passaged and further cultured. Unlike TDSCs there is little research on the optimum culture conditions for LDSC survival and expansion. The rate of proliferation of cells is normally dependent upon the culture conditions, however oxygen tension appears to have a variable effect on LDSC proliferation with one study observing similar proliferation rates at 21% and 2% oxygen, although stem cell marker expression was increased in hypoxia (Zhou *et al*., 2014) and another showing a significant increase in cell proliferation and clonogenicity after pre-treatment at 2% oxygen (Yu *et al*., 2016). In addition, pre-treatment at lower oxygen tensions has been shown to enhance LDSC populations and increase stem cell marker expression (Kawasaki *et al*., 2015). The reason for the discrepancy is most likely due to differences in culture systems. Zhou and others (2014) cultured PDLSCs in hypoxia immediately after digestion and seeding, whereas Yu and others (2016), and Kawasaki and others (2015) cultured cells in normoxia until 24 hours prior to analysis when cells were transferred to a hypoxic environment. Although this approach is useful to analyse the effects of hypoxia on PDLSCs, it is not

physiologically relevant and the duration of the effects of the hypoxic pre-treatment are unknown and may be transient.

The age of the donor seems to have an impact on the morphology and proliferation of PDLSCs as cells from younger donors exhibited a spindle-shaped morphology, however cells from older donors appeared larger (Wu *et al*., 2015b). In addition, cells from younger donors formed increased numbers of colonies and these colonies contained increased numbers of cells. Finally, PDLSCs from younger donors had decreased proliferation times compared with cells from older donors (Wu *et al*., 2015b). Stem cells isolated from ruptured human ACL remnants demonstrated similar characteristics to cells from healthy tissue. Cells had a similar morphology to healthy cells and were also able to form colonies (Fu *et al*., 2015). This indicates that age rather than injury has an impact on human LDSC function.

1.5.1.3 Stem cell differentiation capacity

As a subpopulation of MSCs, LDSCs should be multipotent and have the potential to differentiate into various cell lineages, including osteogenic, adipogenic and chondrogenic lineages (Dominici *et al*., 2006). Many studies have shown the ability of human PDLSCs to form osteogenic, adipogenic and chondrogenic cells *in vitro* (Gay *et al*., 2007; Kim *et al*., 2012; Prateeptongkum *et al*., 2015; Seo *et al*., 2004). The multipotency of the cells was confirmed by staining with alizarin red and for alkaline phosphatase for osteogenic differentiation assays, oil red O for adipogenic differentiation assays and alcian blue and toluidine blue for chondrogenic differentiation assays, as well as western blotting and PCR for analysis of lineage specific proteins and genes respectively. The multipotency of PDLSCs has also been shown in canine PDL, with the PDLSCs able to differentiate into osteogenic, adipogenic and chondrogenic cells (Sedigh *et al*., 2010; Wang *et al*., 2012). LDSCs isolated from human interspinous ligaments demonstrated differentiation down all three lineages (Kristjánsson *et al*., 2016). Similarly, human ACLDSCs have been shown to differentiate into osteogenic, adipogenic and chondrogenic cells (Cheng *et al*., 2009; Steinert *et al*., 2011), as have LDSCs isolated from equine suspensory ligaments (Shikh Alsook *et al*., 2015). PDLSCs isolated from

rabbit ligament however, have only been shown to differentiate into osteogenic and adipogenic cells as chondrogenesis was not assessed (Su *et al*., 2015).

The environment of the LDSCs has a significant impact on their multipotency, for example hypoxia increases the differentiation potential of human PDLSCs (Zhou *et al*., 2014). Transient exposure to 5% oxygen has been shown to increase osteogenic differentiation of PDLSCs after reoxygenation at 21% oxygen (Kawasaki *et al*., 2015; Yu *et al*., 2016), whereas anoxic conditions enhanced tenogenic/ligamentogenic differentiation when compared with cells cultured at 21% oxygen (Kawasaki *et al*., 2015). No differences in adipogenic (Yu *et al*., 2016) and chondrogenic potential were observed between groups (Kawasaki *et al*., 2015). Growth factors also play a role as bone morphogenetic protein-7 and -9 both increase osteogenic differentiation of human PDLSCs (Torii *et al*., 2014; Ye *et al*., 2014).

Age seems to have a variable effect on LDSC multipotency, which is most likely dependent on ligament type. Age has been shown to impact the ability of PDLSCs to differentiate down different lineages, with cells from older donors exhibiting decreased calcium nodule and lipid droplet formation when compared with cells from younger donors (Wu *et al*., 2015b), whereas LDSCs isolated from aged ACL demonstrated similar multipotency to those from younger donors (Lee *et al*., 2015b). Ligament injury appears to have less of an effect on LDSCs than age as cells derived from ruptured human ACL remnants were able to differentiate down all three lineages (Fu *et al*., 2015).

Besides the cell lineages originally derived from the mesenchyme, PDLSCs have been shown to transdifferentiate into cell lineages derived from the endoderm. Lee and others (2014a) induced human PDLSCs to differentiate into pancreatic cells by culturing the cells in defined conditions with specific growth factors and substrates (Lee *et al*., 2014a). In addition, two studies have induced canine PDLSCs to differentiate into neural cells, again with the use of specific conditions and growth factors (Dapeng *et al*., 2014; Li *et al*., 2010). These studies emphasise the plasticity of LDSCs with implications for potential treatments for a range of conditions.

1.5.2 The use of LDSCs in ligament repair

Recent studies have investigated the use of PDLSCs in tissue engineering approaches for the repair of periodontal cementum/bone and ligament (Fujii *et al*., 2008; Lee *et al*., 2014c; Su *et al*., 2015), however little research has been conducted into the use of LDSCs for other forms of ligament repair.

Human PDLSC pellets in combination with fibrinogen and thrombin were transplanted subcutaneously into the dorsal surfaces of SCID mice, where they formed cementum/bone-like structures. Gene expression and histological analysis revealed cementum/bone formation and the development of PDL-like structures (Fujii *et al*., 2008). A similar study transplanted a 3D complex of human PDLSCs embedded in their ECM into SCID mice which formed a cementum-PDL complex-like structure. Further analysis confirmed the presence of cementum and ligament tissue formation in the mice (Yang *et al*., 2009). PDLSCs implanted into pigs also produced periodontal structures. Periodontal lesions were created in pigs, and after one month autologous PDLSCs were transplanted into the defect along with gelatin membranes. The resultant tissue growth resembled cementum and imaging and histological staining revealed osteogenic differentiation (Liu *et al*., 2008).

Scaffolds have been found to aid tissue regeneration of periodontal tissues (Lee *et al*., 2014b; Lee *et al*., 2014c; Rodríguez-Lozano *et al*., 2014). For example, fibronectin and calcium phosphate scaffolds promoted *in vitro* attachment of canine PDLSCs to the scaffold and to canine dental roots. When the canine teeth were replanted *in vivo* with the constructs, functional new periodontal attachments were seen (Lee *et al*., 2014b). Similarly, human PDLSCs grown on polydopamine film showed increased osteogenic activity compared with controls, indicating polydopamine may be a potential substrate for tissue engineering strategies (Lee *et al*., 2014c). Scaffolds containing silk fibroin and graphene oxide have been shown to have a beneficial effect on PDLSCs with an increase seen in cell proliferation and viability, although no changes in stem cell phenotype were noted (Rodríguez-Lozano *et al*., 2014). The use of nanotubes has been shown to benefit tissue engineering strategies (Gao *et al*., 2015). Nanotubes (25-90 nm) with PDLSC sheets, BMSCs, titanium sheets and hydroxyapatite all in combination together, promoted PDLSC

adhesion and spread as well as increasing collagen production. Dense collagen fibres were well aligned and there was formation of blood vessels and cementum-like tissue with the regenerated tissue mimicking that of natural PDL (Gao *et al*., 2015). These studies suggest that PDLSCs hold potential for treating periodontal trauma, therefore it is possible that LDSCs from other sites may also be used for ligament repair strategies.

A recent study has used rabbit stem cells derived from the medial collateral ligament (MCL) in order to repair an experimentally induced MCL injury (Jiang *et al*., 2015). When introduced to the injury site MCLSCs alone induced repair and remodelling with expression of collagen type I and the formation of aligned collagen fibres as well as vascularisation. Functional recovery was also assessed with the use of MCLSCs aiding the formation of ligament that was better able to withstand mechanical strain and failure loads (Jiang *et al*., 2015). However, the addition of CD34+ cells derived from human umbilical cord blood in combination with MCLSCs produced an even greater effect than MCLSCs or CD34+ cells alone. Collagen expression was increased, the fibre alignment scores were higher, vascularisation was increased and also increased loads were tolerated before failure (Jiang *et al*., 2015). The study highlights the potential use of LDSCs for repair of ligament injuries outside of the field of dentistry. In addition, this research demonstrates that although LDSCs can promote repair of ligament injury, this healing effect can be increased by the addition of other cell types (Jiang *et al*., 2015).

As mentioned previously, LDSCs positive for CD34 have also been isolated from ACL tissue and one study has used these cells to investigate the effects of angiogenesis on ACL repair strategies (Takayama *et al*., 2015). CD34+ ACLDSCs were transfected with vascular endothelial growth factor (VEGF) and grown into cell sheets that were wrapped around tendon grafts. These grafts were then implanted into rat knee joints in place of previously excised ACLs. The grafts in combination with the cell sheets exhibited increased tensile strength and load to failure when compared with tendon grafts alone (Takayama *et al*., 2015). In addition, the tendon-bone healing was increased with the use of the LDSC sheets with improved collagen fibre alignment and increased collagen production. The main focus of the study was to assess vascularisation of healing tissue and with the use of LDSC sheets

angiogenesis was significantly increased compared with tendon graft only controls. A VEGF antagonist was used to show the expected decrease in angiogenesis in the absence of VEGF and in addition there was a decrease in tendon-bone healing (Takayama *et al*., 2015). The study demonstrates the potential of vascular-derived LDSCs in treatment strategies for ligament repair but also the importance of angiogenesis during ligament healing.

LDSCs have also been used in tissue engineering strategies for the formation of periodontal bone/cementum (Manescu *et al*., 2015; Su *et al*., 2015). One study isolated PDLSCs from human patients and seeded the cells onto collagenated porcine bone scaffolds. The LDSCs differentiated into osteogenic cells with clear formation of newly synthesised mineralised bone (Manescu *et al*., 2015). A second study transfected rabbit PDLSCs with human osteoprotegerin (hOPG) and seeded these cells on to a scaffold composed of beta-tricalcium phosphate (β-TCP) (Su *et al*., 2015). PDLSCs with and without hOPG transfection adhered and proliferated on the β-TCP scaffold, however hOPG-transfected PDLSCs demonstrated earlier and increased mineralisation and bone formation and these cells contributed to the synthesis of new bone (Su *et al*., 2015). This highlights the potential of LDSCs for treatment of not only ligament injuries but also bone defects and possibly other tissue pathologies.

1.5.3 Canine LDSCs

The majority of non-human LDSC studies have utilised a canine model, however, as with humans, many of the canine studies also focus on periodontal ligament (Lee *et al*., 2014b; Wang *et al*., 2012). The reason for the use of dogs in many LDSC studies is that they provide a useful and translatable model for human work due to the similarities in structure and function of the ACL between the two species (Cook *et al*., 2010). In addition, there are over 300 different dog breeds with significant physical differences between breeds. The strict breeding strategies have led to specific populations with stable phenotypes and predispositions for certain disorders, which produce animals with interesting genetic and phenotypic traits (Innes and Clegg, 2010). Some dog breeds are particularly prone to ACL (or cranial

cruciate ligament (CCL) (Fig.1.4)) damage and rupture, which is normally a non-contact injury in the dog. Although ACL injury is commonly associated with acute trauma, often during sporting activity, there is a growing trend of non-contact related ACL injuries in humans, with 70% of all ACL injuries being caused by non-contact events (Cimino *et al*., 2010). For example, OA patients with no previous knee injuries have been found to have absent ACLs after MRI scans (Hill *et al*., 2005) and patients undergoing total knee replacements have also shown signs of ACL degeneration (Trompeter *et al*., 2009). This research suggests that with the current ageing population, non-contact ACL injuries are becoming more common. Therefore the dog could provide a useful model for studying such non-contact ligament injuries. In addition, CCL/ACL non-contact injury induced degeneration occurs in a similar manner in both dogs and humans (Comerford *et al*., 2011; Hasegawa *et al*., 2013). Matrix metalloproteinase-2 (MMP-2) has been found to be upregulated in dog breeds prone to CCL rupture (Comerford *et al*., 2005), suggesting a role for this proteolytic enzyme in CCL degeneration. Similarly in humans MMP-2, as well as a range of other MMPs, have also been shown to be upregulated in injured ACL tissue (Tang *et al*., 2009).

As well as providing a useful model for translation of research into humans, investigating canine CCL injuries and degeneration is an important area of research in itself due to the prevalence of CCL injuries in the canine population. CCL rupture is the predominant cause of canine lameness (Wilke *et al*., 2006), and since 1970 the number of reported CCL injuries has tripled (Witsberger *et al*., 2008). This may be due to a number of factors, including improvements in veterinary medicine leading to an increased lifespan in dogs. Advancing age can lead to CCL disease as CCL degeneration has been found in some studies to increase with age (Witsberger *et al*., 2008). In addition, neutering female dogs has been suggested as a factor in the number of CCL injuries, and the number of these procedures performed has increased over the years (Whitehair *et al*., 1993; Witsberger *et al*., 2008). Obesity is a growing trend in the canine population and increased body weight applies more pressure to the stifle joint leading to CCL degeneration and rupture (Adams *et al*., 2011) which again may account for the increase in the prevalence of CCL disease. Finally, the genetic predisposition of certain dog breeds to CCL rupture may be

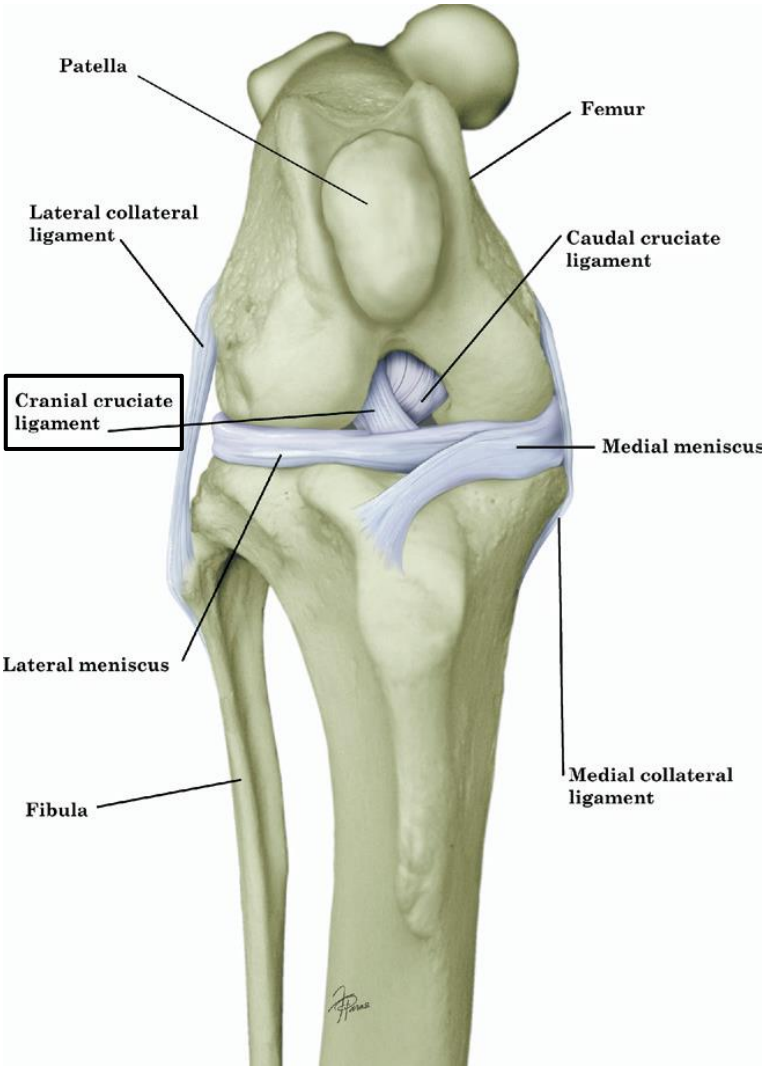


Figure 1.4. Schematic diagram of a canine stifle joint, showing the location of the cranial cruciate ligament (CCL). Adapted from (Canapp Jr, 2007).

exacerbated by inbreeding (Baird *et al*., 2014a; Baird *et al*., 2014b). For example the Rottweiler, Newfoundland, Labrador and Staffordshire terrier are all particularly high risk breeds, with CCL rupture rates being as high as 22% (Wilke *et al*., 2006).

As with humans, the treatment options for canine CCL injury can be unrewarding and they include rest, rehabilitation, anti-inflammatories, glucosamine supplements, ultrasound therapy, shockwave therapy as well as surgical options which are often associated with short and long term side effects such as osteoarthritis (Bergh *et al*., 2014; Duerr *et al*., 2014; Moles *et al*., 2009; Mölsä *et al*., 2014; Wolf *et al*., 2012). Therefore, alternative therapies are currently being sought. Amongst other things, stem cell therapies are under investigation. MSCs have been shown to successfully engraft on to injured CCL tissue with no adverse side effects (Linon *et al*., 2014), however to date only safety studies have been conducted and no studies investigating efficacy have yet been published. With the identification of stem cells in canine periodontal ligament (Chang *et al*., 2009), it is possible that future research and treatment strategies could utilise LDSCs, rather than MSCs, as LDSCs derived from canine periodontal ligament have been used successfully to treat periodontal ligament damage (Lee *et al*., 2014b) and are a readily accessible source of cells.

1.6 The stem cell niche

The stem cell environment or 'niche' has been found to be integral for stem cell survival and viability in a range of stem cell populations and sites (Calvi *et al*., 2003; Garcion *et al*., 2004). The stem cell niche consists of a number of different factors, including ECM composition, cellular interactions as well as secreted and mechanical factors (Lane *et al*., 2014) (Fig.1.5). For example, neural stem cells are regulated by several ECM proteins including tenascin C, which orchestrates neural stem cell development (Garcion *et al*., 2004). The structure of the ECM has also been shown to contribute to neural stem cell development, with the presence of fractones, branched ECM structures, being necessary for stem cell function (Kerever *et al*., 2007) (Fig.1.6.A). These findings indicate that both the composition and the

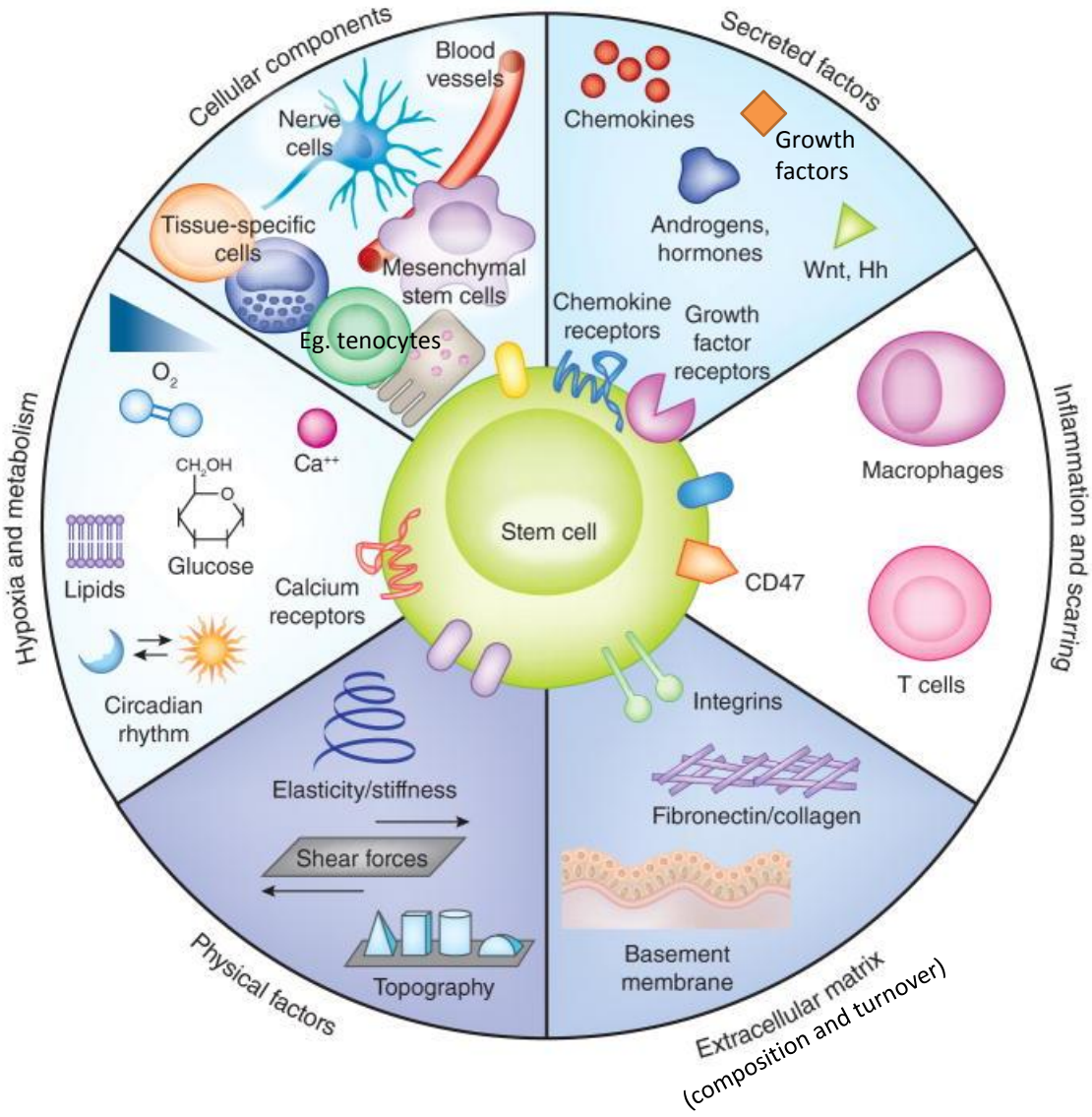


Figure 1.5. The various factors affecting the stem cell niche. Adapted from (Lane *et al*, 2014).

structure of the ECM are crucial for stem cell fate. In addition to the ECM, resident cells within the niche can influence stem cell survival and behaviour. For example, in the haematopoietic stem cell niche osteoblasts release biological mediators, including angiopoietin, which have an impact on stem cell number and quiescence (Calvi *et al*., 2003). Other mediators and growth factors may be transported to the niche via the vasculature, as seen in the haematopoietic stem cell niche where the presence of blood vessels is vital (Nakamura-Ishizu and Suda, 2013) (Fig.1.6.B).

These studies emphasise the importance of the stem cell niche for stem cell survival and function, which suggests that the tendon and ligament stem cell niche may also play a vital role in tendon and ligament stem cell survival and development.

1.6.1 Tendon stem cell niche

The importance of the stem cell niche in tendon tissue has been highlighted by several studies that have used tendon extracts to induce tenogenic differentiation of MSCs derived from non-tenogenic tissue (Voss *et al*., 2016; Yang *et al*., 2013). Bovine tendon ECM was extracted and added to human MSCs which displayed increased tenogenic differentiation and also increased the mechanical strength of the tendon ECM/MSC constructs when compared with controls (Yang *et al*., 2013). Similarly, MSCs were extracted from human bone marrow and grown on human rotator cuff tendon which promoted tenogenic differentiation of the cells (Voss *et al*., 2016). These studies emphasise the importance of the tendon environment and ECM in tenogenic differentiation, suggesting a positive role of the tendon stem cell niche in TDSC viability (Fig.1.7).

1.6.1.1 Extracellular matrix

As previously discussed the tendon ECM has been shown to promote tenogenic differentiation in non-tenogenic cell lines. Bi and others (2007) have shown that the tendon ECM also promotes the tenogenic differentiation of TDSCs

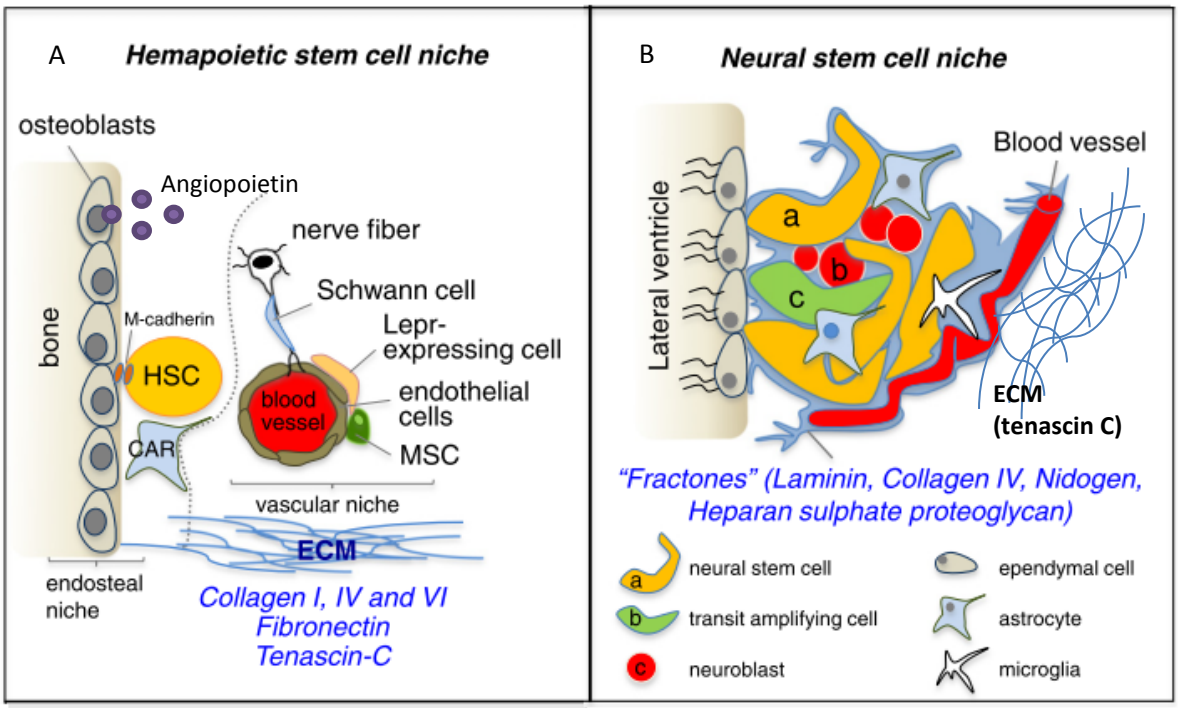


Figure 1.6. Schematic diagrams of the neural (A) and haematopoietic (B) stem cell niche. Adapted from (Gattazzo *et a*l., 2014).

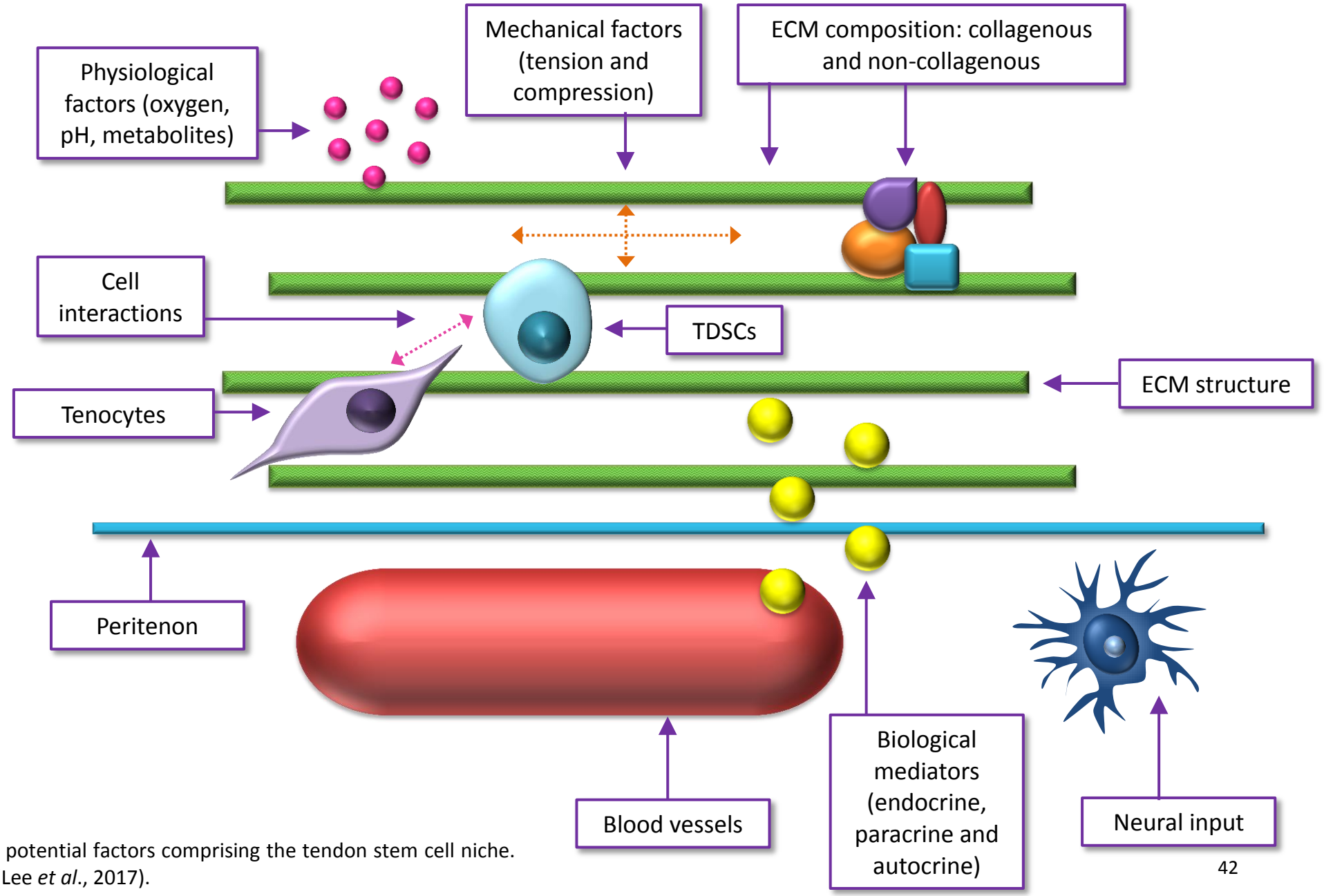


Figure 1.7. The potential factors comprising the tendon stem cell niche. Adapted from (Lee *et al*., 2017).

(Bi *et al*., 2007). The study analysed certain components of the TDSC niche and the effects of removing those components on TDSC characteristics, in particular they investigated two components of the tendon ECM; fibromodulin (Fmod) and biglycan (Bgn). Bi and others (2007) noted that the TDSCs were located between the collagen fibrils and surrounded by ECM suggesting that the ECM was involved in TDSC development and survival. They hypothesised that alterations in the TDSC niche could lead to dysregulation of TDSCs. Genetic inactivation of Fmod and Bgn resulted in abnormal tendon development with the tendon being thinner and more cellular than wild-type mice (Bi *et al*., 2007). The number of TDSC colonies and the proliferation rate increased, however their capacity to differentiate into tenocytes was decreased with reduced expression of tenogenic markers such as scleraxis. This was also evidenced by the reduction in collagen production and the disorganisation of the small amount of collagen produced. The lack of Fmod and Bgn also led to ossification of tendon tissue and markers of osteogenic and chondrogenic cell lineages were also identified (Bi *et al*., 2007). Bi and others (2007) have demonstrated that the absence of fibromodulin and biglycan from the TDSC niche increased stemness and non-tenogenic differentiation, however tenogenic differentiation was reduced, highlighting the role of these two ECM components in promoting tenogenic differentiation.

Two recent studies have also shown similarly that the TDSC ECM is beneficial for TDSC function (Yin *et al*., 2013; Zhang *et al*., 2011a). The use of decellularised tendon matrices increased both the stemness of TDSCs and the proliferation rate when compared with control cells grown on plastic culture surfaces, as well as increasing expression of stem cell markers and multipotency (Zhang *et al*., 2011a). TDSCs grown on porcine decellularised tendon matrices also had the capacity to repair a rat Achilles tendon injury with the quality of the repaired tendon tissue being greater with the use of TDSCs grown on the matrix, as determined by increased collagen deposition, improved fibre structure and arrangement, fewer inflammatory cells as well as increased stiffness and maximum force (Yin *et al*., 2013).

The structure of the ECM also affects the function of TDSCs, as one study found that aligned nanofibres encouraged the growth of spindle-shaped cells which

were able to produce tendon tissue *in vivo* (Yin *et al*., 2010). In addition, TDSCs grown on nanofibres were able to differentiate into tenocytes and had a higher expression of tenogenic markers than cells grown on randomly orientated nanofibres. TDSCs grown on randomly orientated nanofibres differentiated into osteocytes, however the aligned nanofibres prevented this, indicating that the correct ECM structure is vital for TDSC function (Yin *et al*., 2010).

1.6.1.2 Oxygen tension

As discussed previously, hypoxia has been shown to promote TDSC proliferation, however its effects on multipotency are varied (Lee *et al*., 2012; Zhang and Wang, 2013b). It is likely that tendon has a low oxygen tension *in vivo* as it is poorly vascularised (Franchi *et al*., 2007), therefore it would be expected that a hypoxic environment would promote TDSC viability *in vitro.* Despite some discrepancies within the literature it seems clear that TDSCs cultured in a hypoxic environment show increased stem cell characteristics.

1.6.1.3 Biological mediators

There are certain biological mediators that have an impact on TDSCs and their survival and function, for example BMP-2 (Lui and Wong, 2013; Rui *et al*., 2013b). BMP-2 stimulated adipogenic, osteogenic and chondrogenic differentiation and inhibited tenogenic differentiation of rat TDSCs. BMP-2 also promoted glycosaminoglycan (GAG) deposition and aggrecan expression however the expression of other ECM components, such as fibromodulin, decorin and biglycan, were inhibited by BMP-2 (Rui *et al*., 2013b). BMPs bind to cell surface receptors which trigger a signalling pathway involving a Smad complex, an intracellular signalling protein complex (Lui and Wong, 2013). A recent study found that in tendon injury the sensitivity of the BMP/Smad pathway was increased and there was increased expression of several BMPs, including BMP-2 (Lui and Wong, 2013). Insulin-like growth factor-1 (IGF-1) has been found to induce adipogenic differentiation of TDSCs through PI3K/Akt pathway signalling and PPARγ2 activation (Liu *et al*., 2014a), however this effect of IGF-1 only occurs in the presence of BMP-2. Similarly, the study also noted that BMP-2 mediated adipogenic differentiation

only occurs in the presence of IGF-1 (Liu *et al*., 2014a). Therefore, it appears that expression of both BMP-2 and IGF-1 is required for adipogenic differentiation of TDSCs and the overexpression of these proteins may be a cause of tendinopathy. Connective tissue growth factor (CTGF) has been shown to select for CD146+ TDSCs and enrich this cell population (Lee *et al*., 2015a). The selected cells exhibited normal TDSC characteristics and successfully contributed to tendon regeneration in rats with increased alignment of collagen fibres and tensile stiffness seen with the use of CTGF, compared with controls. In addition, CTGF stimulated TDSC proliferation and tenogenic differentiation causing such effects via FAK and ERK1/2 signalling (Lee *et al*., 2015a). Another study has shown a link between CTGF and BMP-12 signalling where CTGF has been found to promote tenogenic differentiation of TDSCs and BMP-12 has also been shown to promote tenogenesis, with both proteins increasing TDSC expression of scleraxis, tenomodulin and type I collagen. The study also demonstrated the interaction between BMP-12 and CTGF in causing these effects (Liu *et al*., 2015a). As discussed previously both EGR-1 (Tao *et al*., 2015) and FGF-2 (Tokunaga *et al*., 2015) also promote tenogenic differentiation of TDSCs.

1.6.1.4 Tenocytes

The predominant cell type within tendon is the tenocyte (tendon fibroblast) and tenocytes are responsible for producing and maintaining the ECM (Birch *et al*., 2013). They therefore have an important role in the survival and function of TDSCs, however it is possible that tenocytes have a direct effect on TDSCs through cell-cell contact or through the secretion of growth factors and other soluble mediators. Although no studies have shown such a link, the use of tenocytes to promote tenogenic differentiation of other stem cell types has been extensively studied. Tenocytes have stimulated tenogenic differentiation of human MSCs (Kraus *et al*., 2013), canine MSCs (Schneider *et al*., 2011), rat MSCs (Luo *et al*., 2009) and ovine amniotic epithelial cells (Barboni *et al*., 2012). These studies utilised a range of techniques involving both direct and indirect contact of the stem cells and tenocytes indicating that tenocytes may release biological factors that promote

tenogenic differentiation. The importance of cell-cell contact between TDSCs and tenocytes has yet to be determined.

1.6.1.5 Mechanical loading

Physical factors also play an important role in tendon development and TDSC function. The application of mechanical load to rabbit tendon tissue increased TDSC proliferation with higher proliferation rates seen with increased load. Mechanical stretching of 4% promoted tenogenic differentiation of TDSCs, however mechanical stretching of 8% also induced adipogenic, osteogenic and chondrogenic differentiation (Zhang and Wang, 2010d) indicating that extreme mechanical loads can be detrimental to TDSC function. Two studies have investigated the role of exercise on TDSC function with the use of a treadmill model in mice (Zhang *et al*., 2010; Zhang and Wang, 2013a). The first study found that TDSC proliferation was increased in mice that had been subjected to exercise compared with control caged mice. In addition, they found that collagen production by TDSCs was increased after exercise compared with control mice (Zhang *et al*., 2010). The second study used varying levels of exercise, with moderate exercise promoting tenogenic differentiation, and intensive exercise promoting both tenogenic and non-tenogenic differentiation (Zhang and Wang, 2013a). The study by Zhang and Wang (2013a) supports the theory that moderate exercise and mechanical loading is beneficial to tendon whereas intensive exercise and loading can be harmful. A possible cause for non-tenogenic differentiation of TDSCs at high mechanical loads may be due to the increased expression of BMP-2 seen during mechanical stimulation (Rui *et al*., 2011c). Other signalling pathways have also been implicated in non-tenogenic differentiation of TDSCs after mechanical loading. Wnt5a-RhoA signalling (Shi *et al*., 2012b) as well as Wnt5a/Wnt5b/JNK signalling (Liu *et al*., 2015b) have been shown to be involved in osteogenic differentiation of TDSCs after uniaxial mechanical tension which may be the cause of tendon ossification in athletes. In addition, the upregulation of tendon matrix proteins seen in human tendon after 8% mechanical stimulation of TDSCs is regulated by ERK1/2 kinases and p38 (Popov *et al*., 2015). It is clear that mechanical loading has a significant impact on TDSC function and this is mediated via a complex array of signalling pathways.

1.6.1.6 Vasculature and innervation

As discussed previously the vascularisation and innervation of tendon varies considerably between species, tendon types and location in the tendon. However, it is likely that these factors play a role in the tendon stem cell niche, particularly the vasculature, as blood vessels provide a steady flow of oxygen and biological mediators to the peritenon. It has also been proposed that the vasculature may actually be a source of TDSCs, as perivascular cells from the supraspinatus tendon expressed stem cell, haematopoietic and tenogenic markers (Tempfer *et al*., 2009). In contrast, other studies have shown null expression of haematopoietic and endothelial markers in TDSCs (Bi *et al*., 2007; Rui *et al*., 2010) and one group has shown that TDSCs isolated from poorly vascularised tendon show enhanced stem cell properties compared with cells from the highly vascularised paratenon (Mienaltowski *et al*., 2013). Therefore, it is possible that there are two sources of TDSCs within tendon suggestive of an important role of the vasculature within the stem cell niche.

1.6.2 Ligament stem cell niche

There is currently very little known about the ligament stem cell niche, however as ligament is structurally and functionally similar to tendon (Benjamin and Ralphs, 1998; Birch *et al*., 2013; Rumian *et al*., 2007) it is likely that those niche factors which are essential for TDSC viability are also necessary in the ligament stem cell niche. As with the majority of research focussing on LDSCs, studies investigating the ligament stem cell niche have mainly focussed on periodontal ligament.

1.6.2.1 Extracellular matrix

The composition and structure of the ECM have been found to have significant effects on various stem cells and some studies also indicate that the ECM has a large impact on LDSC viability and function (Chockalingam *et al*., 2013; Lee *et al*., 2014b; Yang *et al*., 2009). When human PDLSCs embedded in endogenous ECM were implanted into SCID mice they formed a cementum-PDL like structure,

indicative of new tooth growth (Yang *et al*., 2009). PDLSC tissue engineering approaches have also used a number of different ECM-based scaffolds such as fibronectin which have proven successful in repairing periodontal ligament tissue *in vivo* (Lee *et al*., 2014b). Tenascin-C, an ECM protein found in various stem cell niches, has been shown to be increased in human and canine synovial fluid after injury to the joint (Chockalingam *et al*., 2013). It is possible that this ECM protein has a role in stem cell viability but also in tissue degeneration. As seen in tendon (Ni *et al*., 2012; Rui *et al*., 2013a), stem cells in ligament may promote healing of acute injury, however they may contribute towards further degradation through erroneous differentiation in chronic injuries. Tenascin-C may be a cause or an effect of such tissue degeneration.

1.6.2.2 Oxygen tension

As discussed previously, hypoxia has been shown to promote cell proliferation and the expression of stem cell markers as well as promoting differentiation of human PDLSCs into osteogenic, adipogenic and chondrogenic cell lines (Yu *et al*., 2016; Zhou *et al*., 2014).

1.6.2.3 Biological mediators

The presence and quantity of certain biological mediators also has an impact on LDSC properties (Cheng *et al*., 2014b; Li *et al*., 2010; Yu and Wang, 2014). For example, the presence of platelet-derived growth factor (PDGF), nerve growth factor and all-*trans* retinoic acid cause the differentiation of PDLSCs into Schwann cells (Li *et al*., 2010), which is mediated by Erk1/2 signalling (Dapeng *et al*., 2014). These studies indicate that the presence of these growth factors in the ligament stem cell niche could cause erroneous differentiation. In contrast, a study investigating the effects of growth factors on the viability of canine PDLSCs found that the cocktail of endogenous growth factors isolated from whole blood promoted proliferation and osteogenic differentiation of PDLSCs. The cocktail included PDGF, transforming growth factor-beta (TGF-β) and BMP, however the exact composition was not stated (Yu and Wang, 2014). More specifically BMP-7 (Torii *et al*., 2014) and -9 (Ye *et al*., 2014) have been found to induce

cementogenesis/osteogenesis of human PDLSCs in other studies. For stem cells derived from ACL certain media formulations and the presence of specific growth factors, for example FGF and TGF-β1, influence various stem cell properties including proliferation and multipotency (Cheng *et al*., 2014b). ACLDSCs transfected with BMP-12 and -13 demonstrate increased ECM production (Haddad-Weber *et al*., 2010) indicating the importance of these mediators in tissue homeostasis.

1.6.2.4 Neighbouring cells

Ligamentocytes (ligament fibroblasts) are the predominant cell type within ligament and they are responsible for maintenance of ECM homeostasis (Frank, 2004). As discussed previously, components of the ECM are vital for the survival and function of LDSCs, therefore ligamentocytes are an integral part of the LDSC niche. It is possible that ligamentocytes have a direct effect on LDSCs and although no studies have shown such a link, the use of ligamentocytes to promote ligamentogenic differentiation of MSCs has been studied. Bone marrow-derived MSCs have been shown to differentiate into ACL cells when indirectly co-cultured with ACL fibroblasts in combination with mechanical stimulation (Lee *et al*., 2007). This influence of ACL fibroblasts on MSC differentiation may indicate their role in LDSC differentiation. Other cell types have been shown to influence LDSC survival and function, for example in inflammatory environments PDLSC function may be impaired and this loss of function appears to be rescued by the addition of dental follicular cells (Liu *et al*., 2014b). These findings suggest that certain cell types may play important roles within specific ligament stem cell niche environments.

1.6.2.5 Vasculature and innervation

As with tendon, ligament is poorly vascularised and innervated, however it is likely that these two factors still play a role within the stem cell niche. As mentioned above, one study found CD34+ stem cells within human ACL tissue, which possess all the properties found in other LDSCs (Matsumoto *et al*., 2012). The study suggested that LDSCs may be derived from pericytes and the vasculature. Therefore, as a possible source of LDSCs, the vasculature may be a vital part of the ligament stem cell niche. However other studies have shown no expression of

haematopoietic and endothelial markers in LDSCs (Cheng *et al*., 2009; Singhatanadgit *et al*., 2009). Similar to tendon it is possible that there are two sources of LDSCs.

1.6.3 Therapeutic manipulation of the stem cell niche

Manipulation of stem cells *in vivo* via modulation of the stem cell niche holds therapeutic potential for the treatment of a number of conditions. Although stem cell therapies show promising results and successful outcomes, there are a number of issues associated with cell transplantation therapies (Lane *et al*., 2014). The use of autologous stem cells and the associated isolation, culture and implantation of cells can be time consuming and expensive often involving invasive and painful techniques in order to extract cells from the patient (Corso *et al*., 2013). Allogeneic stem cells can be used to avoid some of these issues, however immunosuppression is then required which can result in problematic long-term side effects (Correa *et al*., 2016; Luo *et al*., 2016). In addition, whether autologous or allogeneic cells are used, the survival of these cells when introduced into wounds or diseased tissue is low (Farrar *et al*., 2013; Lane *et al*., 2014). There are also problems associated with the culture of these cells, as phenotypic changes occur when cells are cultured *in vitro* (Mueller *et al*., 2016).

An alternative to stem cell transplantation therapies is modulation of stem cells *in vivo,* which can be achieved via manipulation of the stem cell niche. This manipulation can promote survival, proliferation and differentiation of stem cells. Costly and time consuming laboratory processes could be avoided as well as invasive surgical techniques and the requirement for immunosuppressants. Several clinical trials are currently underway or have recently been completed investigating the safety and efficacy of stem cell niche modulation therapies (Cianfarani *et al*., 2006; Olnes *et al*., 2012). For example, the administration of a thrombopoietin mimetic to patients with severe aplastic anaemia has been shown to increase HSC numbers as well as differentiation of these cells (Olnes *et al*., 2012; Qian *et al*., 2007), demonstrating the benefit of targeting secreted factors for stem cell niche

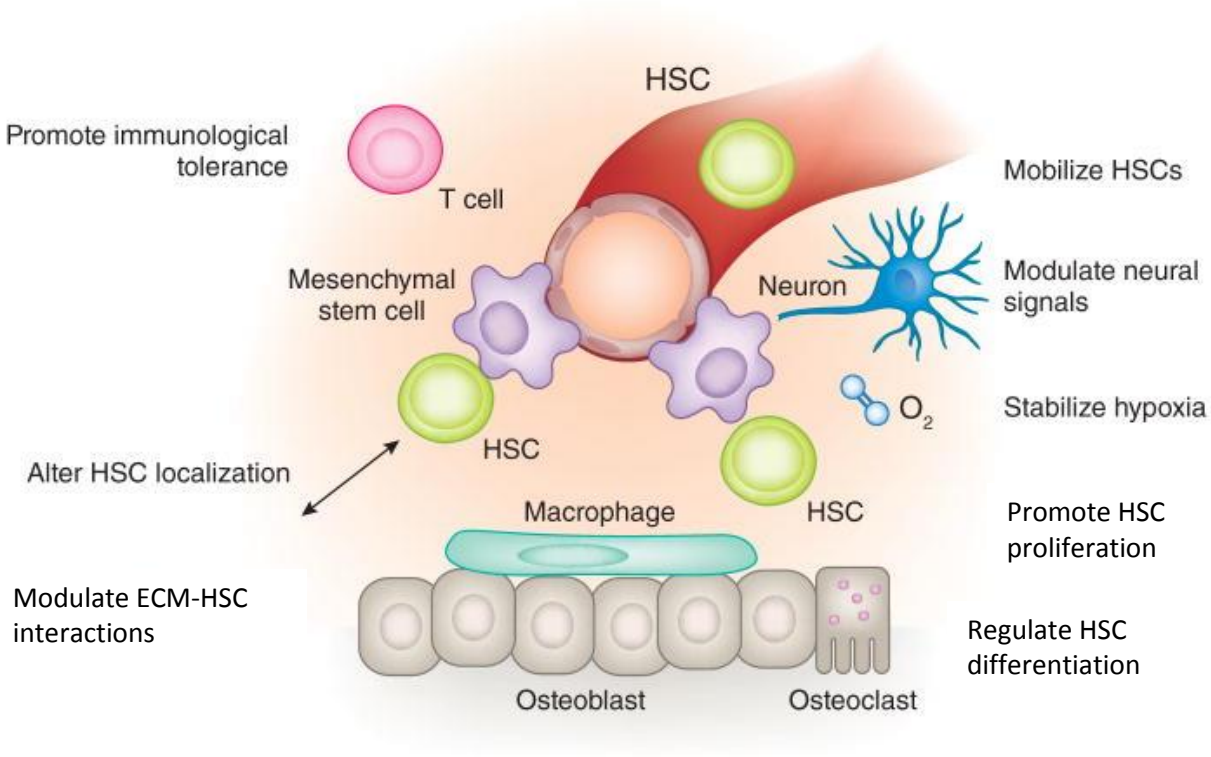


Figure 1.8. Potential targets for the manipulation of the haematopoietic stem cell niche. Adapted from (Lane *et al* 2014).

modulation. Other potential targets for the manipulation of the HSC niche are of shown in Fig.1.8. Granulocyte/macrophage colony stimulating factor (GM-CSF) has been shown to regulate survival, proliferation and differentiation of a number of stem cell types. When injected into chronic skin ulcers GM-CSF causes modulation the stem cell niche by increasing vascularisation (Cianfarani *et al*., 2006), indicating the potential advantages of targeting other cell types within the stem cell niche.

Therefore it is possible that modulation of the tendon and ligament stem cell niche could also influence the behaviour of tendon and ligament stem cells *in vivo* and provide potential therapeutic benefits. As the quantity of research surrounding TDSCs, and to a lesser extent LDSCs, increases, it may be possible to identify specific factors within their stem cell niche which can be targeted to produce a desired change in phenotype and/or function. This approach could provide a new treatment strategy to treat tendon and ligament injury and degeneration.

1.7 Summary

Tendon- and ligament-derived stem cells have been well characterised in certain species for certain tendon/ligament types, however there are still large gaps in the knowledge base. Previous studies have highlighted differences in TDSCs/LDSCs between species and tendon/ligament types, therefore further research into different species and tendon/ligament types is necessary. In addition, clarification of tendon/ligament markers is vital to enable consistency in cell isolation and identification as well as to aid the use of stem cells in tendon and ligament repair. The stem cell niche is essential for maintenance and function of TDSCs/LDSCs and holds potential for treatment of tendon/ligament injuries and degeneration, however further characterisation of the niche is needed.

1.8 Hypothesis

This study will address the hypothesis that stem cells reside within equine superficial digital flexor tendon and canine cranial cruciate ligament and such cells possess traditional MSC properties, however they differ in phenotype to native tissue fibroblasts. It is further hypothesised that the ECM composition of the niche differs between stem cells and native tissue fibroblasts.

1.9 Aims

1. To characterise equine TDSCs and tenocytes isolated from the SDFT; including analysis of stem cell and tenogenic marker expression, colony-forming ability, cell proliferation and multipotency. Further, to compare the differences in phenotype between TDSCs and tenocytes.
2. To characterise canine LDSCs and ligamentocytes isolated from the CCL; including analysis of stem cell and tenogenic marker expression, colony-forming ability, cell proliferation and multipotency. Further, to compare the differences in phenotype between LDSCs and ligamentocytes.
3. To characterise the TDSC/LDSC and tenocyte/ligamentocyte niche; including analysis of gene and protein expression of ECM components. Further, to compare the differences in niche composition between TDSCs/LDSCs and tenocytes/ligamentocytes.

Chapter 2

Materials and Methods

2.1 Materials

Materials are listed below and grouped by supplier:

Thermo Fisher (Massachusetts, USA): DMEM, trypsin, amphotericin B, L-glutamine, penicillin/streptomycin, glycoblue, TE buffer, DNase I, 10 x DNase I reaction buffer, phenol:chloroform:IAA, ammonium acetate, Novex SDS sample buffer, Novex MES running buffer, Novex transfer buffer, NuPage Bis-Tris (4-12%) precast gels

Sigma: foetal calf serum, phosphate-buffered saline, goat serum, donkey serum, dexamethasone, β-glycerophosphate, ascorbic acid, indomethacin, human insulin, IBMX, ITS+3, alizarin red, oil-red-O, alcian blue, safranin-O, SigmaFast BCIP/NBT tablets, tween 20, isopropanol, acetic acid, ethanol, papain, sodium acetate, EDTA, L-cysteine, chondroitin sulphate C, 1-9 dimethyl methylene blue, sodium formate, formic acid, xylene, tri-reagent, BCP, sodium chloride, tris-HCl, β-apn, β-mercaptoethanol, methanol, paraformaldehyde, sodium hydroxide, hexane, hydrogen peroxide, trizma base, DAB 3,3'-diaminobenzidine tablets, haematoxylin, DPX mounting medium, HRP-conjugated goat anti-mouse secondary antibody, crystal violet solution, gluteraldehyde, SILAC media, ammonium bicarbonate, [^{12}C]lysine, [^{12}C]arginine, [^{12}C]proline, [^{13}C]lysine, [^{13}C]arginine, [^{13}C]proline, BCA protein assay, 660nm protein assay, GelCode stain, Silver Stain kit

Worthington Biochemicals (New Jersey, USA): collagenase type 1 and 2

PeproTech (New Jersey, USA): FGF-2, TGF-β

Millipore (Darmstadt, Germany): human fibronectin, Accutase

Abcam (Cambridge, UK): anti-CD90 antibody, anti-fibronectin antibody, anti-collagen I antibody, anti-collagen II antibody, anti-collagen VI antibody, HRP-conjugated goat anti-rabbit secondary antibody

AbD Serotec (California, USA): anti-CD105 antibody

BD Pharmingen (New Jersey, USA): anti-CD73 antibody

Santa Cruz (Texas, USA): anti-tenascin C antibody

Jackson ImmunoResearch (Pennsylvania, USA): FITC-conjugated donkey anti-mouse secondary antibody

Promega (Wisconsin, USA): random primers, M-MLV reverse transcriptase, M-MLV reverse transcriptase 5x buffer, RNAsin plus, GoTaq qPCR master mix, dNTP mix

Eurogentec (Liège, Belgium): qPCR primers

PerkinElmer (Massachusetts, USA): [^{14}C]lysine, [^{14}C]arginine, [^{14}C]proline, [^{14}C]molecular weight marker, nitrocellulose membrane, Western Lightnening-Plus ECL chemoluminescence solution

Roche (Basel, Switzerland): mini protease inhibitor cocktail with EDTA

GE Healthcare (Illinois, USA): Ficoll-Paque Plus

Cell Path (Newtown, Wales): OCT embedding matrix

Waters (Massachusetts, USA): Rapigest SF Surfactant

2.2 Methods

2.2.1 Cell isolation

2.2.1.1 Isolation of equine tendon-derived stem cells (TDSCs) and tenocytes

Equine superficial digital flexor tendon (SDFT) (Fig.2.1) was harvested from equine cadavers obtained from the abattoir. Tendon tissue was grossly normal upon post-mortem examination. The mid-substance tendon tissue (Fig.2.2) was dissected into chunks and digested overnight at 37°C in 125 U/ml collagenase type II. The resulting cell suspension was strained and then centrifuged at 1200 g for 10 minutes and the supernatant discarded. The cells were resuspended in complete DMEM (DMEM supplemented with 10% foetal calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin and 2 µg/ml amphotericin B) and counted using a haemocytometer. The same batch of foetal calf serum was used for all experiments (113M 3395, Sigma). For tenocyte isolation the cells were seeded at $1x10^6$ cells in T75 culture flasks ($1.3x10^4$ cells/cm^2) and for TDSC isolation the cells were seeded at 100 cells per well of a 6-well plate (10 cells/cm^2). The cells were cultured at 37°C, 5% CO_2 and either 21% or 5% O_2 for 10-12 days until they formed colonies. The colonies were isolated using cloning cylinders and local application of trypsin and transferred to T25 culture flasks. Alternatively TDSCs were isolated using differential adhesion to fibronectin substrates. For differential adhesion cells were seeded after digestion at 12,000 cells per well of a 6-well plate (1200 cells/cm^2), previously coated with 20 µg/ml human fibronectin, and the media (complete DMEM + 5 ng/ml FGF-2) replaced after 20 minutes. After 6-8 days the cells were confluent and transferred to a T25 culture flask. Cells were used at passage 2-3 for all experiments.

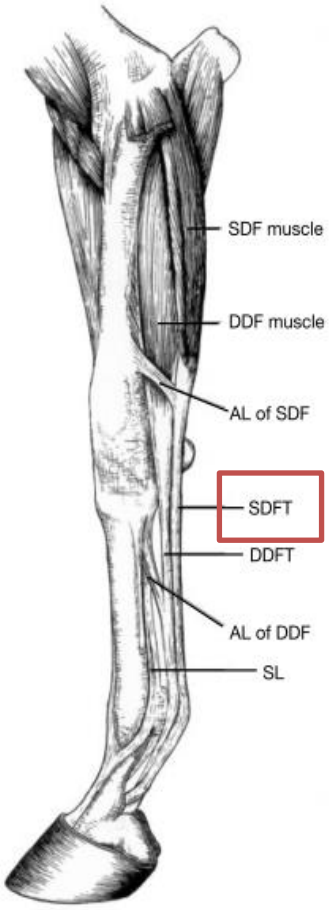


Figure 2.1. Schematic diagram of an equine forelimb indicating the location of the SDFT. Adapted from (Wilson *et al*., 2001).

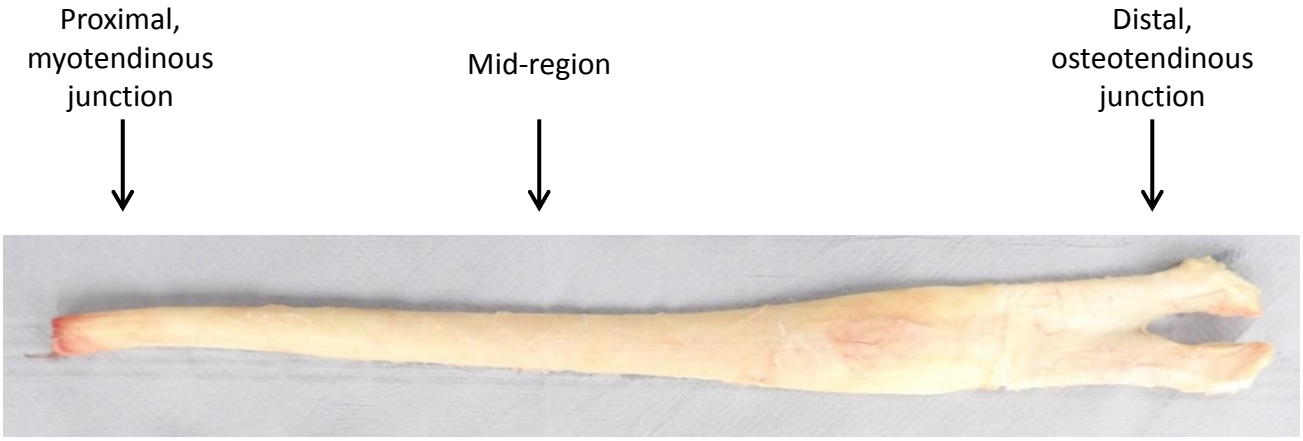


Figure 2.2. Equine SDFT indicating the different regions: proximal, middle and distal.

2.2.1.2 Isolation of murine tendon-derived stem cells (TDSCs), tenocytes and MSCs

Murine tails (C57BL/6; genotype: G4 CTGF x HuRfl, HuRErt2luc, HuRPchu94lacZ; HuRErt2luc, Fl+/+, Cre +/-) were donated by Dr. Simon Tew and Kirsty Johnson (University of Liverpool) as spare tissue from other projects. The tendon tissue was extracted from the tail and digested for 3 hours at 37°C in 20 ml 375 U/ml collagenase type I and 1 x trypsin. The resulting cell suspension was strained and then centrifuged at 1200 g for 10 minutes and the supernatant discarded. The cells were resuspended in complete DMEM (DMEM supplemented with 10% foetal calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin and 2 µg/ml amphotericin B) and counted using a haemocytometer. The same batch of foetal calf serum was used for all experiments (113M 3395, Sigma). For tenocyte isolation the cells were seeded at $1x10^5$ cells in T25 culture flasks ($4x10^3$ cells/cm^2) and for TDSC isolation the cells were seeded at 100 cells per well of a 6-well plate (10 cells/cm^2). The cells were cultured in complete DMEM + 20% FBS at 37°C, 5% CO_2 and 21% O_2 for 10-12 days until they formed colonies. The colonies were isolated using cloning cylinders and local application of trypsin and transferred to T25 culture flasks. Cells were used at passage 2-3 for all experiments. Murine MSCs were purchased from Cyagen, cells were isolated from bone marrow of C57BL/6 mice and used at passage 8-9.

2.2.1.3 Isolation of canine ligament-derived stem cells (LDSCs) and ligamentocytes

Canine cranial cruciate ligament (CCL) was harvested from the stifle joint (Fig.2.3-2.6) of canine cadavers which were euthanased for purposes not related to this study and were clinical waste material donated to the University of Liverpool. Ethical approval for use of this material in this project was granted by the local ethics committee (VREC159). The tissue was dissected into chunks and digested overnight at 37°C in 125 U/ml collagenase type II. The resulting cell suspension was

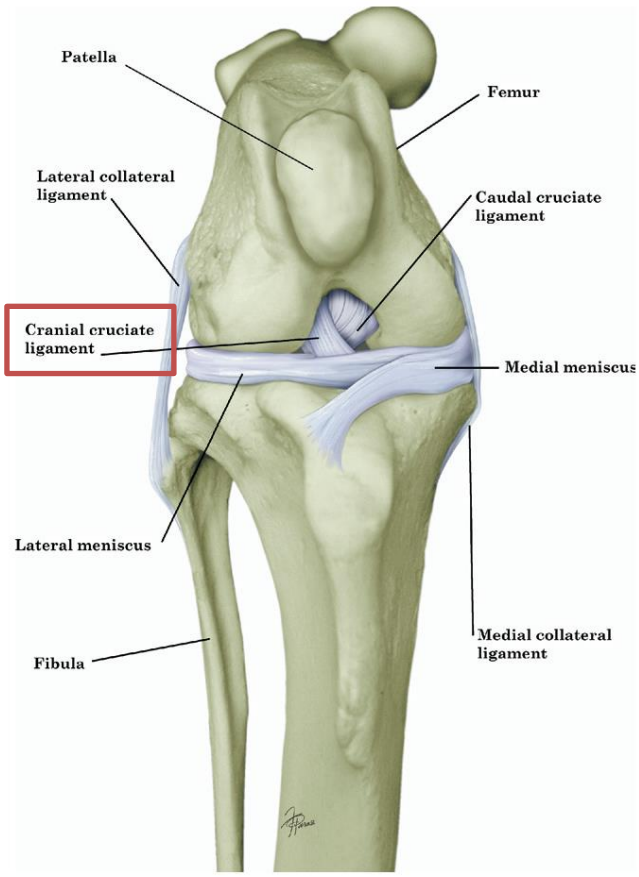


Figure 2.3. Schematic diagram of a canine stifle joint indicating the location of the CCL. Adapted from (Canapp Jr, 2007).

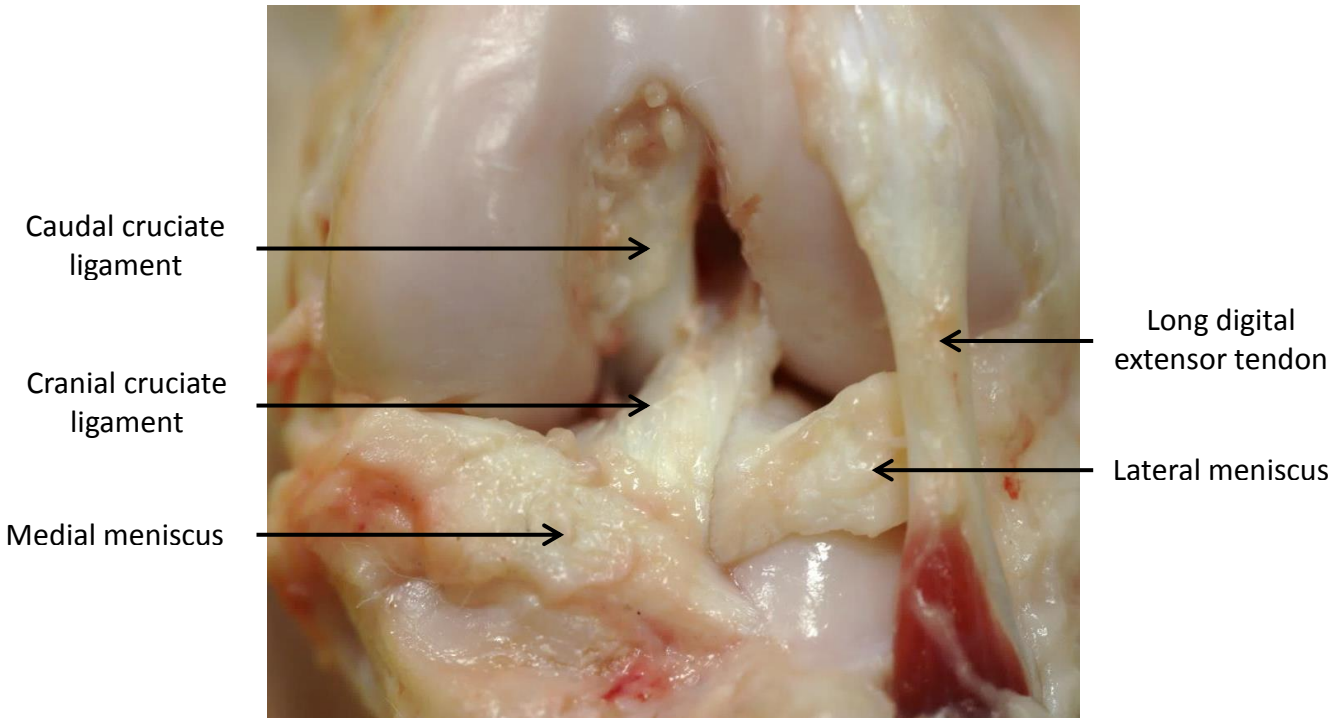


Figure 2.4. Enlarged image of a canine stifle joint highlighting the location of the major ligaments and tendons.

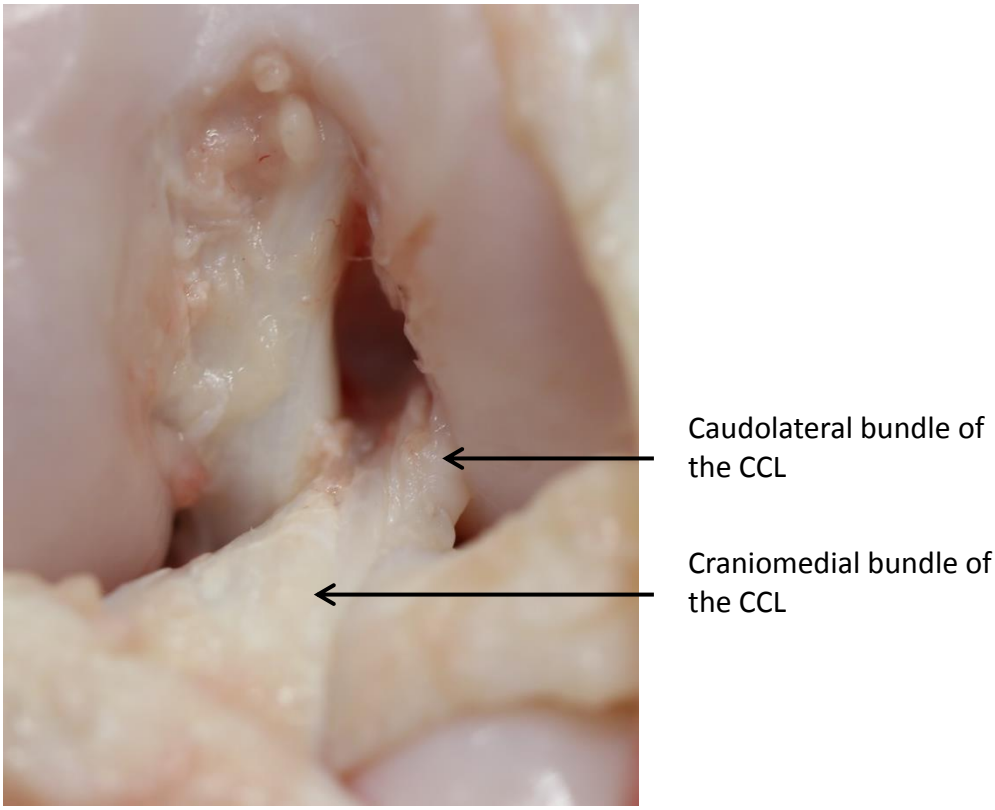


Figure 2.5. Canine stifle joint indicating the location of the caudolateral and craniomedial bundles of the CCL.

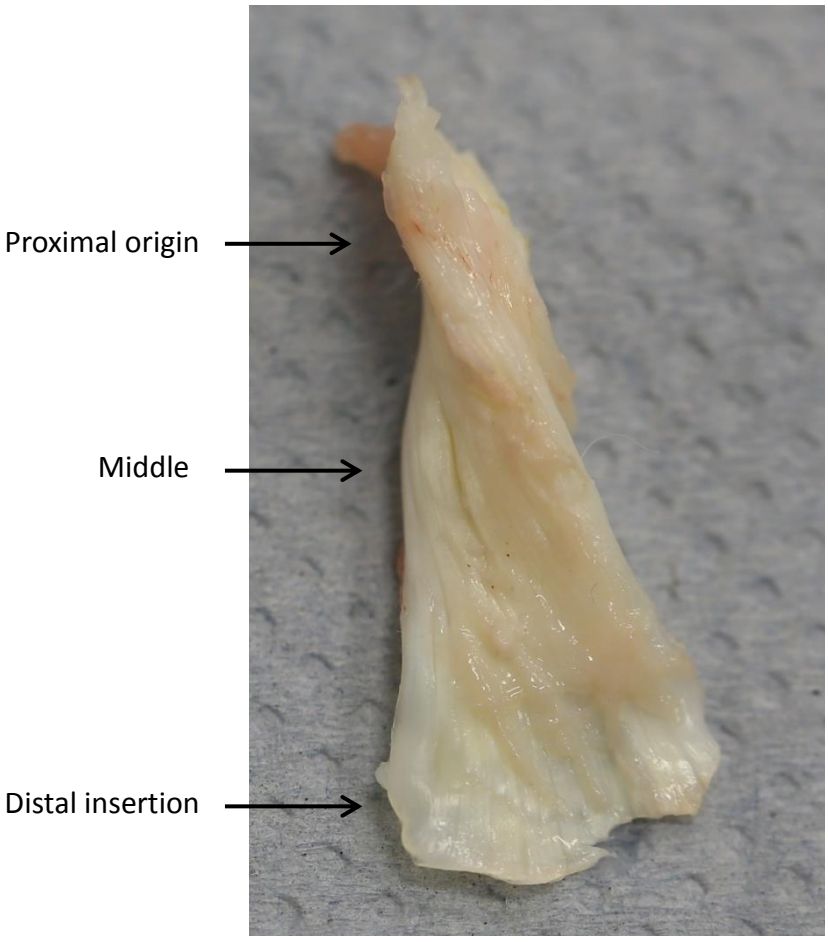


Figure 2.6. Canine CCL indicating the different regions: origin, middle and insertion.

strained and then centrifuged at 1200 g for 10 minutes and the supernatant discarded. The cells were resuspended in complete DMEM (DMEM supplemented with 10% foetal calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin and 2 µg/ml amphotericin B) and counted using a haemocytometer. The same batch of foetal calf serum was used for all experiments (113M 3395, Sigma). For ligamentocyte isolation the cells were seeded at $1x10^6$ cells in T75 culture flasks ($1.3x10^4$ cells/cm^2) and for LDSC isolation the cells were seeded at 100 cells per well of a 6-well plate (10 cells/cm^2). The cells were cultured at 37°C, 5% CO_2 and 5% O_2 for 10-12 days until they formed colonies. The colonies were isolated using cloning cylinders and local application of trypsin and transferred to T25 culture flasks. Alternatively LDSCs were isolated using differential fibronectin adhesion. For differential adhesion cells were seeded after digestion at 12,000 cells per well of a 6-well plate (1200 cells/cm^2), previously coated with 20 µg/ml human fibronectin, and the media (complete DMEM + 5 ng/ml FGF-2) replaced after 20 minutes. After 6-8 days the cells were confluent and transferred to a T25 culture flask. Cells were used at passage 2-3 for all experiments.

2.2.1.4 Isolation of canine mesenchymal stromal cells (MSCs)

To obtain MSCs, bone marrow was harvested from canine subjects undergoing total hip arthroplasty at the Small Animal Teaching Hospital, University of Liverpool. Ethical approval for use of this material in this project was granted by the local ethics committee (VREC63ab). A Jamshidi needle was inserted into the femur and used to extract the bone marrow. The bone marrow aspirate was layered on to Ficoll-Paque Plus and centrifuged for 1500 rpm for 30 minutes. The straw-coloured buffy layer was removed and added to complete DMEM before being centrifuged again at 1200 rpm for 10 minutes. The cell pellet was resuspended in complete DMEM and transferred to T25 culture flasks. Cells were used at passage 2-3 for all experiments.

2.2.2 Stem cell characterisation

2.2.2.1 Cell proliferation assay

Cells were seeded at 10,000 cells in T25 culture flasks at day 0. At 80% confluency the cells were counted and the doubling time calculated using the formula below.

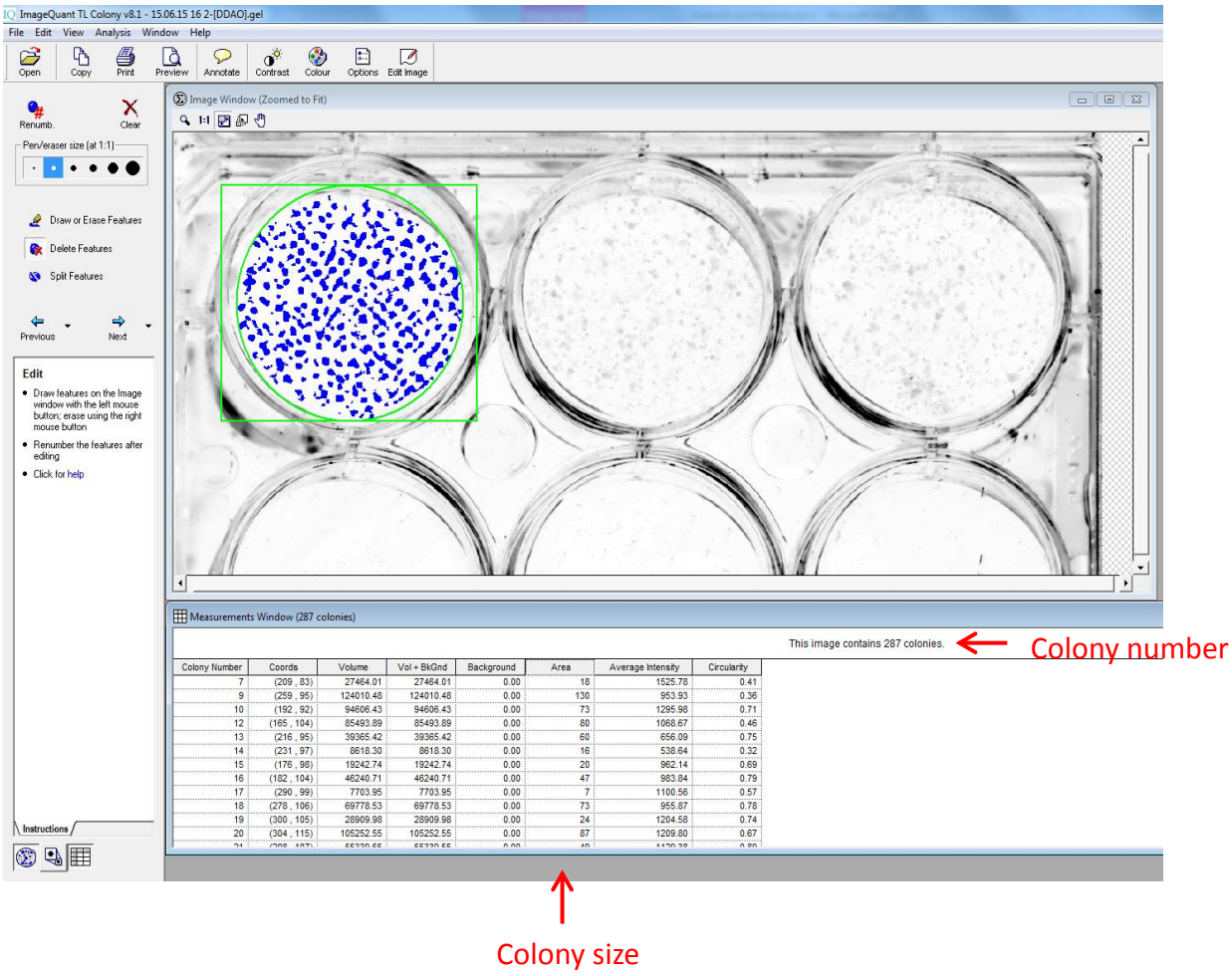
$(LOG_{10}(\text{cell number after proliferation})-LOG_{10}(\text{initial seeding density}))/LOG_{10}(2)$

2.2.2.2 Colony formation assay

Cells were seeded at 100 cells/cm^2 in 6-well cell culture plates. After 7 days in culture the cells were washed and then fixed with 6% gluteraldehyde and stained with 0.5% crystal violet solution. The cells were washed again and imaged using a biomolecular imager (Typhoon FLA 7000, GE Healthcare) and analysed using ImageQuant software (GE Healthcare) for colony number and size (Fig 2.7).

2.2.2.3 Flow cytometric analysis of stem cell marker expression

TDSCs were detached using Accutase, a sensitive solution for removal of cells, and counted. Aliquots containing $1x10^6$ cells were blocked with 10% normal donkey serum in FACS buffer (2.5% FBS in PBS) for 20 minutes before washing. Cells were resuspended in either fluorescently conjugated or unconjugated primary antibodies for 45 minutes at 4°C. Cells were washed and either analysed by flow cytometry (BD Acurri C6 flow cytometer, BD Biosciences) or if required incubated with a secondary antibody (donkey anti-mouse IgG FITC-conjugated antibody) for a further 45 minutes at 4°C before washing and analysis. Cells in the absence of antibody and in the presence of the secondary antibody only were used as negative controls (Radcliffe *et al*., 2010). Anti-CD90 (ab225, Abcam), anti-CD105



Colony Number	Coords	Volume	Vol + BkGnd	Background	Area	Average Intensity	Circularity
7	(209 , 83)	27464.01	27464.01	0.00	18	1525.78	0.41
9	(259 , 95)	124010.48	124010.48	0.00	130	953.93	0.36
10	(192 , 92)	94606.43	94606.43	0.00	73	1295.98	0.71
12	(165 , 104)	85493.89	85493.89	0.00	80	1068.67	0.46
13	(216 , 95)	39365.42	39365.42	0.00	60	656.09	0.75
14	(231 , 97)	8618.30	8618.30	0.00	16	538.64	0.32
15	(176 , 98)	19242.74	19242.74	0.00	20	962.14	0.69
16	(182 , 104)	46240.71	46240.71	0.00	47	983.84	0.79
17	(290 , 99)	7703.95	7703.95	0.00	7	1100.56	0.57
18	(278 , 106)	69778.53	69778.53	0.00	73	955.87	0.78
19	(300 , 105)	28909.98	28909.98	0.00	24	1204.58	0.74
20	(304 , 115)	105252.55	105252.55	0.00	87	1209.80	0.67

Figure 2.7. Illustration of colony formation analysis using ImageQuant software (GE Healthcare) in order to determine colony number and size.

(MCA1557A488T, AbD Serotec) and anti-CD73 (550256, BD Pharmingen) antibodies were used in this study. Average fluorescence values were recorded and a threshold gating out 99.5% of control cells was used. For samples including primary antibody the percentage of positive cells was calculated as that exceeding this threshold.

2.2.2.4 Tri-lineage differentiation assays

Cell monolayers were cultured for 21 days in osteogenic (complete DMEM containing 100 nM dexamethasone, 10 mM β-glycerophosphate and 50 mM ascorbic acid) (Jaiswal *et al*., 1997) and adipogenic (complete DMEM containing 1 µM dexamethasone, 100 µM indomethacin, 10 µg/ml insulin and 500 µM IBMX) induction media (Cheng *et al*., 2009). Cell pellets (containing $5x10^5$ cells) were cultured for 21 days in chondrogenic (complete DMEM containing 100 nM dexamethasone, 25 µg/ml ascorbic acid, 10 ng/ml TGF-β and ITS+3 supplement) induction media (Murdoch *et al*., 2007). Control cells for all treatments were cultured in complete DMEM (phenol red-free). After culturing the cells were stained with alizarin red and BCIP/NBT (5-Bromo-4-chloro-3-indolyl phosphate/Nitro blue tetrazolium) to assess osteogenic differentiation, Oil Red O to assess adipogenic differentiation, or alcian blue for chondrogenic differentiation, as described in the PromoCell MSC application notes (http://www.promocell.com/downloads/application-notes/). Briefly, for alizarin red staining: cell monolayers were washed with PBS before fixing in 10% formalin for 30 minutes. Cells were washed in distilled water, followed by addition of 2% alizarin red S solution (pH 4.1-4.3) for 45 minutes in the dark. Cells were then washed 4 times in distilled water before imaging. For BCIP/NBT staining: cell monolayers were washed with PBS before fixing in 10% formalin for 60 seconds. Cells were washed in 0.05% Tween 20 in PBS, followed by addition of BCIP/NBT substrate solution for 5-10 minutes in the dark. Cells were then washed in 0.05% Tween 20 in PBS before imaging. For oil red O staining: cell monolayers were washed with PBS before fixing in 10% formalin for 30 minutes. Cells were washed in distilled water, followed by addition of 60% isopropanol for 5 minutes. 0.18% oil red O solution was then added to cells for 15 minutes. Cells were then washed 2 times in distilled water before the

addition of haematoxylin for 1 minute. Cells were then washed 4 times with distilled water before imaging. For alcian blue staining: cell monolayers were washed with PBS before fixing in 10% formalin for 60 minutes. Cells were washed twice in distilled water, followed by addition of 0.01% alcian blue solution overnight in the dark. Cells were then washed 3 times in destaining solution (60% ethanol, 40% acetic acid) before imaging. Chondrogenic pellets were also paraffin embedded and 4 µm sections taken (processed by the Department of Veterinary Pathology, Institute of Veterinary Science, University of Liverpool) which were rehydrated and further stained with either 1% Alcian blue solution for 30 minutes or 0.1% Safranin O solution for 5 minutes. Cell pellets were also digested in 10 U/ml papain solution for 3 hours at 60°C before the total sulphated glycosaminoglycan (GAG) content was quantified. Dimethylmethylene blue (DMMB) dye was added to each sample and the absorbance read immediately at 570 nm. The GAG content was calculated from a standard curve produced using chondroitin sulphate standards (Fig.2.8) (Farndale *et al*., 1986). RNA was extracted from all assays to analyse lineage-specific gene expression (see Section 2.2.2.5).

2.2.2.5 RNA extraction and quantitative real time-polymerase chain reaction (qRT-PCR)

RNA was extracted from all cell types by firstly applying Trizol to cell monolayers and using a cell scraper to aid cell detachment. 100 µl BCP was added to samples before vortexing and incubation at room temperature for 3 minutes. Samples were then centrifuged at 14,000 g for 15 minutes at 4°C. The upper aqueous phase was transferred to a new tube and 2 µl of 50 µg/ml glycoblue and 250 µl of 100% isopropanol were added to precipitate the RNA by incubation on ice for 30 minutes. Next the samples were centrifuged at 14,000 g for 20 minutes at 4°C, and the supernatant removed and discarded. 1 ml of 75% ethanol was added to wash the pellet by centrifugation at 14,000 g for 5 minutes at 4°C. The ethanol was removed and discarded and the pellets dried before resuspension in 21.2 µl of Tris-EDTA (TE) buffer. The quantity and quality of RNA was assessed using a NanoDrop spectrophotometer (Thermo Fisher), 260/280 values between 1.8-2.1 were deemed

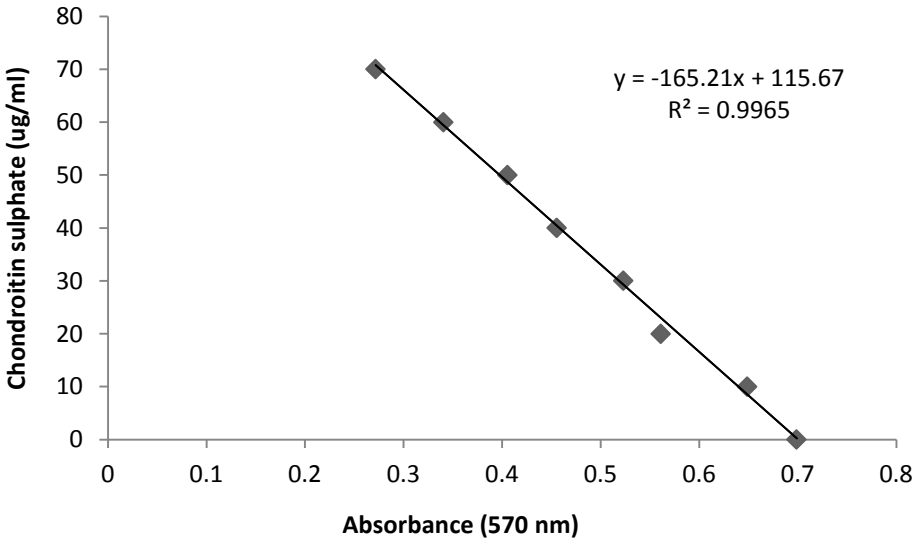


Figure 2.8. Representative standard curve produced using chondroitin sulphate standards, ranging in concentration from 10-70 ug/ml, to enable quantification of sulphated GAG content from the linear equation.

of a sufficient RNA quality. 2 µl of 4 U DNase was then added to the samples for 30 minutes at 37°C to remove DNA, after which time 50 µl of phenol:chloroform:IAA was added to each sample and centrifuged at 14,000 g for 5 minutes at 4°C. 1 µl of 50 µg/ml glycoblue, 10 µl of 5 M ammonium acetate and 330 µl of 100% ethanol were then added to precipitate the RNA by incubation on ice for 30 minutes. The samples were then centrifuged, washed in ethanol and the RNA assessed as described above. This method was adapted from previous studies (McDermott *et al*., 2016; Reynolds *et al*., 2016).

cDNA was synthesised in a 25 µl reaction from 1-2 µg of total RNA. The conditions for cDNA synthesis were: incubation at 5 minutes at 70°C, 60 minutes at 37°C and 5 minutes at 93°C with 0.6 µl of 1 U/µl RNasin ribonuclease inhibitor, 5 µl of 2 mM PCR nucleotide mix, 1 µl of 8 U/µl M-MLV reverse transcriptase and 1 µl of 0.02 µg/µl random-hexamer oligonucleotides per reaction. qRT-PCR was conducted using a GoTaq(R) qPCR Master Mix which included SYBR Green DNA intercalating dye, and in a 25 µl reaction 10 ng of cDNA was amplified in an AB 7300 Real Time PCR System (Applied Biosystems). After an initial denaturation for 10 minutes at 95°C, 40 PCR cycles were performed consisting of 15 seconds at 95°C and 1 minute at 60°C. Relative gene expression was calculated according to the comparative C_t method. These methods were adapted from previous studies (Livak and Schmittgen, 2001; McDermott *et al*., 2016; Peffers *et al*., 2015a; Reynolds *et al*., 2016).

Equine, murine and canine specific primers were used (Table 2.1) and GAPDH was used as an internal control. Primers were designed using Primer-BLAST (NCBI) and the quality of each primer was tested using NetPrimer (Premier Biosoft). In addition, each primer was subjected to a BLAST (NCBI) search to ensure specificity. The best housekeeping genes were determined and all primers were tested for efficiency (see Appendix B).

Species	Gene	Forward	Reverse
Equine	GAPDH	GCATCGTGGAGGGACTCA	GCCACATCTTCCCAGAGG
	CD90	TGCTCCGAGACAAACTGGT	CCGAGGTGTGTGAGGGATTG
	CD73	CCAGGAAGTGGGGAGAACAC	CCAAGGTAATGGTGCCGTTG
	TNC	GTTTCAGATGCCACCCCAGA	AGCCCATAGCTGTTGTTGCT
	SCX	TCTGCCTCAGCAACCAGAGA	TCCGAATCGCCGTCTTTC
	MKX	GATGACGCTAGTGCAGGTGT	CCCCCTTCGTTCATGTGGTT
	EGR-1	CCACCATGGACAACTACCCT	ATGTCAGGAAAAGACTCTGAGG
	OCT-4	GAGAAGGACGTGGTACGAGTG	GTGCCAGGGGAAAGGATACC
	NANOG	CAGGGGATCTTCACCAGTGC	GGAAGGCAGAGGAGAGACAGT
	TNMD	ACGTGACCATGTATTGGATCAATC	CACCATCCTCCTCAAAGTCTTGT
	THBS4	AATCCTGACAGACCCCACCC	GGTAGCGGAGGATGGCTTTGTT
	CD34	CACTAAACCCTCTACATCATTTTCTCCTA	GGCAGATACCTTGAGTCAATTTCA
	CD144	ACTATGAACGCATCCGGCAA	GGAGTATCCAATGCTCCGCC
	RUNX2	GTGGACGAGGCAAGAGTTTC	TGAGGCGGTCAGAGAACAAA
	OMD	CAAATTCATCAACCCCTGAAA	CTTCATCTGGCTCTTGGTCA
	ALP	GGGTAGCAGCCAGTTCAGTT	GGGATCTTTCTCTTTCTCTGGCA
	COL1A1	CATGTTCAGCTTTGTGGACCT	TGACTGCTGGGATGTCTTCTT
	DCN	GTCACAGAGCAGCACCTACC	TCACAACCAAGGAACCTTTTAATCC
	Osteocalcin	GAGGTGCAGCCTTCGTGTC	AGCCAATGATCCAGGTAGCG
	Osterix	CCAGATCCCCCAGGAGGAAG	TTTGCCCAGTGTCGTTGAGT
	Osteopontin	CGCCTATGCCCTTCCAGTTA	TGGTCATGGCTTTCGTTGGA
	FABP4	CAGAGGGTCAGAGCACCTTC	GCCCACTCCCACTTCTTTCA
	PPARγ	ATGGGTGAAACTCTGGAAGATT	GGTAATTTCTTGTGAAGTGCTTGC
	LEP	CATTGAAGCTGTGCCCATCC	AGACTGACTGCGTGTGTGAAA
	SOX9	AGCAGACACACATCTCCCCC	GCGAGGAATGAGCCTACAAGGT
	COL2A1	TGAGCCATGATACGCCTCG	CTCCTTTCTGTCCCTTCGGT
	ACAN	GCGGTACGAGATCAACTCCC	CTTGTAGCTGGCGGGGTC
	COL1A2	GCACATGCCGTGACTTGAGA	CATCCATAGTGCATCCTTGATTAGG
	COMP	GGTGCGGCTGCTATGGAA	CCAGCTCAGGGCCCTCAT
	BIGLYCAN	TCACCTTCCAGCCCCTAGAGT	AGAAGCAGCCCCTCCTCAA
Murine	GAPDH	GAGAGGCCCTATCCCAACTC	GTGGGTGCAGCGAACTTTAT
	CD90	GGATGAGGGCGACTACTTTTGT	TTGGAGCTCATGGGATTCG
	CD73	TGGTTCACCGTTTACAAAGG	CGCTCAGAATTGGAAATTTAAC
	TNC	AGGCGATCCCAGCCAGTCAGT	ATGGACGGGGCACCTCCTGTC
	SCX	AAGTTGAGCAAAGACCGTGACA	TGTGGACCCTCCTCCTTCTAAC
	MKX	AGTAAAGACAGTCAAGCTGCCACTG	TCCTGGCCACTCTAGAAGCG
	Sca-1	GTTTGCTGATTCTTCTTGTGGCCC	ACTGCTGCCTCCTGAGTAACAC
	NANOG	AGGGTCTGCTACTGAGATGCTCTG	CAACCACTGGTTTTTCTGCCACCG
	TNMD	AACTCCACCTCAGCAGTAGTCC	TTTCTTGGATACCTCGGGCCAGAA
	THBS4	TCCTCCGCTACCTGAAGAATGATGG	TTCAATGGACTCTGGGTTCTGGGTG

	CD34	AGGCTGATGCTGGTGCTAG	AGTCTTTCGGGAATAGCTCTG
	CD45	AGTTAGTGAATGGAGACCAGGAA	TCCATAAGTCTGCTTTCCTTCG
	RUNX2	ATGCGTATTCCTGTAGATCCG	TTGGGGAGGATTTGTGAAGAC
	ALP	CCAACTCTTTTGTGCCAGAGA	GGCTACATTGGTGTTGAGCTTTT
	COL1A1	TTCTCCTGGCAAAGACGGACTCAA	AGGAAGCTGAAGTCATAACCGCCA
	DCN	TGAGCTTCAACAGCATCACC	AAGTCATTTTGCCCAACTGC
	Osteocalcin	CTCTGTCTCTCTGACCTCACA	CAGGTCCTAAA AGTGATACC
	Osterix	GAAAGGAGGCACAAAGAAG	CACCAAGGAGTAGGTGTGTT
	Osteopontin	CATGAGATTGGCAGTGATTTGC	TGCAGGCTGTAAAGCTTCTCCT
	FABP4	GAAGCTTGTCTCCAGTCAAAA	AGTCACGCCTTTCATAACACAT
	PPARγ	CTCCGTGATGGAAGACCACTC	AGACTCGGAACTCAATGGC
	LEP	CTTCACCCCATTCTGAGTTTGT	TTCTCCAGGTCATTGGCTATCT
	SOX9	TGGCAGACCAGTACCCGCATCT	TCTTTCTTGTGCTGCACGCGC
	COL2A1	GGTTTGGAGAGACCATGAAC	TGGGTTCGCAATGGATTGTG
	ACAN	TTGCCAGGGGGAGTTGTATTC	GACAGTTCTCACGCCAGGTTTG
Canine	GAPDH	CTGGGGCTCACTTGAAAGG	CAAACATGGGGGCATCAG
	CD90	TGTGCTCAGAGACAAACTGGT	CAGCCAGTCACAGGGAGATG
	CD73	ATGGCTCCACTCAATCCTGC	TCCCAGGTAATTGTGCCGTT
	CD105	GACGCCGAGGTGACATACAT	GCTCTGACAGCTCCCTTGAG
	CD44	ACCTTCCAACTGCATACCCG	TCGTGGTCTTTGGTAATGGGG
	SCX	GTCCAGCTACATCTCGCACC	GTCCAGCTACATCTCGCACC
	MKX	GCGACCCCGGAGTTCTTC	CGCGGTCCTCAAAAAGCAC
	OCT-4	GAGGCTCTGCAGCTCAGTTT	AGCCCAGAGTGGTGACAGAC
	TNMD	CCCACTCTAATAGCAGTTTCAGA	TCCTCACTTGCTTGTCTGGT
	RUNX2	GAACCCAGAAGGCACAGACA	ACTTGGTGCAGAGTTCAGGG
	Osteocalcin	AGGGCACGACCCAGAGTAT	GCTTGGACACGAAGGTTGC
	Osteopontin	CTCTGAGGAAAAGCAGAATGC	GTTGCTGGAATGTCAGTGGG
	FABP4	ATCAGTGTAAACGGGGATGTG	GACTTTTCTGTCATCCGCAGTA
	PPARγ	TTCTTTTAATGGATTGGTCTTTTGC	TGGTAGTTTCTTGTGAAGTGCT
	LEP	TATCTGTCCTGTGTTGAAGCTG	GTGTGAAAATGTCATTGATCCTG
	SOX9	CTCTGGAGGCTGCTGAACG	AAGATGGCGTTGGGGGAAAT
	COL2A1	AGCTAAAGGATCTGCTGGCG	CTTGTTCGCCTTTGAAGCCA
	ACAN	ACAATGCCCAAGACTACCAGTGGA	TTCTCGTGCCAGATCATCACCACA

Table 2.1. Primer sequences for equine, murine and canine genes.

2.2.3 Stem cell niche characterisation

2.2.3.1 Histology

SDFT tissue was fixed overnight in 4% paraformaldehyde at 4°C before paraffin embedding. 4 µm thick sections were cut, dried and washed before the following stains were performed: Haematoxylin and Eosin (H&E) and Masson's trichrome (processed by the Department of Veterinary Pathology, Institute of Veterinary Science, University of Liverpool).

2.2.3.2 Immunohistochemistry

Whole SDFT tissue sections were frozen in OCT cryo-embedding compound using hexane and dry ice. Hexane was cooled on dry ice before the dry ice was added to the hexane. The tissue samples were fixed to cork disks and covered in OCT before being added to the hexane. Once samples were completely frozen they were removed and stored at -80°C. 4 µm sections were cut from the blocks using a cryostat, after which the sections were fixed in methanol for 20 minutes. 3% hydrogen peroxide was then added to all sections for 10 minutes to block endogenous peroxidase activity. After washing 3 times each in distilled water and Tris-buffered saline (TBS) (20 mM Trizma Base and 0.14 M NaCl in water) for 5 minutes the sections were blocked for one hour in 10% goat serum and then for a further hour in primary antibody at room temperature. After washing, the secondary antibody (goat anti-mouse IgG horseradish peroxidase-conjugated antibody) was added to the sections for one hour at room temperature, followed by further washing steps. Next, 3,3'-Diaminobenzidine (DAB) substrate was added to the sections for 30 seconds and then another washing step. The sections were then counterstained with haematoxylin for 10 seconds before washing. Finally the sections were dehydrated using ethanol and xylene before mounting with DPX mountant and imaging. This method was adapted from the Abcam Immunohistochemistry (IHC-Fr) – Frozen sections protocol

(http://www.abcam.com/ps/pdf/protocols/ihc_fr.pdf) and the Abcam IHC-Paraffin protocol (IHC-P) (http://www.abcam.com/ps/pdf/protocols/ihc_p.pdf). Anti-tenascin C (sc-59884, Santa Cruz) and anti-decorin (kindly donated by Professor Bruce Caterson, Cardiff University) antibodies were used in this study, as well as anti-CD90 (ab225, Abcam) and anti-CD73 (550256, BD Pharmingen) antibodies.

2.2.3.3 Extracellular matrix (ECM) extraction

For equine SDFT stem cell niche characterisation experiments, the ECM was extracted from cultured cells in a monolayer by initially extracting the cells using trypsin. The ECM was then extracted using a guanidine extraction buffer (4 M guanidine HCl, 50 mM sodium acetate, 65 mM DTT, containing Roche mini protease inhibitor cocktail tablets), for 48 hours at 4°C. The resultant protein suspension was then centrifuged at 14,000 g for 15 minutes at 4°C and the soluble fraction removed and stored at -80°C until required (Kharaz *et al*., 2016).

For canine CCL proteomics validation experiments, the ECM was extracted from cultured cells in a monolayer using a number of different methods. Cells were initially extracted using either 4.8 mM EDTA; 1x trypsin or 20 mM NH_4OH in 0.5% (w/v) Triton X-100 (detergent) (Choi *et al*., 2011; Soteriou *et al*., 2013). The ECM was then extracted using a urea extraction buffer (7 M urea, 0.15 M NaCl, 50 mM Tris-HCl, pH 6.5, EDTA containing Roche mini protease inhibitor cocktail tablets) and incubated at 4°C for 48 hours. Alternatively, the ECM was extracted using a 2x reducing sample buffer (50 mM Tris-HCl, pH 6.8, 10% (w/v) glycerol, 4% (w/v) SDS, 0.004% (w/v) bromphenol blue, 8% (v/v) β-mercaptoethanol) which was added to the plates and the ECM removed immediately using a cell scraper (Soteriou *et al*., 2013). The final method of ECM extraction was the use of a Rapigest buffer (0.06% (w/v) Rapigest diluted in 25 mM ammonium bicarbonate) where the plates were incubated at room temperature for 30 minutes and 80°C for 10 minutes (Peffers *et al*., 2015b). Rapigest is a solution used to promote enzymatic digestion of proteins, which is often used prior to mass spectrometry proteomic analysis (Little *et al*., 2014; Peffers *et al*., 2015b).

All experiments were performed in 6-well plates and 1 ml of cell/ECM extraction buffer was added to each well, except for the Rapigest method where only 180 µl of buffer was added to 35 mm petri dishes (to maintain consistency with the methodology used by the Centre for Proteome Research, University of Liverpool). The protein extractions from all experiments were stored at -80°C until required.

2.2.3.4 Electrophoresis

The quantity of protein in ECM samples was analysed using a 660 protein assay as per manufacturer's instructions. The protein quantity was calculated from a standard curve produced from albumin standards (Fig.2.9). Samples were then diluted to the same concentration as the sample containing the lowest protein concentration to ensure equal loading, before the addition of 2 x SDS sample buffer containing β-mercaptoethanol to each sample. After boiling for 5 minutes the samples were loaded on to 4-12% precast Bis-Tris gels and run at 200 V for 35 minutes. After running the gels were fixed and stained with GelCode or a Silver Stain kit as per the manufacturer's instructions.

2.2.3.5 ^{14}C metabolic labelling of ECM proteins

SDFT cells were subjected to ^{14}C metabolic labelling in 24 well plates. Cells were incubated in SILAC (stable isotope labelling with amino acids in cell culture) media supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin and 2 µg/ml amphotericin B, 0.2 mM ascorbate and 0.4 mM β-APN (complete SILAC media), as well as 0.8 mM [^{12}C]L-lysine 2HCl, 0.4 mM [^{12}C]L-arginine HCl and 0.4 mM [^{12}C]L-proline, for 1 hour at 37°C. The media was replaced with complete SILAC media supplemented with 2.5 µCi/ml [^{14}C]L-lysine and [^{14}C]L-arginine or 2.5 µCi/ml [^{14}C]L-proline, for 24 hours at 37°C. After which the cells and ECM were extracted and analysed using electrophoresis as described previously (Section 2.2.3.3 and 2.2.3.4).

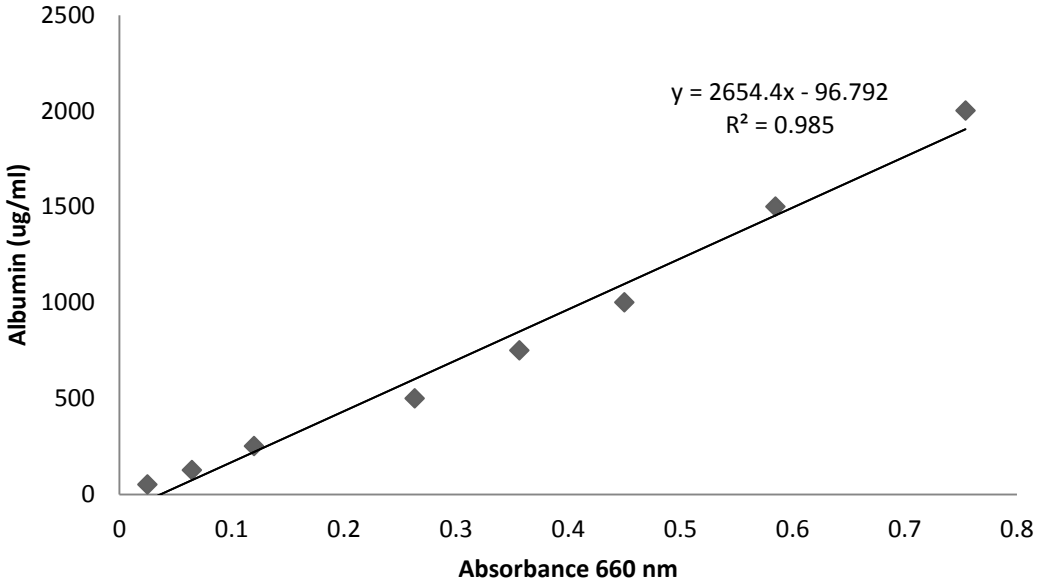


Figure 2.9. Representative standard curve produced using albumin standards, ranging in concentration from 50-2000 ug/ml to enable protein quantification from the linear equation.

Following this the gels were stained with GelCode, dried under vacuum for 90 minutes at 80°C and exposed to a phosphorimaging plate. After 24 hour exposure the plate was processed using a phosphorimager (Typhoon FLA 7000, GE Healthcare) and the images analysed using ImageJ (NIH).

2.2.3.6 ^{13}C metabolic labelling of ECM proteins for proteomic analysis

LDSCs and ligamentocytes were subjected to ^{13}C lysine metabolic labelling. Cells were incubated in complete SILAC media (Section 2.2.3.5) supplemented with 0.8 mM [^{12}C]L-lysine 2HCl, 0.4 mM [^{12}C]L-arginine HCl and 0.4 mM [^{12}C]L-proline (unlabelled media) for 1 hour. The media was then exchanged for labelled media (complete SILAC media supplemented with 0.8 mM [^{13}C]L-lysine 2HCl, 0.4 mM [^{12}C]L-arginine HCl and 0.4 mM [^{12}C]L-proline) for 4, 24 and 48 hours. After which the cells were removed using trypsin and the plates washed with PBS, before transfer to the Centre for Proteome Research, University of Liverpool for protein extraction and proteomic analysis.

2.2.3.7 Proteomic analysis

The experiments and methods described in Sections 2.2.3.7.1 and 2.2.3.7.2 were conducted by Dr Deborah Simpson of the Centre for Proteome Research, University of Liverpool.

2.2.3.7.1 Sample preparation for proteomic analysis

Rapigest solution (0.06% (w/v) solution in 25 mM NH_4HCO_3) was applied to each plate and the surface covered by manual rotation. The plates were placed on an orbital shaker for 30 minutes (200 rpm) at room temperature then incubated at 80°C for 10 minutes and allowed to cool. Protein extracts were transferred to low-bind tubes and samples were reduced by the addition of 60 mM DTT in 25 mM NH_4HCO_3 followed by sample incubation at 60°C for 10 minutes. Alkylation was carried out by the addition of 180 mM iodoacetamide in 25 mM NH_4HCO_3 and the

sample incubated at room temperature for 30 minutes in the dark. 0.05 µg/µl trypsin was added to samples before incubation at 37°C overnight. Digests were terminated by the addition of trifluoroacetic acid (TFA) and incubated at 37°C for 45 minutes, before centrifugation at 17,200 g for 30 minutes and transfer of the clarified digest to fresh low-bind tubes.

2.2.3.7.2 LC-MS/MS proteomic analysis

Data-dependent liquid chromatography tandem mass spectrometry (LC-MS/MS) analyses were conducted on a QExactive HF quadrupole-Orbitrap mass spectrometer (Scheltema *et al*., 2014; Williamson *et al*., 2016) coupled to a Dionex Ultimate 3000 RSLC nano-liquid chromatograph. Sample digest (8 µl) was loaded onto a trapping column (Acclaim PepMap 100 C18, 75 µm x 2 cm, 3 µm packing material, 100 Å) using a loading buffer of 0.1% (v/v) TFA, 2% (v/v) acetonitrile in water for 7 minutes at a flow rate of 9 µl min^{-1}. The trapping column was then set in-line with an analytical column (EASY-Spray PepMap RSLC C18, 75 µm x 50 cm, 2 µm packing material, 100 Å) and the peptides eluted using a linear gradient of 96.2% A (0.1% [v/v] formic acid):3.8% B (0.1% [v/v[formic acid in water:acetonitrile [80:20] [v/v]) to 50% A:50% B over 30 minutes at a flow rate of 300 nL min^{-1}. This was followed by washing at 1% A:99% B for 5 minutes and re-equilibration of the column to starting conditions. The column was maintained at 40^{o}C and the effluent introduced directly into the integrated nano-electrospray ionisation source operating in positive ion mode. The mass spectrometer was operated in data dependent acquisition (DDA) mode with survey scans between *m*/*z* 300-2000 acquired at a mass resolution of 70,000 (FWHM) at *m*/*z* 200. The maximum injection time was 250 ms and the automatic gain control was set to 1e6. The 10 most intense precursor ions with charge states of between 2+ and 5+ were selected for MS/MS with an isolation window of 2 *m*/*z* units. The maximum injection time was 100 ms and the automatic gain control was set to 1e5. Fragmentation of the peptides was by higher-energy collisional dissociation using a normalised collision energy of 30%. Dynamic exclusion of *m*/*z* values to prevent repeated fragmentation of the same peptide was used with an exclusion time of 20 seconds.

2.2.3.7.3 Proteomic data analysis

For protein identification and label-free quantification raw data files were imported into PEAKS v.8 (Bioinformatics Solutions Inc) (Zhang *et al*., 2012) and *de novo* and database PEAKS searches were carried out using the UniProt canine protein database. The instrument configuration was set to Q Exactive, and the following parameters were used for each search: Enzyme: trypsin; Fragment mass error tolerance: 0.01 Da; Parent mass error tolerance: 10 ppm; Precursor mass search type: monoisotopic; Non-specific cleavage: none; Maximum missed cleavages: 1; Fixed modifications: carbamidomethyl 57.02; Variable modifications: oxidation (M) 15.99, oxidation or hydroxylation 15.99; Maximum variable PTM per peptide: 3. Search results were filtered using the following parameters: False discovery rate: 1%; Proteins -10lgP ≥ 15; Unique peptides ≥ 2; De novo only ALC (%) ≥ 50. For label-free quantification the following parameters were also used: RT range: 0-90; Fold change ≥ 2; Quality ≥ 0.2; Charge: 2-5; Confident in 3 samples. LDSC and ligamentocyte groups were compared using the PEAKS compare function. Protein identifications and gene ontology were analysed using the UniProt canine protein database and the Matrisome Project database (Naba *et al*., 2016) and protein interaction network analysis was performed using STRING software (SIB). Differential protein expression was compared between LDSC and ligamentocyte groups and protein identification and gene ontology were analysed using the UniProt canine protein database.

For analysis of heavy isotope metabolic labelling, peptides for ECM proteins labelled with ^{13}C lysine were initially identified using MASCOT (Matrix Science). The following parameters were applied for all MASCOT searches: Enzyme: trypsin; Maximum missed cleavages: 1; Fixed modifications: carbamidomethyl; Variable modifications: oxidation (M), Label:13C(6) (K); Mass values: monoisotopic; Fragment mass tolerance: 0.01 Da; Peptide mass tolerance: 10 ppm; Unique peptides ≥ 2; Peptides -10lgP ≥ 15. Raw data files were imported into Xcalibur (Thermo Fisher Scientific) for analysis of extracted ion chromatograms and raw mass spectra. Heavy (H) and light (L) peaks were identified for each labelled peptide based on the observed m/z ratio and the scan number obtained from MASCOT. The area under each peak was recorded and relative isotope abundance (RIA) was

calculated as H/(L+H) and the total protein pool was calculated as H+L. These parameters were plotted against time to produce dynamic protein charts for protein synthesis and turnover.

2.2.4 Statistical analysis

Statistical analysis was performed using SPSS (IBM) and SigmaPlot (Systat Software Inc). To ensure data was normally distributed Shapiro Wilk tests were performed. For normally distributed data parametric tests were used for pairwise comparisons. For data which was not normally distributed Log_{10} data transformations were performed before parametric tests were used. For comparing two groups paired or independent Student's t-tests were used. For comparing three or more groups a one-way ANOVA with Tukey post-hoc test was performed. For comparing three or more groups with multiple factors a two-way ANOVA was used, with Tukey post-hoc test. For comparing three or more groups with multiple factors and repeated measures, a two-way ANOVA with repeated measures and a Holm-Sidak post-hoc test was used. p-values of <0.05 were taken to be statistically significant.

Chapter 3

Equine tendon-derived stem cell characterisation and investigation of the equine tendon stem cell niche

3.1 Introduction

The identification of tendon-derived stem cells (TDSCs) holds potential for the treatment of tendinopathies in both humans and animals. TDSCs are a subset of cells most likely derived from mesenchymal stromal cells (MSCs) due to the similar characteristics possessed by both cell types. TDSCs express similar markers to MSCs, such as Oct-4 and nucleostemin (Zhang and Wang, 2010b) as well as a variety of CD markers (Lee *et al*., 2012). TDSCs also express tenogenic markers which is where they differ from MSCs, these markers include tenomodulin (Bi *et al*., 2007) and tenascin C (Rui *et al*., 2010), however these markers are also expressed by other tendon cells such as tenocytes. Therefore it is necessary to use a panel of markers to isolate TDSCs, as no single marker has yet been identified for effective isolation of these cells. TDSCs are also able to form colonies (Bi *et al*., 2007), which is a basic hallmark of a stem cell. Another feature of TDSCs is their multi-lineage differentiation potential, specifically osteogenic, adipogenic and chondrogenic differentiation (Rui *et al*., 2010), which is a characteristic shared with MSCs. Certain factors affect the growth and function of TDSCs, including age (Zhou *et al*., 2010) and passage number (Tan *et al*., 2012a), as well as the environment of the stem cell, known as the 'niche'. The niche comprises many aspects of the cell's environment and oxygen tension (Lee *et al*., 2012), mechanical strain (Zhang and Wang, 2010d), biological mediators (Rui *et al*., 2013b), additional cell types (Kraus *et al*., 2013) and the protein composition of the niche (Bi *et al*., 2007) have all been shown to affect TDSCs.

TDSCs could provide a potential therapy for aged or degenerated tendon, and current preliminary studies have shown promising results. A number of different tissue engineering approaches involving the introduction of TDSCs to tendon injury have shown increased healing compared with controls (Ni *et al*., 2012; Yin *et al*., 2013; Zhang *et al*., 2011a).

TDSCs have been isolated from a number of different species, including human, mouse (Bi *et al*., 2007), rat (Rui *et al*., 2010), rabbit (Zhang and Wang, 2010b), cow (Yang *et al*., 2016) and horse (Lovati *et al*., 2011), however this study will focus on equine TDSCs. Tendinopathy in horses is a well-documented problem

(Ely *et al*., 2009) and a new, effective treatment strategy is currently being sought, with TDSCs high on the list of possible therapies. In addition, research into equine TDSCs is important for its translatability into humans (Innes and Clegg, 2010). To date, only one study has previously investigated the presence of TDSCs in equine tendon, which demonstrated the restricted differentiation potential of a cell population within equine tendon with limited stem cell properties (Lovati *et al*., 2011). Furthermore, there have been no studies investigating the composition of the equine TDSC niche or the effect of the niche on equine TDSCs.

3.2 Hypothesis

I hypothesise that a native tendon progenitor cell population resides within equine SDFT and which can be differentially isolated from other tendon cell types by low-density plating and/or differential adhesion to fibronectin substrates. This progenitor cell population will demonstrate all of the hallmark properties of stem cells, including clonogenicity, multipotency and stem cell marker expression, as well as expression of tenogenic markers. The progenitor cell population will differ from tenocytes with the latter cell type demonstrating reduced stemness when compared with progenitor cell populations.

3.3 Aims

1. To characterise equine TDSCs and tenocytes isolated from the SDFT; including analysis of stem cell and tenogenic marker expression, colony-forming ability, cell proliferation and multipotency. Furthermore, to compare the differences in phenotype between TDSCs and tenocytes.

2. To characterise the TDSC and tenocyte niche; including analysis of gene and protein expression of ECM components and microscopic examination of SDFT tissue to highlight potential niches within the tissue. Furthermore, to compare differences in niche composition between TDSCs and tenocytes.

3.4 Results

3.4.1 Tenocyte and TDSC morphology and colony formation

Tendon cells were isolated and seeded using three different methods: high density for isolation of tendon fibroblasts (tenocytes); low density for isolation of TDSCs (TDSC-L); and low density on fibronectin substrates also for isolation of TDSCs (TDSC-F) (Chapter 2, Section 2.2.1.1).

For all three cell types there was identification and growth of two phenotypically distinct cell types with a differing morphology and colony forming ability. Fibroblastic cells grew in large, sparse, widely spread colonies, whereas more rounded cells, with a cobblestone-like appearance grew in dense, compact, smaller colonies (Fig.3.1.A). All cell types exhibited the same pattern of colony growth, with similar colony numbers for tenocytes and TDSC-F at 21% and 5% oxygen. TDSC-L demonstrated slightly more varied colony numbers with a difference seen between the two oxygen tensions, although this was not significant. Average colony size was also fairly uniform across all three cell types and both oxygen tensions, with no significant differences seen, although tenocytes demonstrated a slight increase in colony size, and TDSC-L a slight decrease (Fig.3.1.B-C).

A
Fibroblastic cells
(sparse colonies)
Rounded cells
(dense colonies)
21% O2
5% O2
Tenocytes
TDSC-F
TDSC-L
Tenocytes
TDSC-F
TDSC-L

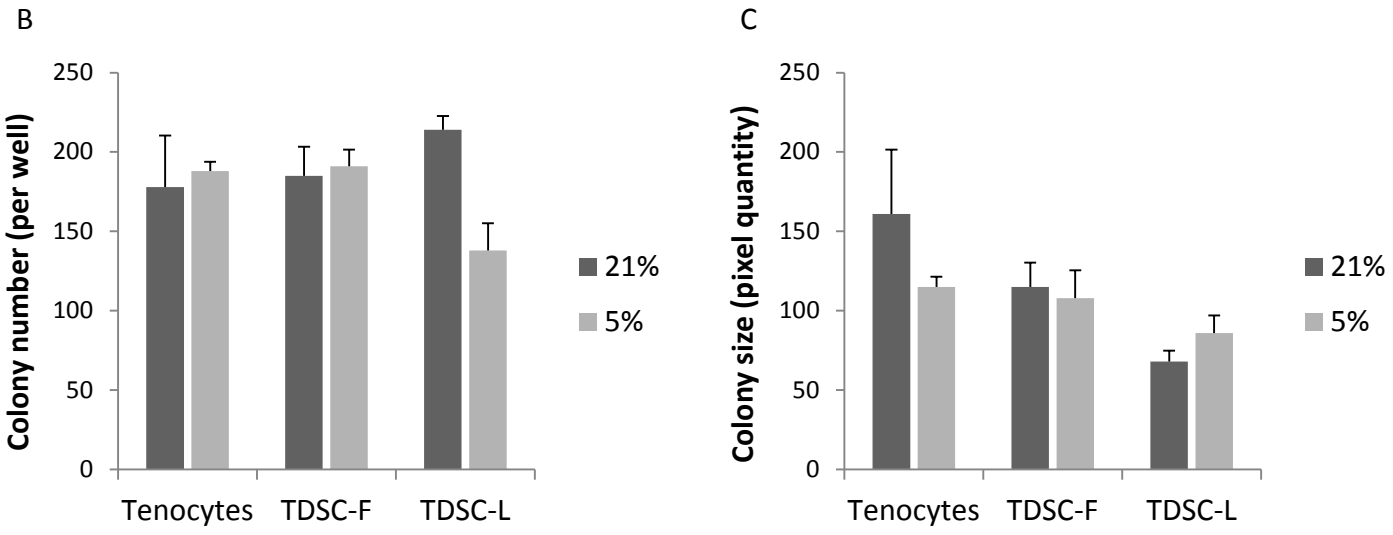


Figure 3.1. Tenocyte and tendon-derived stem cell (TDSC) morphology and colony formation at 21% and 5% oxygen tension. Representative images of cell morphology and colony forming ability are shown. Cells were grown for 7 days before staining with crystal violet and imaging for cell morphology analysis and colony formation. White bars = 100 µm, black bars = 1 cm (A). Colonies were counted (B) and measured (C) using ImageQuantTL software. n=3 biological replicates, error bars shown represent SEM. Pairwise comparisons were performed using a two-way ANOVA with Tukey post-hoc tests.

3.4.2 Tenocyte and TDSC proliferation

Cell population doubling times were similar for all three cell types, particularly at 21% oxygen, with a mean (±SEM) of 33.4 (±3.8), 32.1 (±5.3) and 36.9 (±10.2) hours per cell doubling for tenocytes, TDSC-F and TDSC-L respectively. The doubling times for cells at 5% oxygen were more varied, with means of 35.8 (±4.5), 27.8 (±0.8) and 48.2 (±2.1) hours per doubling for tenocytes, TDSC-F and TDSC-L respectively. There were no statistically significant differences in doubling times between cell types or between oxygen tensions (Fig.3.2).

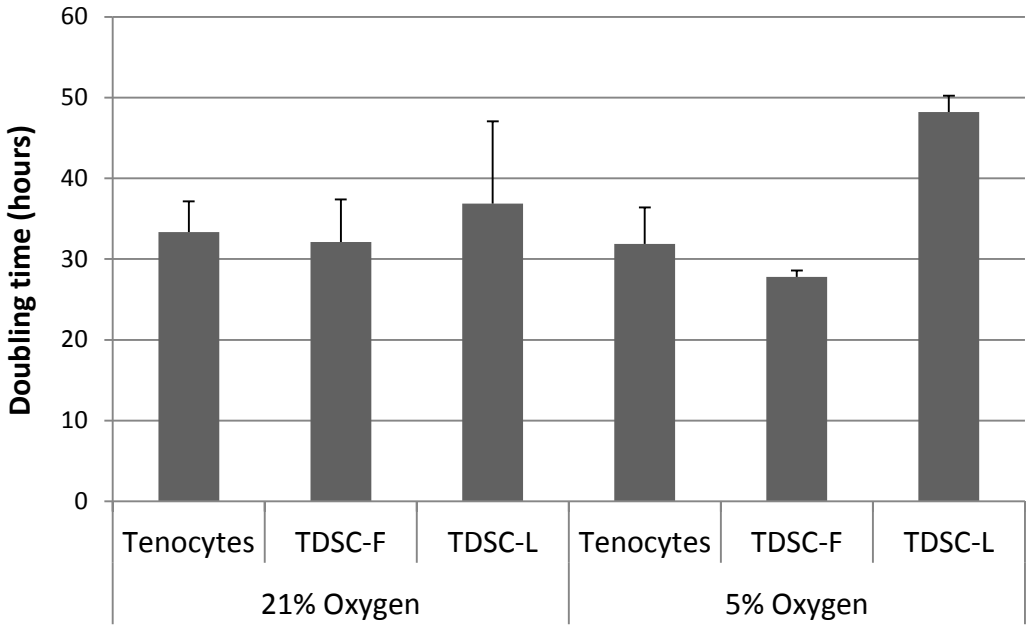


Figure 3.2. Population doubling times for tenocytes and TDSCs at 21% and 5% oxygen tension. n=4 biological replicates for tenocytes and n=3 for TDSCs, error bars shown represent SEM. Pairwise comparisons were performed using a two-way ANOVA with Tukey post-hoc tests.

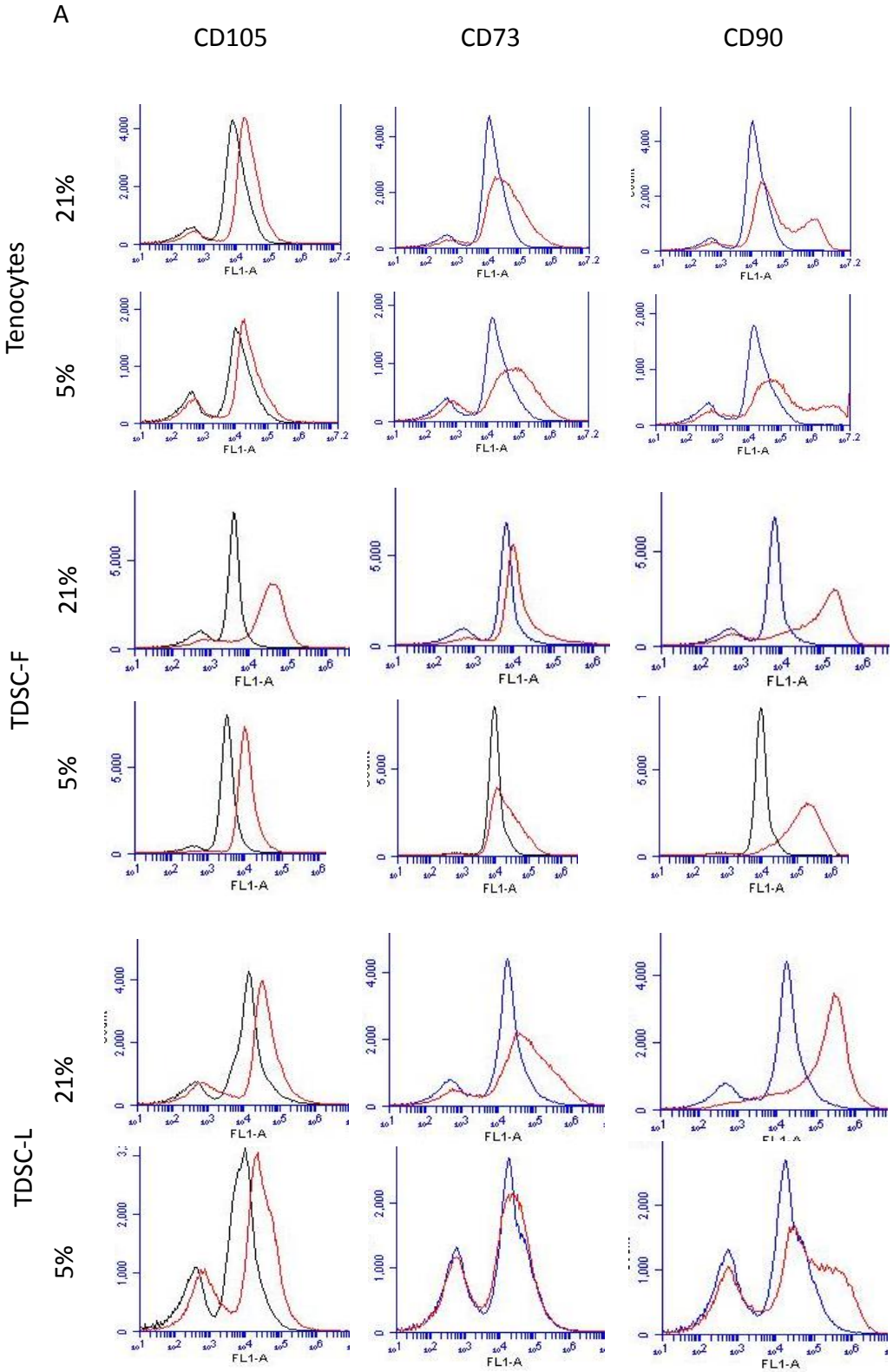
3.4.3 Tenocyte and TDSC marker expression

Tenocyte and TDSC marker expression was assessed using flow cytometry, immunohistochemistry and qRT-PCR for analysis of gene expression.

Flow cytometric analysis showed a slight shift in the fluorescence peak for both CD105 and CD73 in all three cell types (Fig.3.3.A), however when quantified (Fig.3.3.B-D) this was only significant for tenocytes at 21% oxygen. Conversely, CD90 was highly expressed in all three cell types, with statistically significant increases in expression for all cell types at both oxygen tensions, excluding TDSC-L at 21% oxygen, when compared with zero controls. In general there was an increase in marker expression in TDSC-F compared with TDSC-L and tenocytes, particularly CD90 expression.

Gene expression analysis of stem cell markers in tenocytes and TDSCs showed similar expression levels across all three cell types and both oxygen tensions, and no significant differences in gene expression between cell types or oxygen tensions, except for THBS4 (Fig.3.4). The pluripotency markers octamer-binding transcription factor 4 (Oct4) and Nanog showed extremely low gene expression in all three cell types with the highest levels seen in TDSC-F for Oct4. Conversely the lowest expression of Nanog was seen in TDSC-F, and the highest in tenocytes and TDSC-L. The MSC markers CD90 and CD73 were both highly expressed in tenocytes and TDSC-F, with variable expression seen in TDSC-L. The expression of early growth response hormone-1 (EGR-1), a transcription factor involved in stem cell differentiation, showed no clear pattern with varied expression between cell types and oxygen tensions. The early tendon markers Mohawk (MKX) and scleraxis (SCX) were highly expressed in TDSC-F and tenocytes. The early tenogenic marker tenascin C (TNC) showed similar expression between cell types. Other tenogenic markers, thrombospondin-4 (THBS4) and tenomodulin (TNMD) were expressed highly in tenocytes with reduced levels seen in TDSCs, although very low gene expression levels were seen generally for TNMD. The expression of THBS4 in tenocytes was significantly higher than TDSC-F and TDSC-L. Finally the expression of haematopoietic markers CD34 and CD144 were generally low, except in tenocytes and TDSC-F which showed slightly higher expression of CD34.

Immunohistochemical analysis of frozen equine SDFT tissue sections showed no expression of CD73 and abundant expression of CD90. CD90 expression was mainly localised to the IFM, probably due to the abundance of cells in this area, however expression was also seen within fascicles where cells also reside. Not all cells were stained indicating that not all cells within equine SDFT expressed CD90 (Fig.3.5).


A
CD105
CD73
CD90
Tenocytes
TDSC-F
TDSC-L
21%
5%
FL1-A

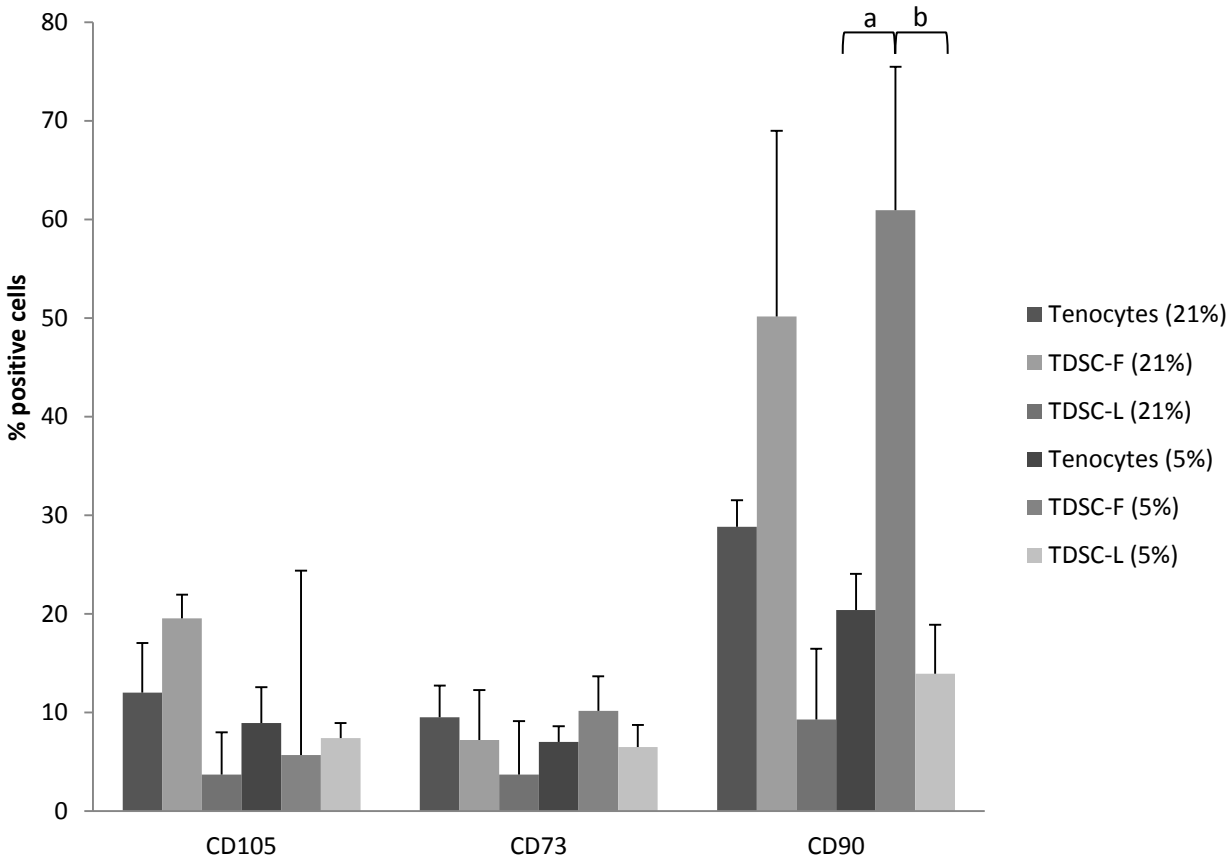


Figure 3.3. Flow cytometric analysis of stem cell markers expressed by tenocytes and TDSCs at 21% and 5% oxygen. (A) shows representative flow cytometry plots. Black/blue peaks represent control samples and red peaks represent samples in the presence of each antibody. This data has been quantified (B) to show percentage of positive cells after subtraction of control fluorescence (99.5%). n=3 biological replicates, error bars shown represent SEM. Pairwise comparisons were performed using a two-way ANOVA. P-values: a=0.036, b=0.024.

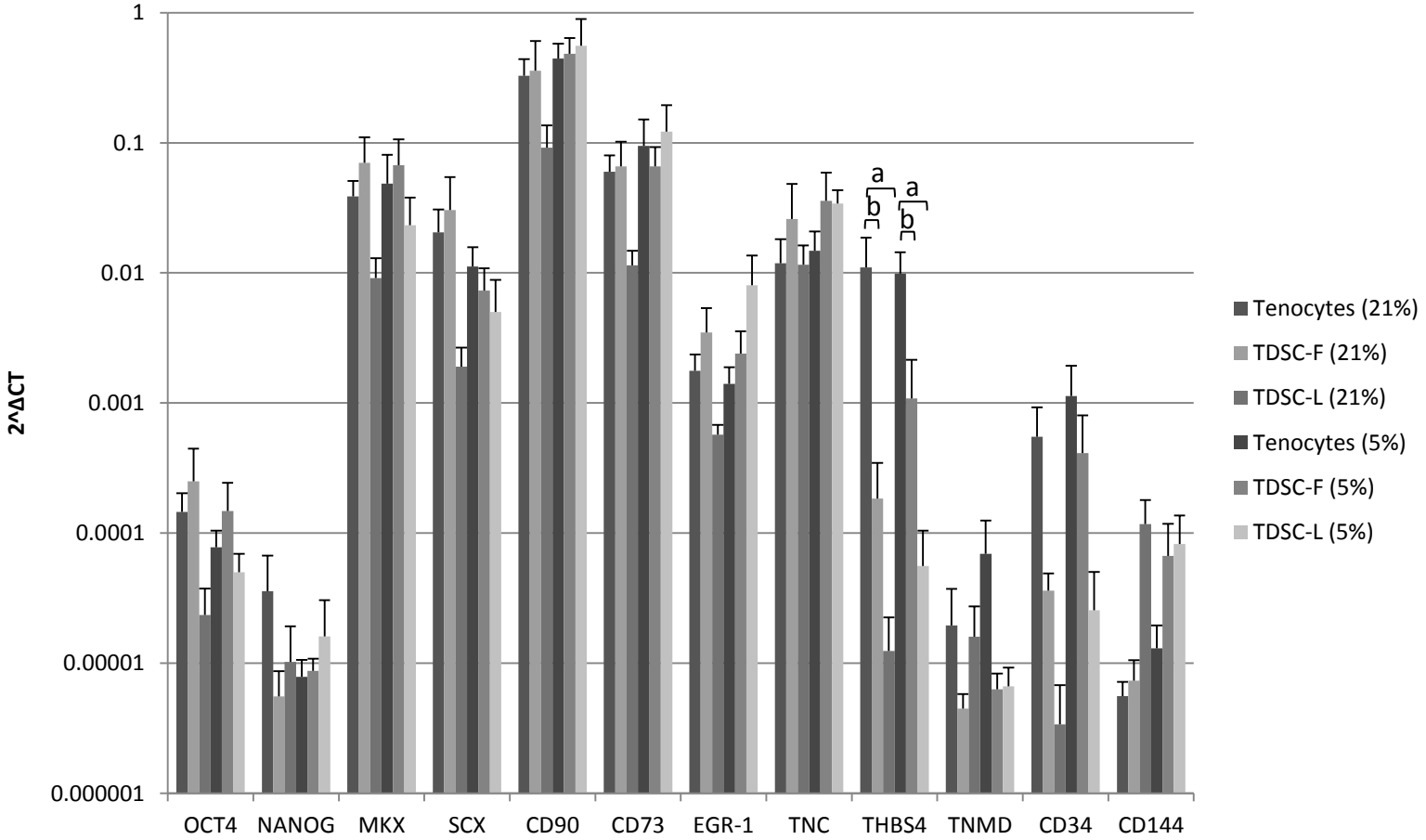


Figure 3.4. Gene expression analysis of stem cell markers in tenocytes and TDSCs at 21% and 5% oxygen. Values are shown on a logarithmic scale and normalised to GAPDH. n=6 biological replicates for tenocytes and TDSC-F at 5% oxygen, n=4 for all other samples. Error bars shown represent SEM. Pairwise comparisons were performed using a two-way ANOVA and Tukey post-hoc test after Log_{10} transformation. P-values: a= 0.042, b=0.036.

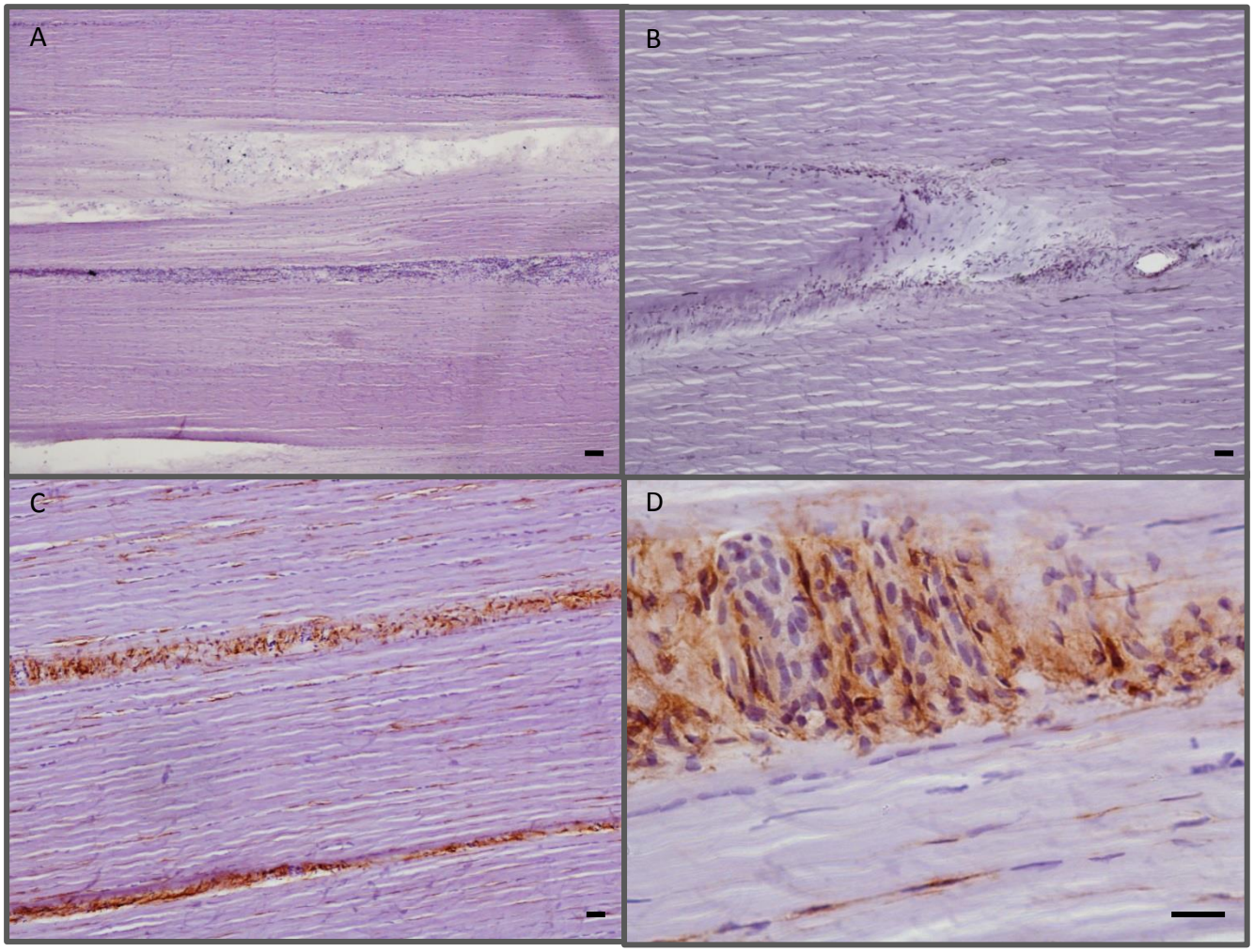


Figure 3.5. Analysis of stem cell marker expression of SDFT by immunohistochemistry. Control frozen sections were incubated with secondary antibody only (A) and mouse IgG and secondary antibody (B). Frozen sections of SDFT were stained with anti-CD90 (C-D) and anti-CD73 (no staining observed so images not shown). Bar = 100 µm.

3.4.4 Tenocyte and TDSC tri-lineage differentiation potential

The ability of tenocytes and TDSCs to differentiate into different cell lineages was analysed by staining, glycosaminoglycan (GAG) assays and qRT-PCR for gene expression analysis following tri-lineage differentiation.

All three cell types showed osteogenic differentiation as assessed by BCIP/NBT and alizarin red (AR) staining. The BCIP/NBT staining procedure stains active alkaline phosphatase enzyme and exhibits as dark purple or black staining as well as the formation of dark precipitate. Alizarin red stains calcium nodules and manifests as a red stain. Conversely no adipogenic differentiation was seen in any cell type as assessed by Oil red O (ORO), which stains oil droplets and which also exhibits as a red stain. Chondrogenic pellets were sectioned and stained with alcian blue (AB) to highlight the presence of GAGs, which was mostly clearly seen in TDSC-F, however the intensity of stain did not alter between the negative (control media) and positive (chondrogenic induction media) pellets for tenocytes or TDSC-L (Fig.3.6). There was a significant increase in GAG production in positive pellets compared with negative pellets for TDSC-F and TDSC-L. For TDSC-F there was an increase in the mean (±SEM) GAG content from 1.5 (±0.3) µg in the negative pellet to 3.8 (±0.3) µg in the positive pellet. Similarly for TDSC-L there was an increase from 0.9 (±0.5) µg to 3.7 (±0.6) µg (Fig.3.7).

Gene expression analysis of lineage specific genes showed a lack of osteogenic differentiation in all three cell types. There were small increases seen for some osteogenic markers when comparing the negative and positive samples, for example runt-related transcription factor-2 (RUNX2), osteocalcin (OC) and particularly osterix, which was significantly increased in tenocytes and TDSC-F. However for many of the osteogenic markers there was no alteration in expression or a decrease was observed; for example COL1A1 and osteoprotegerin (OPG) which showed significant decreases in gene expression in positive samples of tenocytes and TDSC-F. The expression of adipogenic markers was increased in the positive samples compared with the negative samples for all cell types, however these increases were small and not significant. Chondrogenic gene expression varied between cell types, with no significant alterations (Fig.3.8).

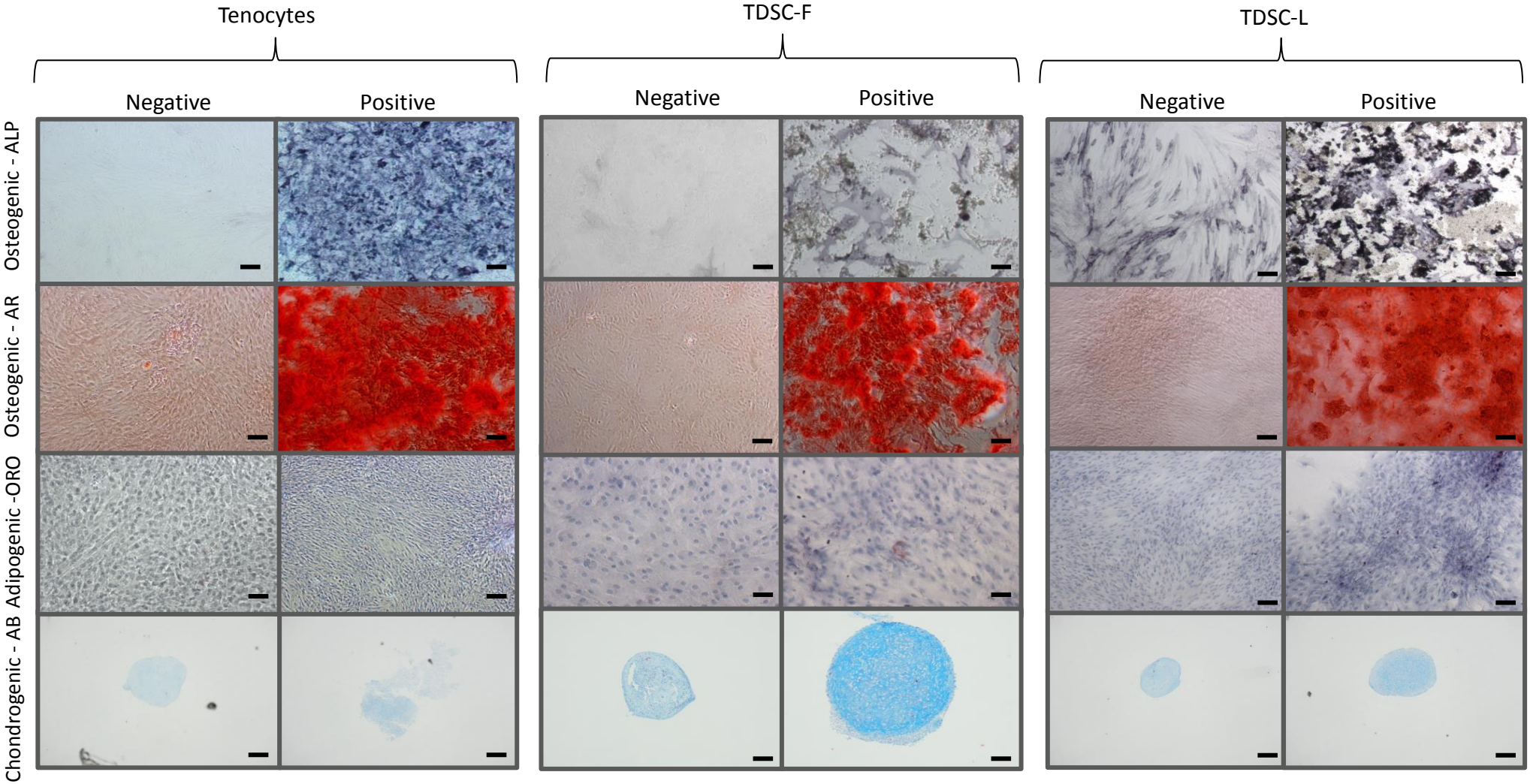


Figure 3.6. Histological analysis of tri-lineage differentiation potential of tenocytes and TDSCs at 5% oxygen. Representative images are shown for each cell type after induction of osteogenic, adipogenic and chondrogenic differentiation (positive) and also for control samples (negative), after appropriate staining. Cells subjected to osteogenic differentiation media were stained for both alkaline phosphatase (ALP) activity and calcium deposits using alizarin red (AR). Cells subjected to adipogenic differentiation media were stained for oil droplet formation using oil red O (ORO), and cell pellets exposed to chondrogenic differentiation media, for GAG formation using alcian blue (AB). Bar = 100 µm. n=3 biological replicates for tenocytes, n=5 for TDSC-F and n=4 for TDSC-L.

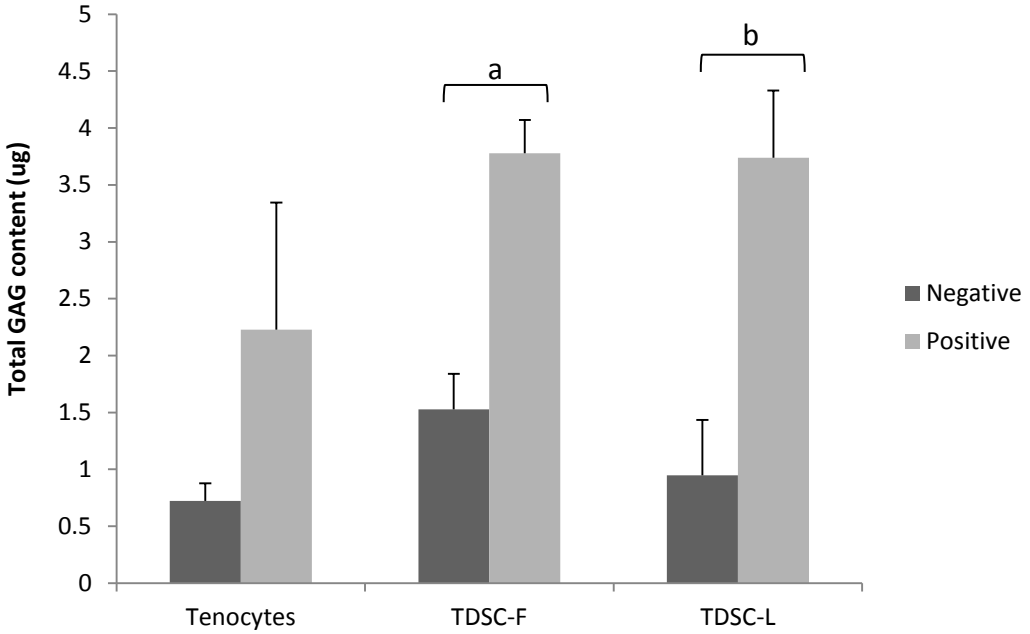


Figure 3.7. Total glycosaminoglycan (GAG) content of cell pellets with (positive) or without (negative) chondrogenic induction at 5% oxygen. n=3 biological replicates for tenocytes, n=5 for TDSC-F and n=4 for TDSC-L, error bars shown represent SEM. Pairwise comparisons were performed using paired Student's t-tests. P-values: a=0.005, b=0.003.

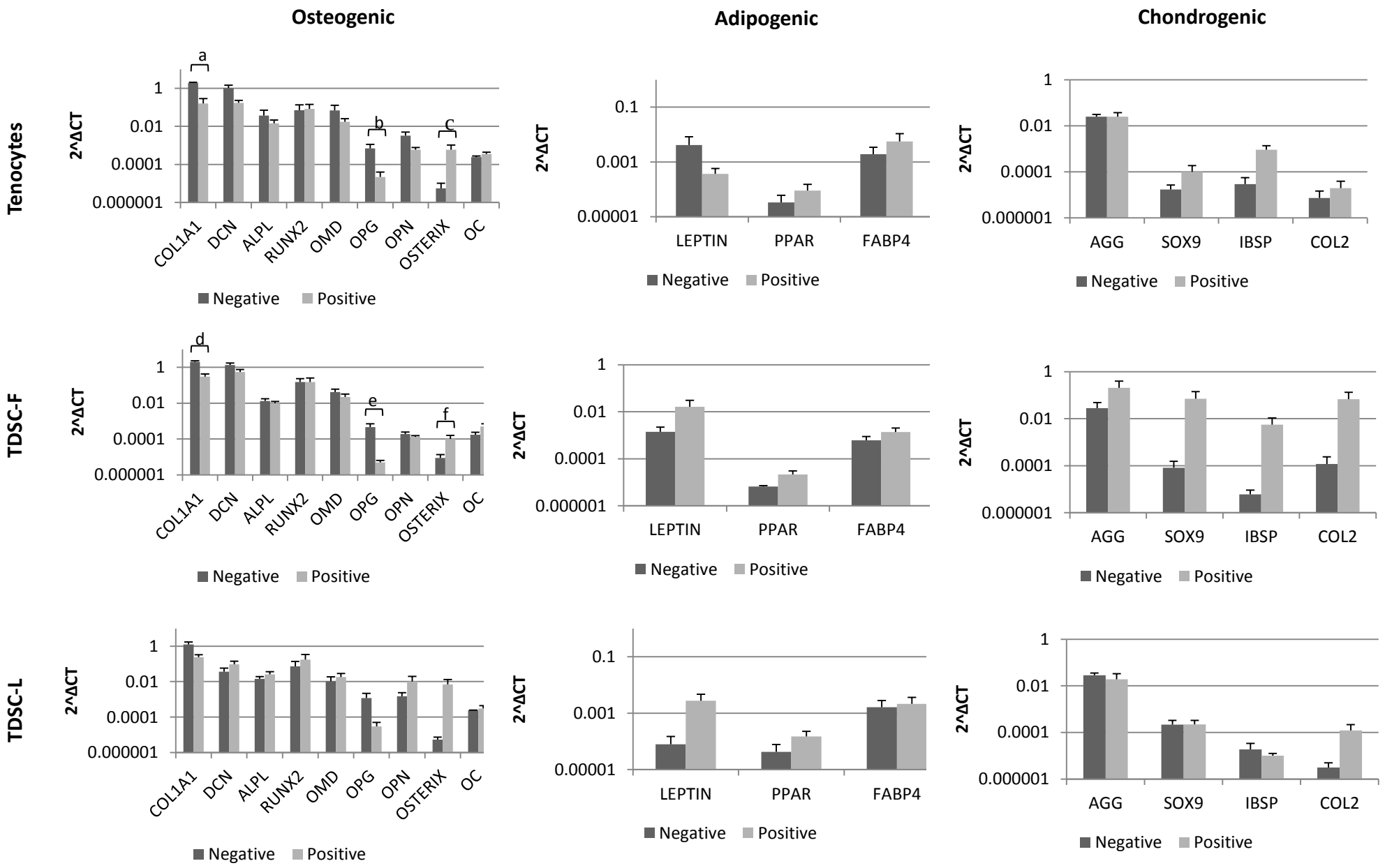

Osteogenic
Adipogenic
Chondrogenic
Tenocytes
TDSC-F
TDSC-L
2^ΔCT
a
b
c
d
e
f
1
0.01
0.0001
0.000001
0.1
0.001
0.00001
COL1A1
DCN
ALPL
RUNX2
OMD
OPG
OPN
OSTERIX
OC
LEPTIN
PPAR
FABP4
AGG
SOX9
IBSP
COL2
Negative
Positive

Figure 3.8. Gene expression analysis of lineage specific markers by tenocytes and TDSCs at 5% oxygen. Values are shown on a logarithmic scale and normalised to GAPDH. n=3 biological replicates for tenocytes, n=4 for TDSC-L and n=5 for TDSC-F, error bars shown represent SEM. Pairwise comparisons were performed after Log_{10} transformations using paired Student's t-tests. P-values: a=0.049, b=0.049, c=0.049, d=0.009, e=0.009, f=0.047.

3.4.5 Tenocyte and TDSC niche composition

Histological analysis of equine SDFT tissue showed distinct regions or 'niches' within tendon. Haematoxylin and eosin staining (3.9.A) showed highly cellular interfascicular matrix (IFM) regions and less cellular fascicular regions. The morphology of the cells also differed between regions with those residing in the IFM having a more rounded shape and those in between the fascicles being flatter and more spindle shaped. In addition, Masson's Trichrome staining (3.9.B) showed that SDFT is poorly vascularised and that the vasculature is confined to the IFM region.

Immunohistochemical analysis revealed obvious staining for tenascin C (3.9.C) which was restricted to the IFM, conversely the large quantities of decorin (3.9.D) seen in SDFT were predominantly found within the fascicular regions, however decorin was also shown to be present within the IFM.

Gene expression analysis of SDFT ECM components showed only small and insignificant differences between cell types and oxygen tensions (Fig.3.10).

Radioactive labelling of equine SDFT ECM proteins showed similar profiles for all three cell types and both oxygen tensions. Due to the reduced incorporation of ^{14}C proline the bands corresponding to collagenous proteins were not as clear as the non-collagenous protein bands, and there appeared to be several bands seen at 21% oxygen which were not seen at 5%, however this pattern was consistent across the cell types. The labelling profile for ^{14}C lysine and arginine appears to be the same for all three cell types and both oxygen tensions.

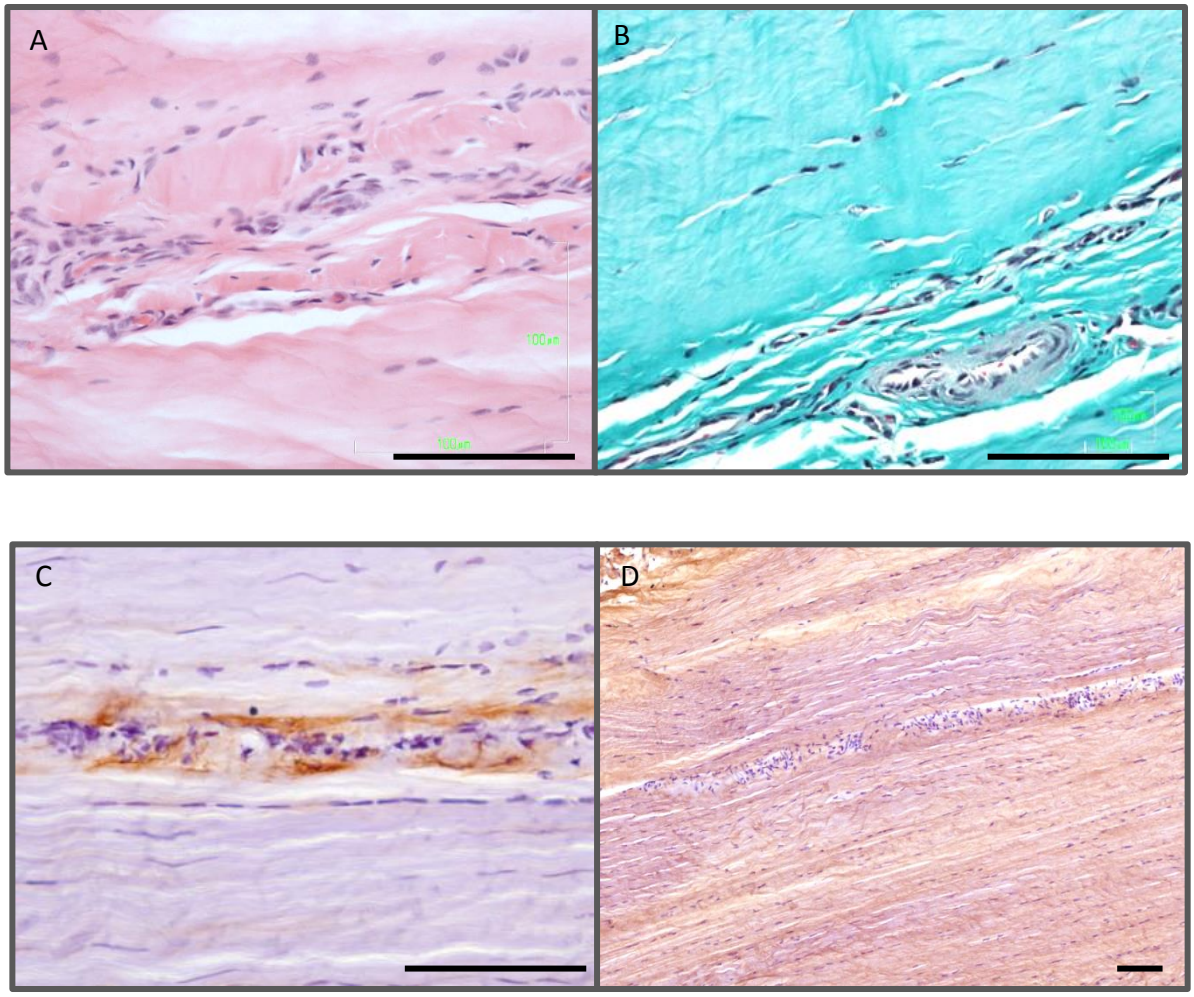


Figure 3.9. Equine SDFT histology (A & B) and immunohistochemistry (C & D) images. Paraffin embedded sections of equine SDFT were stained with haematoxylin and eosin (A) or Masson's Trichrome (B). Frozen sections of equine SDFT were stained with antibodies raised against tenascin C (C) and decorin (D). Representative images are shown. Bar = 100 µm.

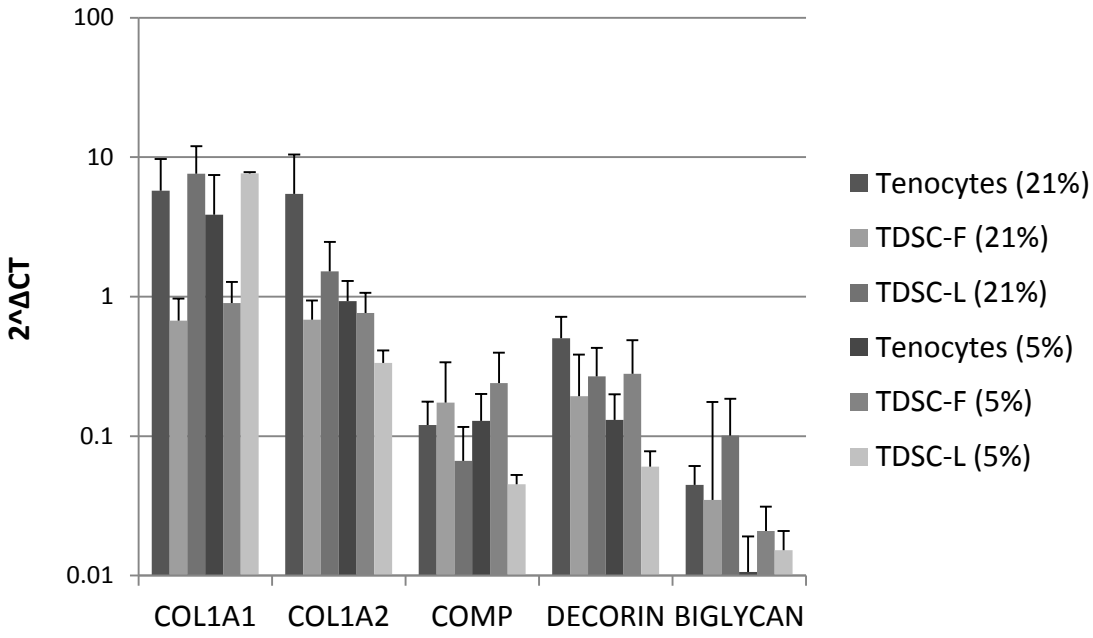


Figure 3.10. Gene expression analysis of ECM components in tenocytes and TDSCs at 21% and 5% oxygen. Values are shown on a logarithmic scale and normalised to GAPDH. n=4 biological replicates for tenocytes and n=3 for TDSCs. Error bars shown represent SEM. Pairwise comparisons were performed using a two-way ANOVA with Tukey post-hoc tests after Log_{10} transformation.

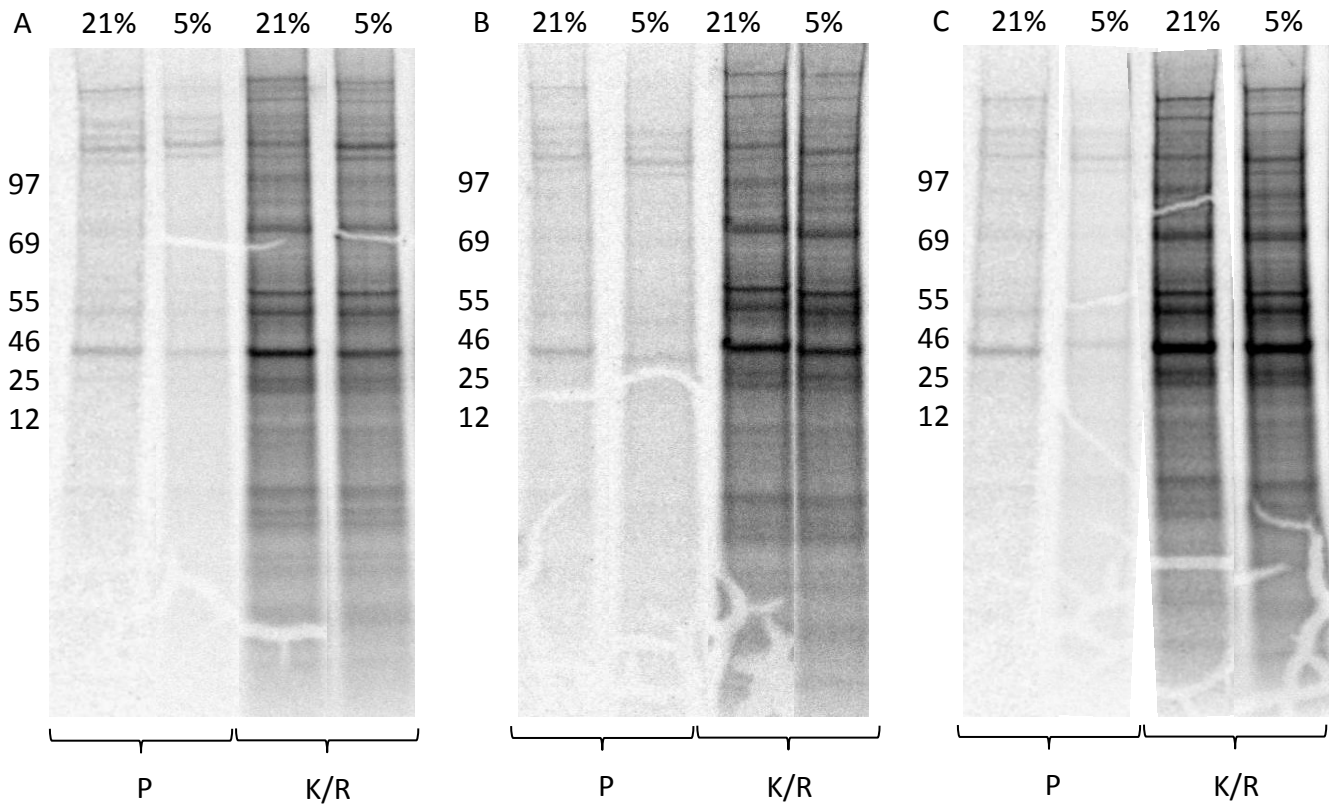


Figure 3.11. ^{14}C labelling of tenocytes (A) TDSC-F (B) and TDSC-L (C). ^{14}C proline (P) labelled collagenous proteins produced by tendon cells and ^{14}C lysine (K) and arginine (R) labelled non-collagenous proteins which were analysed by autoradiography. 21% and 5% represent cells grown at 21% and 5% oxygen tensions respectively. Representative images are shown.

3.5 Discussion

3.5.1 Isolation of equine tendon cells

TDSCs have been isolated from a number of different species, including human, mouse (Bi *et al*., 2007), rat (Rui *et al*., 2010), rabbit (Zhang and Wang, 2010b), cow (Yang *et al*., 2016) and horse (Lovati *et al*., 2011). Species variation may account for some of the differences seen in the properties and phenotype of TDSCs, as may other factors including age and source. The method of TDSC isolation and the conditions in which the cells are cultured is another factor that could potentially have a large impact on TDSC survival and function. The majority of studies to date have used variations of a single method to isolate TDSCs. This method, known as the 'low-density plating method' involves seeding tendon cells after digestion, at very low density (Bi *et al*., 2007), the exact density varies from study to study. The cells are then cultured at 21% oxygen, 5% carbon dioxide and 37°C for varying lengths of time before colonies are selected and propagated (all studies discussed below have used this method). This study was unable to replicate the findings shown in the literature based on this method, the reasons for which will be discussed below. Therefore alternative methods were investigated in order to isolate TDSCs from equine tendon. Hypoxia has been shown to promote the viability of stem cells from a variety of sources, including MSCs from cord blood (Co *et al*., 2014) and TDSCs from tendon (Zhang and Wang, 2013b). In addition, seeding freshly isolated cells on to certain substrates promotes the growth of stem cell-like cells. For example, bovine articular cartilage was digested and seeded on to fibronectin substrates and cells with stem cell-like properties showed differential adhesion to fibronectin, these cells were further cultured (Dowthwaite *et al*., 2004). Endothelial progenitor cells have also been differentially isolated by adhesion to fibronectin substrates (Bueno-Betí *et al*., 2013), indicating the potential use of this method for isolation of a range of progenitor cells.

This study aimed to characterise stem/progenitor cells derived from equine superficial digital flexor tendon and provide a comparison with tenocytes (tendon fibroblasts). Several properties of these cells were assessed in order to determine

whether the cells were true stem cells or a form of progenitor cell. TDSCs are clonogenic, multipotent and express both stem cell and tenogenic markers (Bi *et al*., 2007). All of these properties are hallmarks of MSCs, as outlined by the International Society for Cellular Therapy (ISCT) and are the specific criteria by which MSCs are identified and characterised (Dominici *et al*., 2006). As a subset of MSCs, TDSCs can also be identified and characterised using the same criteria.

3.5.2 Clonogenic potential and morphology of equine tendon cells

Three cell types were investigated in this study: tenocytes; TDSCs isolated by adhesion to fibronectin substrates (TDSC-F); and TDSCs isolated by the conventional low-density plating method (TDSC-L), and all cell types were analysed after culture at both 21% and 5% oxygen tension. All cell types demonstrated a similar pattern of growth, with similar morphologies and colony forming ability (Fig.3.1.A). For all three cell types there appeared to be growth of two different cell populations, directly after seeding and at further passages. Cells with a fibroblast-like spindle-shaped morphology, typical of fibroblasts, grew in large, sparse colonies, whereas cells with a more rounded, cobblestone-like appearance grew in smaller, very dense clusters (Fig.3.1.A). Tenocytes have consistently been shown to demonstrate a typically fibroblastic morphology (Lohberger *et al*., 2015; Wang *et al*., 2003; Zhang and Wang, 2010b), consistent with this study. In contrast, there are mixed reports on the morphology of TDSCs, with some studies showing a tenocyte-like morphology (Nagura *et al*., 2016; Salingcarnboriboon *et al*., 2003; Shi *et al*., 2012a), some a rounded, cobblestone appearance (Tao *et al*., 2015; Zhang and Wang, 2010b, 2014) and others demonstrating an intermediate fusiform morphology (Yang *et al*., 2016). Equine TDSCs have been shown to demonstrate a fibroblastic morphology (Lovati *et al*., 2011), which is partially consistent with this study, however there have been no reports of rounded, cobblestone-like cells in equine tendon. In addition, studies characterising equine MSCs have also reported a fibroblast-like cell morphology for these cells (Hillmann *et al*., 2015; Lovati *et al*., 2012; Violini *et al*., 2009).

The studies discussed above only noted one cell population in their tendon cell cultures, however I have demonstrated the presence of two distinct cell morphologies in equine tendon cell cultures. The previous literature has reported the requirement for low seeding densities when isolating stem cells, as cell contact has been shown to regulate cell differentiation in cortical stem cells (Tsai and McKay, 2000). Additionally, for TDSCs low density plating has been found to promote stem cell growth, proliferation and viability, as well as producing pure cell cultures (Lee *et al*., 2012; Rui *et al*., 2010; Tan *et al*., 2012a). Therefore in this study I seeded tendon cells at a very low density, either on plastic (TDSC-L) or fibronectin (TDSC-F) in order to select for TDSCs. Any tenocytes present would not survive due to the low density and the requirement for cell-cell contact (Güngörmüş and Kolankaya, 2008; Schulze-Tanzil *et al*., 2004). According to the literature this method should provide a pure culture of TDSCs. Conversely I seeded cells at a high density in order to select for tenocytes, as any TDSCs present would be unable to proliferate and would differentiate into tenocytes (Güngörmüş and Kolankaya, 2008; Schulze-Tanzil *et al*., 2004), which should provide a pure culture of fibroblast cells. However this was not the case and two distinct cell populations were seen for all three cell types. This may be expected for tenocyte populations as all cells from the tendon digest were seeded together. However for TDSCs, after initial plating and colony formation, individual colonies were isolated leaving behind any contaminating tenocytes. Regardless of this precaution, fibroblastic cells, which based on previous literature I identify as tenocytes, appear to have contaminated rounded, cobblestone-like TDSC populations. Ideally fluorescently activated cell sorting (FACS) or a similar technique would be employed to definitively isolate TDSCs from tenocytes, however there are several problems with this. Firstly, there are no specific markers for TDSCs, so a panel of markers would need to be used which would complicate the isolation procedure. Secondly, it is not known how the phenotype of the elongated fibroblastic cells differs from that of the rounded cobblestone-like cells and therefore it is not determined which markers would be useful to select for separation of these two cell types. Finally, the lack of antibodies for equine research would severely limit which markers could be used, if any. For these reasons I was unable to isolate pure cultures of tendon cells, and the cell

populations used for further analysis are most likely a heterogeneous mixture of cells at different stages of differentiation. One other study has demonstrated the presence of two distinct cell populations in tendon cell cultures. Ruzzini and others (2013) noted growth of both rounded, cobblestone-like cells as well as elongated fibroblast-like cells when isolating human TDSCs (Ruzzini *et al*., 2013). They state that the former cells they determined to be TDSCs and the latter cells tenocytes. They separated these two cell populations based on CD44 expression and all further analysis was performed on CD44+ cells (Ruzzini *et al*., 2013). Although in theory this is the most logical protocol to adopt, CD44 expression is not specific to TDSCs or stem cells in general. Tenocytes have also been reported to express CD44 (Crockett *et al*., 2010), which means the cells isolated by Ruzzini and others (2013) may still have been contaminated with tenocytes and therefore also a heterogeneous mixture of cells.

The first criterion for the characterisation of MSCs is the ability of the cells to form colonies (Dominici *et al*., 2006) and in this study both tenocytes and TDSCs formed heterogeneous colonies (Fig.3.1.A). Previous studies have all demonstrated the ability of TDSCs to form colonies, although the appearance of these colonies has been shown to differ between studies. Bi and others (2007) found mouse TDSCs form heterogeneous cell colonies, in terms of colony size, shape and cell density, whereas human TDSCs formed more homogenous, dense colonies (Bi *et al*., 2007). Similarly, other studies have also reported heterogeneity in colony size as well as cell density and morphology (Mienaltowski *et al*., 2013; Rui *et al*., 2010; Zhang and Wang, 2010b), consistent with this study. Ruzzini and others (2013), the only study to identify two tendon cell populations, found that the rounded, cobblestone-like cells formed colonies, whereas the fibroblast-like cells did not (Ruzzini *et al*., 2013) which is partially consistent with my data. Hypoxia has been shown to increase the colony-forming ability of human TDSCs, with increased colony numbers and increased cell density (Lee *et al*., 2012; Zhang and Wang, 2013b). No significant differences between oxygen tensions were observed in this study for colony number or size (Fig.3.1.B-C). This is in contrast to the aforementioned studies; the reason for this discrepancy may be due to species variation, as discussed previously (Bi *et al*., 2007).

3.5.3 Proliferation of equine tendon cells

The current literature shows a great deal of variation in relation to the population doubling times (PDT) of TDSCs. This variation can generally be attributed to species variation as well as differences in tendon types. However extreme variation has also been shown between studies focussing on the same tendon within the same species (Lee *et al*., 2012; Zhang and Wang, 2014), which may be attributable to differences in culture conditions, passage number, patient age (Zhou *et al*., 2010) or patient exercise history (Rui *et al*., 2013a).

This study found no significant differences in PDT for tenocytes and TDSCs (Fig.3.2) and only one other study has compared the properties of these two cell types. Zhang and others (2010b) demonstrated a significant increase in PDT for tenocytes isolated from rabbit patellar and Achilles tendon when compared with TDSCs (Zhang and Wang, 2010b), which is inconsistent with my data. The larger differences seen in their study may be due to species and tendon type differences, as well as variation in culture conditions. The values observed in this study are considerably higher than those reported by Lovati and others (2011) for TDSCs isolated from equine SDFT (Lovati *et al*., 2011). This difference in PDT may be due to the different initial seeding densities used, with this study seeding cells at 100 cells/cm^2 and Lovati and others (2011) seeding at 10,000 cells/cm^2 (Lovati *et al*., 2011). The different cell seeding densities may select for slightly different tendon cell populations. Lee and others (2012) showed a similar PDT for human patella TDSCs in both hypoxic and normoxic conditions (Lee *et al*., 2012), similarly this study found no significant differences between the PDT of cell types grown at 5% and 21% oxygen. In addition, no differences were seen in a study investigating the PDT of human tenocytes at 20%, 10% and 2.7% oxygen (Webster and Burry, 1982).

3.5.4 Stem cell and tenogenic marker expression of equine tendon cells

To date there is no one marker which identifies a cell as a TDSC, therefore a panel of markers need to be used, which include traditional pluripotency markers, MSC markers and tenogenic markers. Together, this panel of markers will ensure

that isolated cells are both stem cell-like and from the tenogenic lineage, rather than from blood or surrounding tissue. In this study marker expression of tenocytes and TDSCs was analysed using flow cytometry and qRT-PCR, as well as investigating marker expression in whole tendon tissue sections using immunohistochemistry.

Flow cytometric analysis of TDSCs and tenocytes showed high expression of CD90 in this study for all cell types at both oxygen concentrations, particularly TDSC-F at 5% oxygen with this cell type expressing significantly higher levels of CD90 than tenocytes and TDSC-L (Fig.3.3). This is in accordance with the literature which also demonstrates high expression of CD90 in TDSCs (Alberton *et al*., 2014; Chen *et al*., 2015c) and equine tenocytes (Cadby *et al*., 2014). I found much lower expression of CD73 and CD105 across all three cell types (Fig.3.3) which is in contrast to previous studies which have demonstrated high expression of both markers in human (Lee *et al*., 2012; Stanco *et al*., 2014), mouse (Alberton *et al*., 2014) and rat (Rui *et al*., 2013a) TDSCs. These differences may be due to the species, however equine tenocytes also highly express CD105 (Cadby *et al*., 2014), as do equine MSCs, although these cells did not express CD73 (Braun *et al*., 2010). Another study found that equine MSCs did not express either CD105 or CD73 (Hillmann *et al*., 2015) which mirrors the results found in this study. The discrepancies between studies may be due to differences in breed or age of the horses, or due to differences in sample collection, for example the first study isolated MSCs from live horses, whereas the latter study isolated from cadavers. Alternatively, the differences in marker expression may be due to variations in the culture conditions of the cells, as one study found significant differences in rat TDSC gene expression with prolonged cell culture periods (Guo *et al*., 2016). Another possibility to explain the discrepancies between this study and others is the use of antibodies. Equine antibodies are poorly defined and validated, and although all of the antibodies used in this study had been carefully selected based on use in previous studies, there is still a possibility that the antibodies chosen were unable to bind the antigen. I was unable to isolate MSCs as a positive control cell type due to material restrictions, which would have aided antibody validation. Across all cell types no significant differences in CD90, CD73 or CD105 marker expression were observed in this study between 21% and 5% oxygen which is in accordance with published data for human

TDSCs (Lee *et al*., 2012). As can be seen on the flow cytometry plots, there are two peaks for many of the samples, particularly TDSC-L and tenocytes. This may be due to cellular debris and other particles, however the use of a threshold gates out any small particles. Therefore it is possible that the two peaks relate to two different cell populations, and that each sample, rather than being a pure culture of TDSCs or tenocytes, is actually a heterogeneous mixture of cells at different stages of differentiation.

As mentioned previously the ISCT have published minimal criteria to define MSCs, the second of these criterion was that the isolated cells expressed CD90, CD105 and CD73 (Dominici *et al*., 2006). As I have shown, this study found high expression of CD90, but low expression of CD105 and CD73 in equine TDSCs, and as TDSCs are believed to be a subset of MSCs it would be expected that these cells conformed to the criteria for defining MSCs. The reason for this discrepancy may be that TDSCs have a slightly different phenotype to MSCs, however many studies have shown expression of all three markers in TDSCs as discussed previously. Another possibility is species variation. The criteria outlined by the ISCT are based on human MSCs, so it is possible that equine MSCs or TDSCs may have a slightly different phenotype to human cells. This may indeed be the case as studies investigating MSC and tenogenic marker expression in horses have produced varying and conflicting results (Burk *et al*., 2014; Radcliffe *et al*., 2010; Ranera *et al*., 2011). In addition, the lack of equine antibodies is also problematic for identification of equine MSC markers, as discussed previously. Another issue with this criterion from the ISCT is the lack of specificity of the three markers. Although CD90, CD105 and CD73 have traditionally been used to identify MSCs, they are also present on a number of other cell types including tendon fibroblasts (Cadby *et al*., 2014; Tsuzaki *et al*., 2005), dermal fibroblasts and hepatocytes (Lupatov *et al*., 2015). For this reason, using only CD90, CD105 and CD73 as markers for positive identification of TDSCs is insufficient and other markers must also be utilised. It is necessary to use stem cell markers, MSC markers and tenogenic markers to ascertain if the isolated tendon cells are indeed TDSCs. Due to the limited number of equine antibodies available, particularly for stem cell markers, I decided to investigate gene expression of a panel of markers. The most interesting outcome from this data was the lack of any

statistically significant differences in gene expression between cell types or oxygen tensions, except for one gene: thrombospondin-4, which may be a false-positive result. This is suggestive of a similar gene expression profile for the three different cell types, or that the cell types are all very similar.

Gene expression of the pluripotency markers Oct4 and Nanog was generally low in all cell types (Fig.3.4), which is consistent with some of the literature, which has shown no expression of Oct4, and low expression of Nanog in TDSCs (Alberton *et al*., 2014) and tenocytes (Cadby *et al*., 2014; Zhang and Wang, 2010b). This is also consistent with reports that Oct4 expression is restricted to embryonic stem cells only and not somatic cells (Lengner *et al*., 2008). However other studies have shown expression of Oct4 in equine TDSCs (Lovati *et al*., 2011) and both Oct4 and Nanog in rat (Tan *et al*., 2013) and human (Zhang and Wang, 2013b) TDSCs. These studies may have been identifying non-functional pseudogenes.

Mohawk (MKX), scleraxis (SCX) and early growth response 1 (EGR-1) are all transcription factors involved in tendon development. They are expressed by tendon progenitor cells and are involved in tendon cell differentiation (Lejard *et al*., 2011; Liu *et al*., 2010; Schweitzer *et al*., 2001; Tao *et al*., 2015). These three markers were more highly expressed than the pluripotency markers, however there were no significant differences (Fig.3.4). SCX has been shown to be expressed in mouse (Alberton *et al*., 2014), rat (Chen *et al*., 2015c) and human (Lee *et al*., 2012) TDSCs and equine tenocytes (Cadby *et al*., 2014). No significant differences in SCX gene expression have been seen at different oxygen tensions (Lee *et al*., 2012), consistent with this study. EGR-1 has been shown to be expressed in rabbit TDSCs, where it promotes tendon differentiation and therefore the expression of tenogenic markers, including SCX and tenomodulin (Tao *et al*., 2015). SCX is expressed in all tendon cells during development, in mature tendon and in terminally differentiated cells, whereas MKX is only expressed by tendon cells during development and TDSCs in mature tendon (Liu *et al*., 2010). The only study to demonstrate MKX expression in TDSCs used bovine foetal tendon, and found that expression declined during later developmental stages (Yang *et al*., 2016). Therefore, the expression of MKX by tenocytes seen in this study is surprising, and may provide further evidence that this cell population is heterogeneous.

Using flow cytometry I have shown that tenocytes and TDSCs express CD90 but only low levels of CD73, however the gene expression of these markers was also analysed. Consistent with the flow cytometry data I found high expression of CD90 in all cell types, however in contrast to the earlier data I also found high expression of CD73 (Fig.3.4). The reason for the high gene expression of CD73 when compared with protein expression may be due to post-transcriptional degradation of mRNA (Greenbaum *et al*., 2003) or possibly due to the equine antibody used, as discussed previously.

Tenascin C (TNC), tenomodulin (TNMD) and thrombospondin-4 (THBS4) are glycoproteins found in the tendon extracellular matrix and are therefore used as tendon markers (Kannus, 2000; Thorpe *et al*., 2013). TNC was highly expressed in all cell types in this study (Fig.3.4), consistent with findings in rat (Chen *et al*., 2012a), human (Lee *et al*., 2012), mouse (Zhang and Wang, 2013b) and rabbit (Zhang and Wang, 2010a) TDSCs as well as human tenocytes (Lohberger *et al*., 2015). Hypoxic environments have been shown to have no impact on TNC expression in human TDSCs (Lee *et al*., 2012), consistent with this study. TNC, whilst predominantly expressed in developing tissues, persists in adult differentiated tendon (Schweitzer *et al*., 2001), and therefore its expression in both tenocytes and TDSCs is to be expected. Expression of TNMD was low in all cell types (Fig.3.4), however high expression of TNMD has been demonstrated in mouse (Alberton *et al*., 2014), rat (Chen *et al*., 2015c), human (Lee *et al*., 2012) and rabbit (Tao *et al*., 2015) TDSCs. This discrepancy is most likely due to species variation, as Cadby and others (2014) have demonstrated very low expression of TNMD in equine tenocytes (Cadby *et al*., 2014), consistent with this study. Hypoxia has been shown to increase the expression of TNMD in human TDSCs (Lee *et al*., 2012), which is inconsistent with my data. As with TNC, TNMD is highly expressed during development, however it is also expressed in adult tendon, where it plays a role in the regulation of tenocyte proliferation (Docheva *et al*., 2005). The low expression of TNMD in this study may account for the increased PDTs, when compared with other equine studies. THBS4 expression was high in tenocytes, however significantly lower in TDSCs (Fig.3.4). THBS4 has been shown to be expressed in rat TDSCs (Ning *et al*., 2015) and tenocytes (Jelinsky *et al*., 2010), although expression levels in tenocyte cell cultures

were considerably lower than expression in tendon tissue. Barsby and others (2014) induced equine embryonic stem cells to differentiate into tenocytes in 3D culture and the resulting cells expressed THBS4 (Barsby *et al*., 2014).

CD34 and CD144 are haematopoietic stem cell (HSC) markers and it is necessary to ensure that any tendon cell cultures are not contaminated with HSCs. As the tendon proper is largely avascular, the likelihood of contamination is low, however this can still occur as the endotenon and peritenon contain blood vessels. CD144 expression in this study was very low for all cell types, however CD34 expression was slightly higher, although the level of expression was still low (Fig.3.4), consistent with the literature (Cadby *et al*., 2014; Mienaltowski *et al*., 2013; Stanco *et al*., 2014).

Whole SDFT tissue sections were stained in order to look at the expression, location and distribution of CD markers. CD90 was expressed predominately in the interfascicular matrix (IFM) (Fig.3.5.C-D) which was to be expected due to the increased cellularity of this part of the tendon. Cells residing between fascicles also expressed CD90, however interestingly not all cells within the IFM or between fascicles expressed CD90, suggesting the presence of at least two different cell phenotypes within equine tendon tissue. In contrast there was no expression of CD73 throughout the tissue (Fig.3.5.E-F). These results are consistent with the flow cytometric analysis of tendon cells, however not with the gene expression data, which demonstrated both CD90 and CD73 expression in tendon cells. The possible reasons for this discrepancy have been discussed previously.

3.5.5 Multipotency of equine tendon cells

The final criterion outlined by the ISCT for positive identification of MSCs is the tri-lineage differentiation potential of the cells; the ability to differentiate into osteogenic, adipogenic and chondrogenic cells (Dominici *et al*., 2006). This study has demonstrated the ability of equine TDSCs and tenocytes to differentiate down osteogenic and chondrogenic lineages, but not adipogenic.

The presence of alizarin red staining, which stains calcium nodules, and BCIP/NBT staining, which stains for alkaline phosphatase, both indicated that

osteogenic differentiation had occurred. Staining was observed for all cell types (Fig.3.6), consistent with the literature for rat (Rui *et al*., 2010; Rui *et al*., 2011b), mouse (Alberton *et al*., 2014) and human (Lee *et al*., 2012; Yin *et al*., 2010) TDSCs. The pattern of staining seen in this study was not wholly consistent with the gene expression data. Although expression of some of the osteogenic marker genes was increased in the positive sample (osteogenic induction media), when compared with the negative (control media), the majority of these increases were not significant (Fig.3.8). In fact only osterix expression was significantly increased in tenocytes and TDSC-F. Osterix is a transcription factor vital for osteoblast differentiation and bone formation (Sinha *et al*., 2014). Expression of many of the osteogenic marker genes was similar between the negative and positive groups, the reason for which may be due to a lack of induction in the positive samples. Alternatively, some of the genes may be expressed during the early stages of osteogenic differentiation, therefore when gene expression was analysed after 21 days the expression of such genes had started to decrease, or return to control levels. This may be the case for Runx2, which is a transcription factor with an early role in osteoblast differentiation, whereas osterix is expressed later in the differentiation process (Sinha *et al*., 2014). However this does not account for the low level expression of genes encoding bone proteins such as osteocalcin (OC) and osteomodulin (OMD). In addition, some of the genes were significantly decreased in the positive samples, compared with the negative, the reasons for which are unclear. A possible explanation may be due to the primer sequences used, however all primers were validated (see Appendix B). The expression of osteogenic marker genes observed in this study is not wholly consistent with the literature. Rat TDSCs have been shown to express significantly increased levels of Runx2, osteocalcin and osteopontin in induced samples, when compared with controls (Rui *et al*., 2010). Similarly, TDSCs isolated from mice demonstrated increased expression of alkaline phosphatase and osterix, as well as osteocalcin and osteopontin, after osteogenic induction (Salingcarnboriboon *et al*., 2003). Rabbit (Zhang and Wang, 2010b) and human (Zhang and Wang, 2013b) TDSCs have also been shown to express increased levels of Runx2 after induction of osteogenic differentiation. The study conducted by Bi and others (2007) demonstrated differences in osteogenic gene expression between species, with no

increase seen in alkaline phosphatase gene expression in mice, but an increase in human TDSCs (Bi *et al*., 2007). They also demonstrated an increase in osteopontin in murine TDSCs after osteogenic induction, but no difference in expression of osteocalcin in human TDSCs (Bi *et al*., 2007). The apparent species variation in osteogenic marker expression may account for the lack of induction of many osteogenic markers genes seen in this study. However, Lovati and others (2011) demonstrated increases in the expression of osteocalcin and osteopontin in equine TDSCs (Lovati *et al*., 2011), although these increases were not quantified, and therefore cannot be directly compared with this study. The data obtained in this study and others may indicate that alizarin red staining is not a completely reliable *in vitro* assay for osteogenic differentiation.

No adipogenic differentiation of tenocytes or TDSCs was observed in this study (Fig.3.6) as assessed by oil red O staining, which stains intracellular oil droplets. This is in stark contrast to the previous literature which has demonstrated the ability of TDSCs to differentiate into adipocytes in a number of different species, including mouse, human (Bi *et al*., 2007), rat (Rui *et al*., 2010), rabbit (Zhang and Wang, 2010b) and horse (Lovati *et al*., 2011). Although it should be noted that oil red O staining is not uniform, and for all the studies referenced above there were only isolated areas of staining, indicating that not all of the cells were adipocytes. There are many possible reasons for the discrepancy between my results and those in the literature. These include differences in species, tendon type, age and exercise history of subjects and culture conditions. This assay was validated in other species (see Chapter 4) therefore technical error cannot account for the absence of oil red O staining seen here. As with the osteogenic assays, the adipogenic staining pattern seen in this study is not entirely consistent with the gene expression data. Generally an increase in all adipogenic marker genes for all three cell types was observed in this study, however none of the increases were significant (Fig.3.8). Previous studies have demonstrated increased adipogenic marker gene expression consistent with their oil red O staining. For example, Rui and others (2010) observed an increase in Peroxisome Proliferator-Activated Receptor Gamma 2 (PPARγ2) in rat TDSCs after adipogenic induction, compared with controls (Rui *et al*., 2010). In mice TDSCs, expression of FABP4 and PPARγ2 was increased after adipogenic induction

(Salingcarnboriboon *et al*., 2003). Similarly, in rabbit TDSCs, expression of PPARγ2 was significantly increased after induction (Zhang and Wang, 2010b).

Chondrogenic differentiation of equine TDSCs was observed with an increase seen in alcian blue staining, which stains for glycosaminoglycans (GAGs), for TDSC-F, however the increase in stain intensity was less for TDSC-L and tenocytes (Fig.3.6). This is consistent with the literature, with staining seen in human (Cheng *et al*., 2014a), mouse (Asai *et al*., 2014) and equine (Lovati *et al*., 2011) TDSCs. The GAG content of the pellets for each cell type was assessed as an indication of the extent of chondrogenic differentiation, and I found an increase in GAG content after chondrogenic induction in all three cell types, however this was only significant in TDSCs (Fig.3.7). This is consistent with the literature which has shown an increase in GAG content in human TDSCs after chondrogenic induction (Stanco *et al*., 2014). Although Lovati and others (2011) did not observe a significant increase in GAG content of equine TDSCs after chondrogenic differentiation (Lovati *et al*., 2011). The discrepancy with this study may be due to experimental variation or differences in age and exercise history of the horses. The chondrogenic staining pattern is fairly consistent with the chondrogenic gene expression data in this study. Small insignificant increases in chondrogenic marker gene expression in tenocytes and TDSC-F were observed, however there was very little change in gene expression in TDSC-L (Fig.3.8). This is inconsistent with the previous literature as increases in COL2, ACAN and SOX9 after chondrogenic induction have all been reported in human (Lee *et al*., 2012) and rat (Rui *et al*., 2010) TDSCs, and COL2 and SOX9 in rabbit (Zhang and Wang, 2010b) TDSCs.

I found that tenocytes exhibited the same differentiation potential as TDSCs: osteogenic and chondrogenic differentiation, but no adipogenic. Previous research conducted on tenocytes isolated from equine SDFT found them to possess osteogenic and adipogenic differentiation potential, however they did not test for chondrogenic differentiation (Cadby *et al*., 2014). Consistent with this study they demonstrated insignificant increases in Runx2 expression, but significant increases in osterix expression. Inconsistent with my study was the adipogenic potential of tenocytes (Cadby *et al*., 2014), the possible reasons for this discrepancy mirror those for TDSCs and are discussed previously. Analysis of the multipotency of rabbit

tenocytes showed no differentiation potential (Zhang and Wang, 2010b) and the reason for this inconsistency is likely due to species differences, as highlighted previously.

3.5.6 Equine tendon stem cell niche

One of the initial aims of this study was to analyse the TDSC niche and investigate the differences between the extracellular matrix (ECM) of tenocytes and TDSCs. Therefore I initially performed histology and immunohistochemistry on SDFT sections in order to analyse the structure of the tendon and identify any potential cell niches. Haematoxylin and eosin staining revealed that the majority of cells were located within the interfascicular matrix (IFM) with sparse cells found between the collagen-rich fascicles (Fig.3.9.A). Masson's trichrome stain again demonstrated the presence of many cells within the IFM, as well as blood vessels (Fig.3.9.B). Immunohistochemistry revealed the presence of tenascin C predominantly within the IFM (Fig.3.9.C). Tenascin C is an ECM glycoprotein with important roles during development. In adulthood tenascin C is restricted to areas of high tensile strength, for example tendon, and tissues with high cell turnover, such as stem cell niches (Chiquet-Ehrismann *et al*., 2014). Tenascin C has been shown to play a role in a number of different stem cell niches including neural (Garcion *et al*., 2004), hair follicle (Tucker *et al*., 2013) and osteogenic (Kilian *et al*., 2008) niches. Therefore, the presence of tenascin C within the IFM indicates a potential location for the TDSC niche. The expression of decorin within tendon tissue was also assessed, which was observed predominantly between fascicles, but also within the IFM (Fig.3.9.D). Decorin is a proteoglycan located between tendon fibres, fibrils and fascicles and links collagen fibrils together (Thorpe *et al*., 2013), therefore its location within the fascicular region of tendon is expected. This demonstrates the different regions within tendon, which could provide a niche for TDSCs.

As discussed previously there were very few differences between tenocytes and TDSCs isolated in this study, therefore I expected to see few differences between the surrounding ECM composition of the different cell types. The gene expression of some of the major known constituents of the tendon ECM was

analysed to compare differences between tenocytes and TDSCs. I found no significant differences in expression of any of the ECM markers between cell types or oxygen tensions (Fig.3.10).

Radioactive labelling of cells allows analysis of proteins that are being actively synthesised during the labelling period. Radioactive proline is primarily incorporated into collagenous proteins, and radioactive lysine and arginine into both collagenous and non-collagenous proteins. The purpose of the experiment was to assess whether there were differences in protein production between tenocytes and TDSCs which would be indicative of their environment and niche. However no differences in protein production between the different cell types or oxygen tensions were observed (Fig.3.11). If tenocytes and TDSCs were different cell types and demonstrated different phenotypes then it would be expected that they would require different environments or niches in which to survive and function, therefore I would expect to see production of different ECM proteins, however this was not the case. This suggests that the cell types analysed in this study are cells with a similar phenotype, or a heterogeneous mixture of tendon cells at different stages of differentiation. Alternatively, synthesis of niche components may not be maintained in *in vitro* culture or beyond certain developmental stages.

3.5.7 Conclusion

In this study I have isolated a population of tendon cells that possess some stem cell properties, including the expression of some stem cell markers, clonogenicity and multipotency. However these cells did not express all of the desired markers and they were unable to differentiate into adipogenic cells. These results are not wholly consistent with the previous literature, which has found TDSCs from a number of different species to express all desired stem cell and tenogenic markers and differentiate down all three cell lineages. There are many possible reasons for these discrepancies, the most obvious of which is species variation. As outlined above there has been considerable variation between species both within (Bi *et al*., 2007) and between studies (Asai *et al*., 2014; Rui *et al*., 2013a). Intraspecies variation is also common when investigating TDSCs (Kohler *et*

al., 2013; Lee *et al*., 2012; Ruzzini *et al*., 2013; Zhang and Wang, 2014). The other possible reasons for these discrepancies include the age of the animal, which has an impact on many aspects of TDSCs, as well as injury. I found no impact of age on tendon cell function, however due to material limitations I did not have sufficient numbers of animals at each age to perform any quantitative analysis (data not shown). Gross examination of each SDFT and forelimb revealed no apparent injuries, however I had no details of the animals' medical background and so cannot completely rule out the possibility of other injuries or illnesses that could affect tendon function. Another factor which can affect the function of TDSCs is tendon mechanics. The forces applied to tendons and the level of stretch they are subjected to can have effects on cell phenotype and function. Throughout the *in vitro* experiments no mechanical forces were applied to the cells, however I had no information relating to the exercise history of the horses used, therefore I do not know what forces the tendons were subjected to during the animals' lifetime. Previous literature has demonstrated the beneficial nature of moderate exercise, and the detrimental effects of intensive exercise. Moderate exercise has been shown to promote tenogenic differentiation, whereas intensive exercise promotes both tenogenic and non-tenogenic differentiation (Zhang and Wang, 2010d, 2013a). It is possible that the horses used in this study were subjected to moderate exercise throughout their lifetimes, which promoted tenogenic differentiation of the resident TDSCs, rather than non-tenogenic differentiation.

One of the aims of the study was to compare the differences between tenocytes and TDSCs, both their phenotype and their extracellular environment. Very few differences between tenocytes and TDSCs were observed during this study, which was unexpected. Unfortunately there is only one study to date that has directly compared tenocytes and TDSCs from the same species in a single experiment (Zhang and Wang, 2010b). Zhang and Wang (2010b) found large differences between the two cell types, with tenocytes unable to differentiate down osteogenic, adipogenic or chondrogenic lineages, as well as being negative for stem cell markers. In addition, tenocytes had an elongated cell morphology, whereas TDSCs were cobblestone-like in appearance (Zhang and Wang, 2010b). However, other studies investigating equine tenocytes have found that they are multipotent,

clonogenic and express stem cell markers (Cadby *et al*., 2014). These results are highly consistent with my own and suggest that tenocytes are not distinct from TDSCs, or alternatively that cell separation and isolation techniques require optimisation. When SDFT cells were plated there was growth of two distinct cell populations, which I attempted to separate using cloning cylinders, however it is possible that either cell type contaminated the other and therefore none of the cell populations were pure and were a mixture of tendon cells at different stages of differentiation. This is supported by the immunohistochemical analysis of CD90 expression in SDFT tissue. Cells within the IFM and between the fascicles stained positively for CD90, however not all of the cells in either region were positive, indicating that neither the IFM nor the fascicular regions contain one distinct cell type. Based on previous literature and some of my preliminary findings I expected to observe TDSCs within the IFM and tenocytes in the fascicular regions. This hypothesis is supported to a certain extent by some of my data, for example the localised expression of tenascin C, known to play a role in stem cell niches, in the IFM. However much of my data is contradictory to this hypothesis, predominantly the lack of any real difference between tenocytes and TDSCs. The differential expression of CD90 throughout SDFT tissue, as well as the remarkably similar protein labelling profile of tenocytes and TDSCs suggests that the cells are not residing within different environments. The most likely explanation is that the cells isolated, both tenocytes and TDSCs, are in fact a mixture of cells at different stages of differentiation. In order to further investigate equine TDSCs and tenocytes and their niche, it is first necessary to isolate the different cell types effectively. The most useful tool to do this would be FACS, or a similar technique, however this method has its own disadvantages as discussed previously. However, possibly the largest problem is the lack of a specific TDSC marker. At present a panel of markers has to be used to correctly identify a cell as being a TDSC, which encompass pluripotency, MSC and tenogenic markers. If one specific marker could be found for identification of TDSCs then this would allow great advances in future research in this field. Similarly, if a standard protocol for isolation of TDSCs could be devised, this would also aid the development of research within this area and promote consistency across studies.

Despite the restricted differentiation potential of the tendon cells isolated in this study, they may still be used therapeutically to treat tendinopathies. Current practices extract MSCs from equine bone marrow and adipose tissue, which can involve complicated and painful procedures, before culturing the cells and implanting them at the site of injury (Carvalho *et al*., 2013; Conze *et al*., 2014; Smith *et al*., 2013). If the equine TDSC niche could be characterised, and those components which promote stem cell viability and function identified, then it may be possible to artificially introduce those components to the site of injury. This would have the effect of promoting and stimulating native TDSCs to repair the injured tendon. The restricted differentiation potential of equine TDSCs may actually provide a benefit during times of injury, possibly reducing the tendency towards non-tenogenic differentiation. This research, as well as having potential implications for veterinary medicine, is also translatable for human research. There have been many analogies demonstrated between human and equine tendon, in structure, function and pathogenesis (Innes and Clegg, 2010). Similarities between the two species have also been demonstrated on a cellular level, with MSCs isolated from equine and human adipose tissue demonstrating similar stem cell characteristics (Hillmann *et al*., 2015).

Further research needs to be conducted into the characterisation of equine TDSCs, commencing with the optimisation of isolation procedures and the identification of suitable markers, however current data is encouraging and may provide the basis for future treatment options for tendinopathy.

Chapter 4

Murine tendon-derived stem cell characterisation

4.1 Introduction

As described in Chapters 1 and 3, tendon-derived stem cells (TDSCs) have the potential to treat a range of tendinopathies in both humans and animals. As a form of tendon progenitor cell they can be induced to, and spontaneously, differentiate into tenocytes, the predominant cellular component of tendon and the manufacturers of extracellular matrix (ECM). Therefore they are likely to play a role in natural tendon healing, but also may be of use in tissue engineering strategies. Before such advances can be made, TDSCs, as well as their environment and the interaction between the two, must be fully characterised.

The main focus of this study was to investigate TDSCs in equine tendon, however, as described in Chapter 3, I was unable to replicate previous studies either for equine TDSCs, or other species. Therefore, I decided to try and replicate some of the earlier published studies that focused on murine TDSCs to ensure that methods and techniques were appropriate and that previous studies were replicable.

There have been numerous studies published characterising murine TDSCs, investigating the effects of specific factors on these cells, as well as their use in tissue engineering strategies. Murine TDSCs have been shown to form colonies, express stem cell markers, as well as differentiate into osteogenic, adipogenic and chondrogenic cell types (Bi *et al*., 2007), all characteristics indicative of mesenchymal stromal cells (MSCs) (Dominici *et al*., 2006).

The majority of studies investigating murine TDSCs have used patellar or Achilles tendons for TDSC isolation, however in this study I was unable to obtain cells from these tendons. Both collagenase digest and cell outgrowth methods were employed however no cell growth was observed. Therefore, for all experiments detailed below, murine tail tendons were used for isolation of TDSCs and tenocytes, and in addition I also analysed murine MSCs as a positive control.

4.2 Hypothesis

I hypothesise that a native tendon progenitor cell population resides within murine tail tendon which can be differentially isolated from other tendon cell types by low-density plating. This progenitor cell population will demonstrate all of the hallmark properties of MSCs, including clonogenicity, multipotency and stem cell marker expression, however the cell population will differ from MSCs with the increased expression of tenogenic markers. The progenitor cell population will differ from tenocytes with the latter cell type demonstrating reduced stemness when compared with progenitor cell populations.

4.3 Aim

1. To characterise murine TDSCs and tenocytes isolated from tails, as well as murine MSCs, as a methodological validation of the study of equine TDSC characterisation. Characterisation will include analysis of stem cell and tenogenic marker expression, colony-forming ability, cell proliferation and multipotency.

4.4 Results

4.4.1 Isolation of murine TDSCs

Murine patellar tendon was isolated immediately post-mortem from individual mice before performing a collagenase/trypsin digest, however no cells were observed 14 days after plating. Murine patellar tendons were also pooled in order to increase cell numbers, however after extraction, digestion and culture of four biological replicates there was still an absence of cell growth. Finally, murine patellar tendons were placed in cell culture dishes with appropriate media to allow outgrowth of cells from tissue explants, however again, there was still an absence of cell growth. All subsequent experiments used murine tail tendon for isolation of tendon cells (Chapter 2, Section 2.2.1.2).

4.4.2 Tenocyte, TDSC and MSC morphology and colony formation

All three cell types exhibited slightly varying cell morphologies. Tenocytes demonstrated a fairly compact cell morphology, neither rounded nor fibroblastic, whereas TDSCs displayed a rounded morphology. MSCs had a more fibroblastic appearance, however there were dense areas of growth where the cells were more rounded (Fig.4.1.A).

All three cell types were able to form colonies, however these colonies were not homogeneous. Tenocytes generally formed large sparse colonies, whereas TDSCs formed more compact, dense colonies. MSCs formed smaller fairly sparse colonies with areas of compact cell growth (Fig.4.1.A). When quantified there was very little difference in colony number between tenocytes and MSCs, however there was a statistically significant difference between tenocytes and TDSCs (Fig.4.1.B). A similar pattern was observed for colony size, with all three cell types exhibiting similar colony sizes, with tenocytes the smallest and MSCs the largest (Fig.4.1.C).

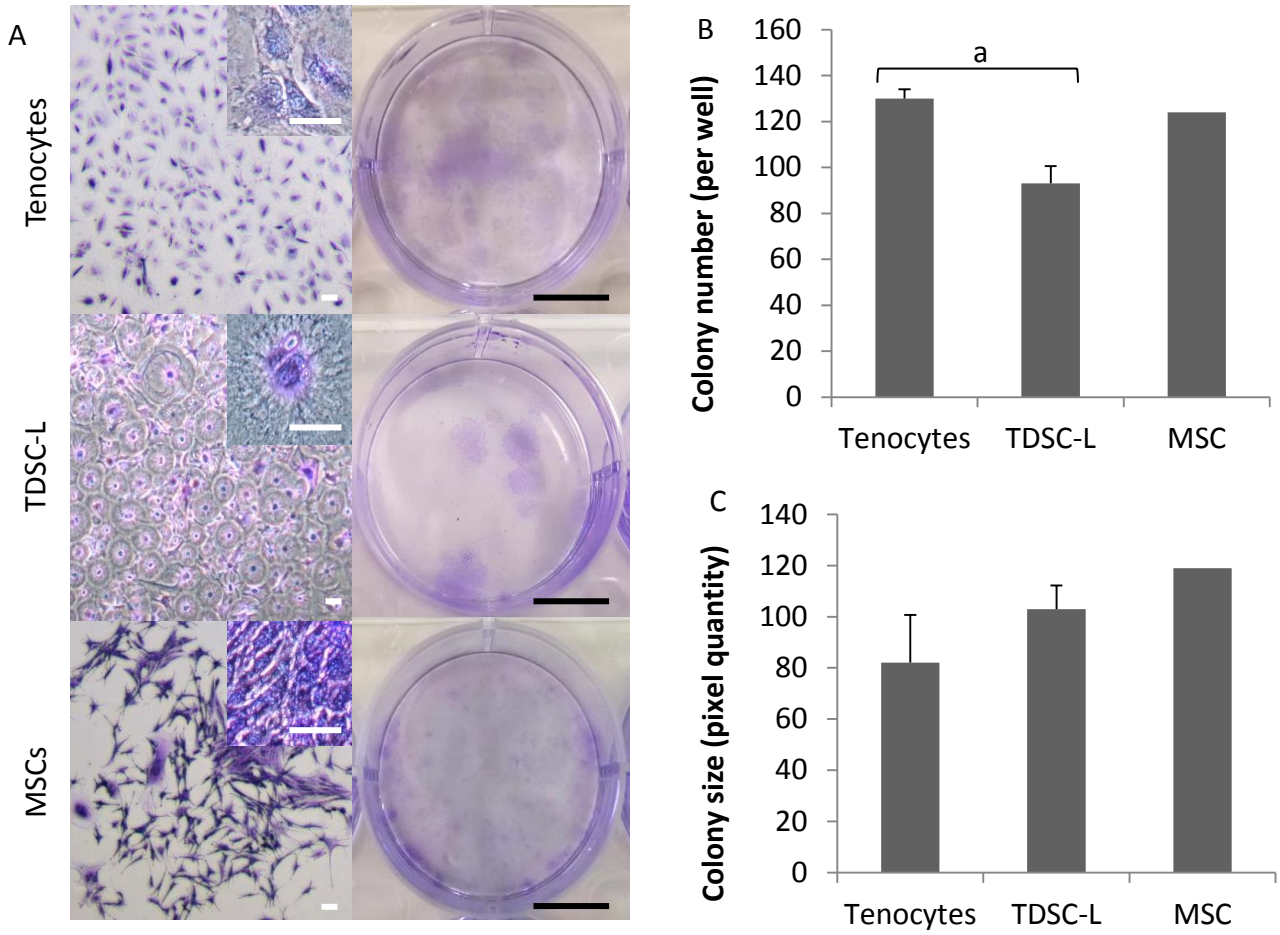


Figure 4.1. Tenocyte, TDSC and MSC morphology and colony formation. Representative images of cell morphology and colony forming ability are shown. Cells were grown for 7 days before staining with crystal violet and imaging for cell morphology analysis and colony formation. White bars = 100 µm, black bars = 1 cm (A). Colonies were counted (B) and measured (C) using ImageQuantTL software. n=3 biological replicates for tenocytes, n=4 for TDSCs and n=1 for MSCs, error bars shown represent SEM. Pairwise comparisons were performed using Student's t-tests. P-values: a=0.01.

4.4.3 Tenocyte, TDSC and MSC proliferation

Tenocytes and TDSCs proliferated very slowly and demonstrated very long population doubling times (PDT) with a mean (± SEM) of 354 (±81) and 508 (±28) hours respectively. In contrast MSCs had a much shorter PDT: 24 hours (Fig.4.2).

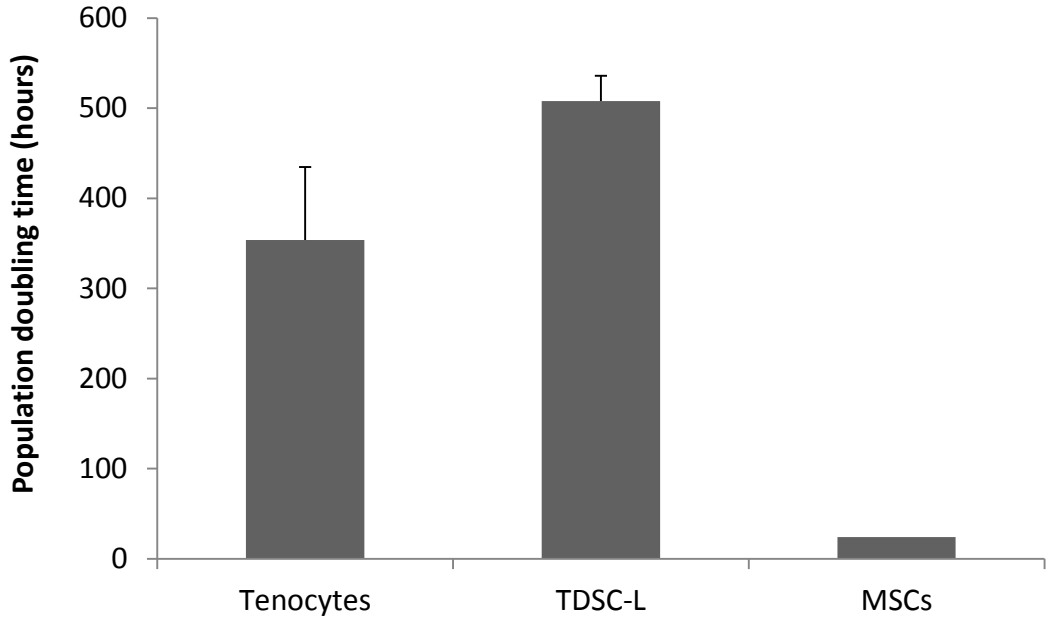


Figure 4.2. Population doubling time for tenocytes, TDSCs and MSCs. n=3 biological replicates, error bars shown represent SEM. Pairwise comparisons were performed using Student's t-tests.

4.4.4 Tenocyte, TDSC and MSC marker expression

The gene expression of stem cell and tenogenic markers was assessed by qRT-PCR (Fig.4.3). The majority of stem cell and early tenogenic markers were more highly expressed in TDSCs when compared with tenocytes and MSCs, and this increase was significant for Nanog, scleraxis, mohawk and CD73, when compared with tenocytes. The stem cell marker Sca-1 was similarly expressed in all cell types, and CD90 was expressed at similar levels in tenocytes and TDSCs, but expression was reduced in MSCs. Markers found in developed tendon, such as tenascin C, thrombospondin-4 and tenomodulin, exhibited higher expression in tenocytes, moderate expression in TDSCs, however expression in MSCs was low. The haematopoietic stem cell (HSC) marker CD34 was highly expressed in all cell types, whereas CD45 demonstrated low expression in all three cell types, with significantly higher levels observed for tenocytes.

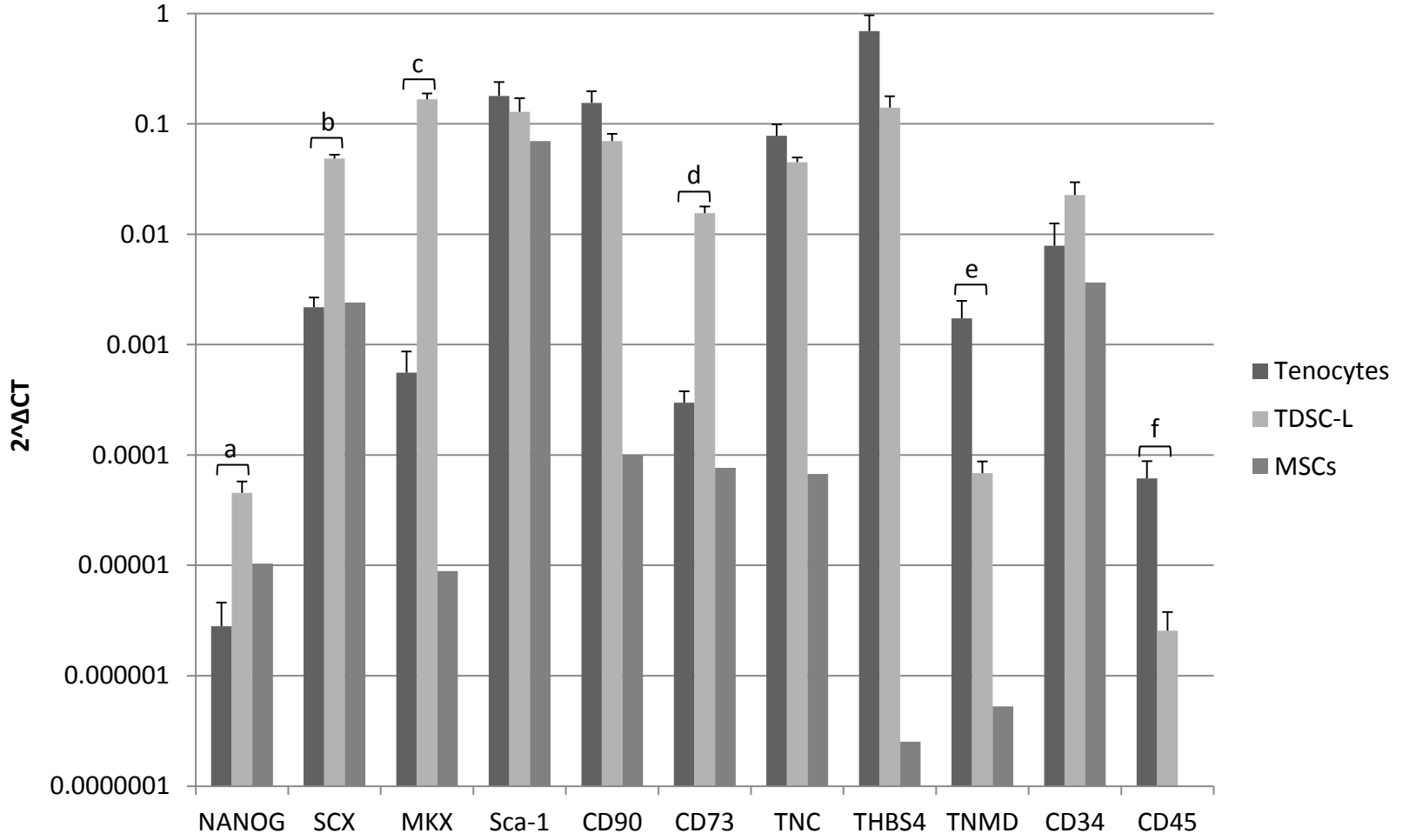


Figure 4.3. Gene expression analysis of stem cell markers in tenocytes, TDSCs and MSCs. Values are shown on a logarithmic scale and normalised to Gapdh. n=6 biological replicates for tenocytes, n=4 for TDSCs and n=1 for MSCs. Error bars shown represent SEM. Pairwise comparisons were performed using Student's t-tests after Log_{10} transformations. P-values: a=0.008, b=0.011, c=0.001, d=0.011, e=0.011, f=0.011.

4.4.5 Tenocyte, TDSC and MSC tri-lineage differentiation capacity

The ability of tenocytes, TDSCs and MSCs to differentiate into different cell lineages was analysed by staining, glycosaminoglycan (GAG) assays and qRT-PCR for gene expression analysis.

All three cell types demonstrated osteogenic differentiation as assessed by alkaline phosphatase levels and alizarin red staining, however MSCs exhibited only small amounts of alizarin red staining (Fig.4.4). No adipogenic differentiation was observed for tenocytes, however some oil red O staining was seen in MSCs, and considerably more for TDSCs (Fig.4.4). Tenocytes demonstrated some chondrogenic differentiation, with an increase in the intensity of safranin O staining, although the intensity of alcian blue staining did not appear to increase following differentiation (Fig.4.4). MSCs demonstrated stronger chondrogenic differentiation with a moderate increase in stain intensity for safranin O (Fig.4.4). Due to low cell numbers, chondrogenic differentiation assays were not performed on TDSCs.

There was an increase in mean GAG formation for tenocytes from 0.25 (±0.12) µg to 0.5 (±0.22) µg, however this was not significant, and for MSCs from 2.5 µg to 3.6 µg. GAG content was not analysed in TDSCs due to low cell numbers (Fig.4.5).

Gene expression analysis of lineage specific genes showed very low level expression of the majority of osteogenic markers for all three cell types, with significant decreases seen in expression of some genes for tenocytes and TDSCs. However there was a significant increase in Runx2 and Opn expression for TDSCs. There were small increases seen for some osteogenic markers for MSCs, however statistical analysis was not performed due to few biological replicates. There were small increases in all adipogenic genes for tenocytes and MSCs, and much larger significant increases for TDSCs. Similarly there was an increase in the majority of chondrogenic markers in tenocytes and MSCs, although these were not significant. Chondrogenic markers were not analysed in TDSCs due to low cell numbers (Fig.4.6).

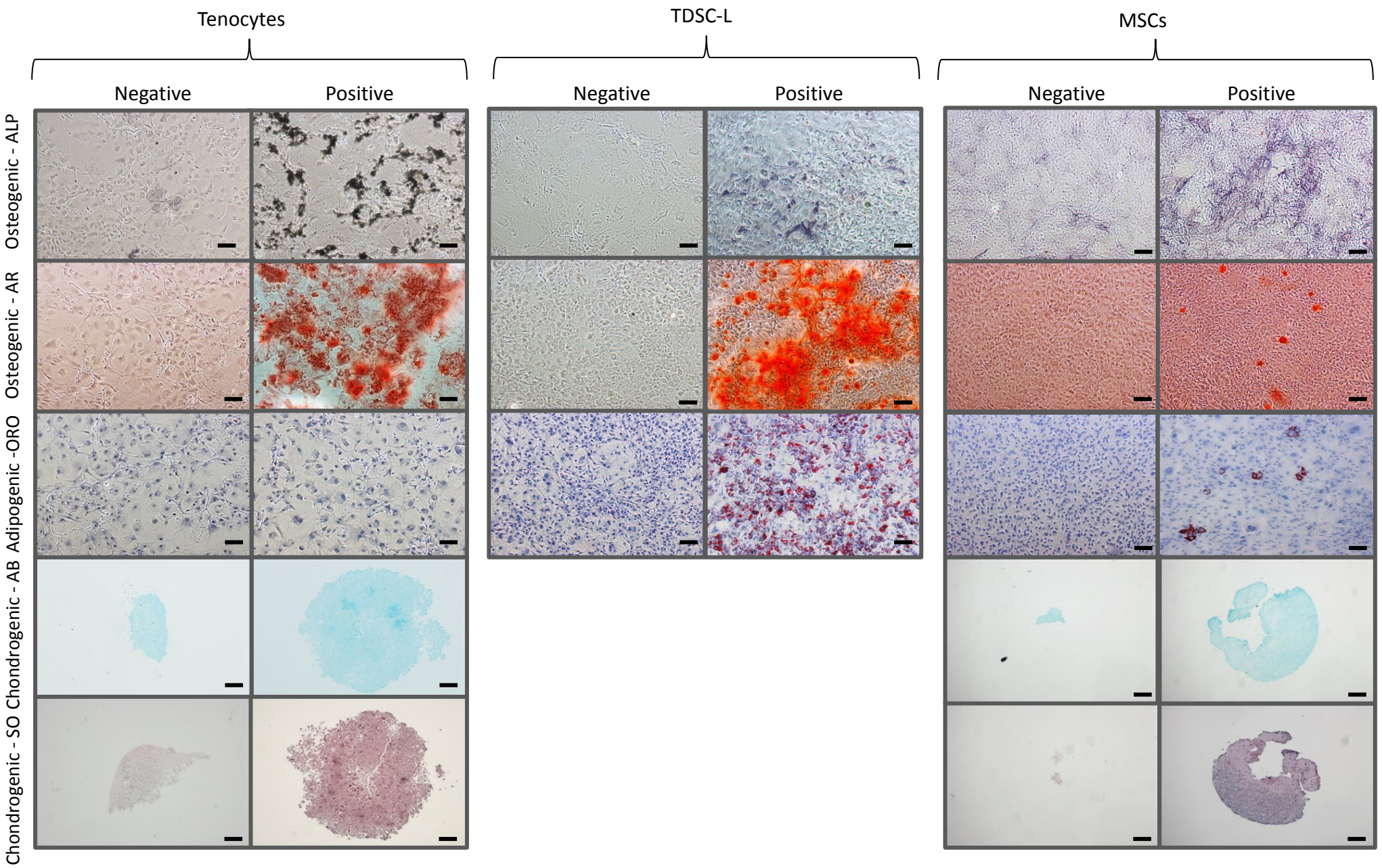

Tenocytes
TDSC-L
MSCs
Negative
Positive
Negative
Positive
Negative
Positive
Osteogenic - ALP
Osteogenic - AR
Adipogenic -ORO
Chondrogenic - AB
Chondrogenic - SO

Figure 4.4. Histological analysis of tri-lineage differentiation potential of tenocytes, TDSCs and MSCs. Representative images are shown for each cell type after induction of osteogenic, adipogenic and chondrogenic differentiation (positive) and also for control samples (negative), after appropriate staining. Cells subjected to osteogenic differentiation media were stained for both alkaline phosphatase (ALP) activity and calcium deposits using alizarin red (AR). Cells subjected to adipogenic differentiation media were stained for oil droplet formation using oil red O (ORO), and cell pellets exposed to chondrogenic differentiation media, for GAG formation using alcian blue (AB) and safranin O (SO). Bar = 100 µm. n=6 biological replicates for tenocytes, n=4 for TDSC-L and n=1 for MSCs.

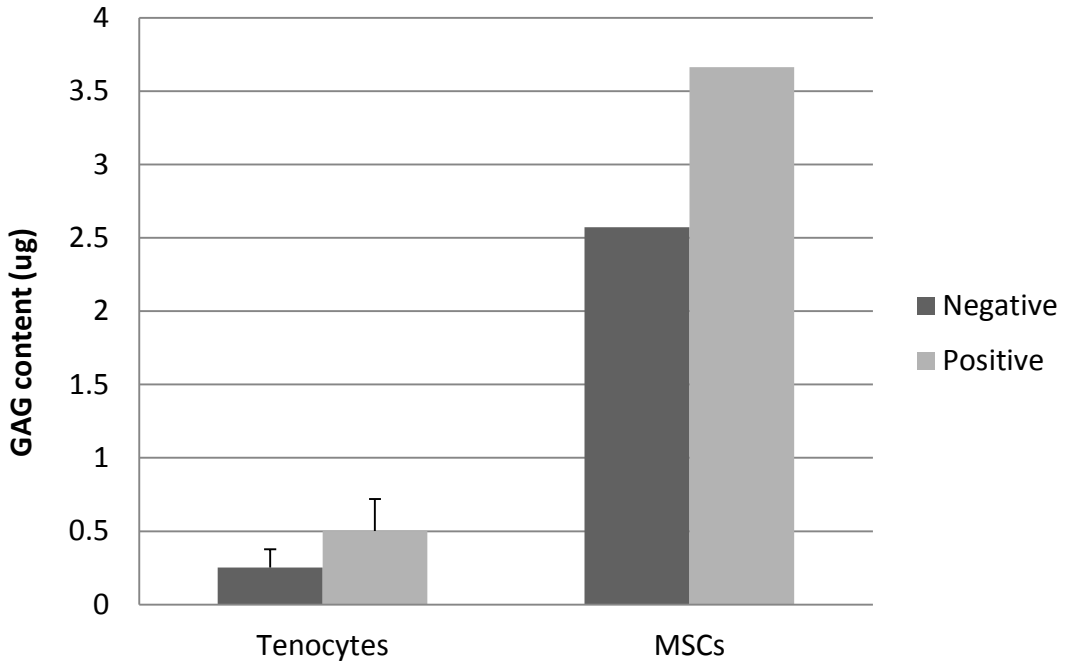


Figure 4.5. Total glycosaminoglycan (GAG) content of cell pellets with (positive) or without (negative) chondrogenic induction. n=6 biological replicates for tenocytes, n=1 for MSCs, error bars shown represent SEM. Pairwise comparisons were conducted using paired Student's t-tests.

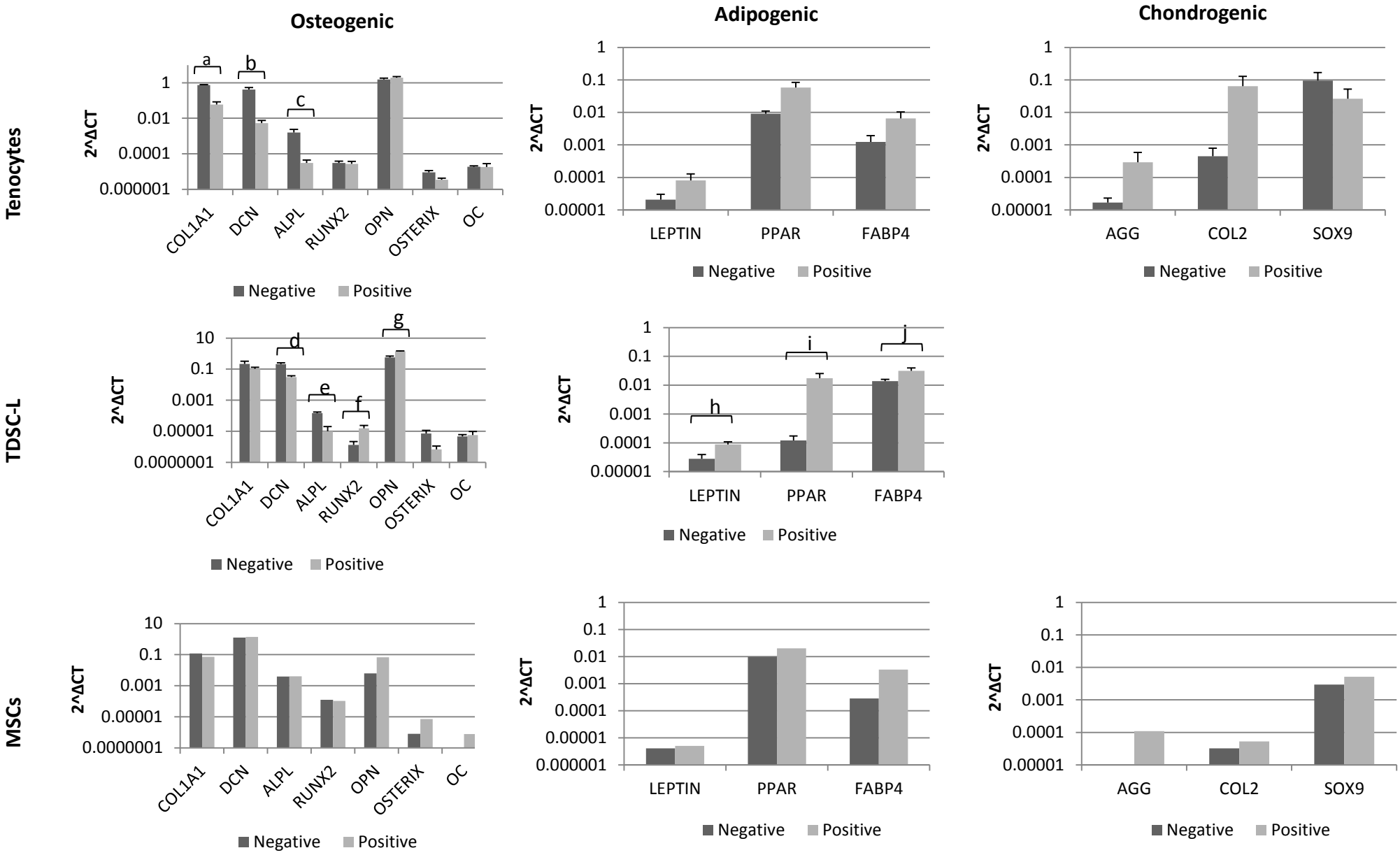

Osteogenic
Adipogenic
Chondrogenic
Tenocytes
TDSC-L
MSCs
2^ΔCT
a
b
c
d
e
f
g
h
i
j
COL1A1
DCN
ALPL
RUNX2
OPN
OSTERIX
OC
LEPTIN
PPAR
FABP4
AGG
COL2
SOX9
Negative
Positive

Figure 4.6. Gene expression analysis of lineage specific markers for tenocytes, TDSCs and MSCs. Values are shown on a logarithmic scale and normalised to Gapdh. n=6 biological replicates for tenocytes, n=4 for TDSC-L and n=1 for MSCs. Error bars shown represent SEM. Pairwise comparisons were performed after Log_{10} transformations using paired Student's t-tests. P-values: a=0.004, b=0.004, c=0.01, d=0.021, e=0.02, f=0.02, g=0.021, h=0.021, i=0.021, j=0.021.

4.5 Discussion

4.5.1 Isolation of murine tendon cells

The initial studies that identified and characterised TDSCs were conducted in mice (Bi *et al*., 2007; Salingcarnboriboon *et al*., 2003). Both of these studies, as well as the majority of subsequent murine TDSC studies, have used patellar or Achilles tendon for TDSC isolation. As detailed above, I was unable to isolate cells from these tendons and there are a number of reasons why this may have occurred, many of which are due to the limitations imposed by the use of spare material. As the purpose of this study was to validate the methods and techniques used for equine research, I used fresh spare murine material from other studies. By obtaining tissue in this manner, and not using additional mice for this study alone, I reduced the number of animals used for scientific research, one of the three R's outlined by the National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs) (Burden *et al*., 2015). For this reason, I had no control over the strain, age, gender, history or number of mice I was able to use. The mice from which I isolated patellar tendon were considerably older than those used previously: 6-31 months in this study, compared with 4-8 weeks in previous studies (Bi *et al*., 2007; Mienaltowski *et al*., 2014; Salingcarnboriboon *et al*., 2003), which may account for reduced cell numbers. In addition, some previous studies pooled as many as eight tendons (Mienaltowski *et al*., 2013), although others used individual tendons (Bi *et al*., 2007) and still observed cell growth. As far as information was available, I followed the isolation procedures provided in previous studies, however it is still possible that experimental and culture conditions may have had an impact on tendon cell growth.

Due to the lack of cells provided by patellar tendon digestion, I used murine tail tendon, as cells had been previously successfully isolated from tail tendon in my group. The mice used for isolation of tail tendon cells were 6-8 weeks old and therefore considerably younger than the mice used for patellar tendon cell isolation. Only one other study has previously isolated TDSCs from murine tail tendon (Alberton *et al*., 2014), therefore I followed their protocol for digestion and

culture, however no cell seeding density was provided. A low cell seeding density was selected based on my own previous work and other studies, however the density selected may be the reason for any differences observed between my findings and those of previous studies. Seeding density has been shown to play a key role in stem cell and tenocyte isolation and growth (Güngörmüş and Kolankaya, 2008; Lee *et al*., 2012; Schulze-Tanzil *et al*., 2004; Tsai and McKay, 2000) and previous studies on TDSC isolation in mice and other species have specified the requirement for low seeding densities (Cheng *et al*., 2014a; Lee *et al*., 2012; Mienaltowski *et al*., 2014; Ni *et al*., 2012; Rui *et al*., 2010), although many studies on murine TDSCs did not specify the seeding density used. Interestingly, the isolation of murine MSCs from bone marrow does not appear to be dependent on seeding density with published methods varying from extremely low densities, akin to TDSC isolation methods (Eslaminejad *et al*., 2006), to extremely high densities, exceeding those used for tenocyte isolation (Soleimani and Nadri, 2009; Sun *et al*., 2003). If TDSCs are a subset of MSCs then it would be expected that isolation methods would be similar. It is possible that higher seeding densities for tendon cells could promote TDSC-tenocyte interactions and promote TDSC differentiation, which necessitates low seeding densities to select for TDSCs only. Whereas the presence of tenocytes is clearly not a factor for MSC isolation and culture, and therefore higher seeding densities are acceptable. Alternatively it is possible that the differences in seeding density reported for TDSCs and MSCs and the observed differences in phenotype could be an indication that these two cell types are more distinct from each other than originally thought. This is supported by previous studies (Bi *et al*., 2007) as well as my own and could account for the differences observed between these two cell types.

The lack of information on optimal seeding densities for murine tail TDSC isolation may be the reason why I was unable to replicate the findings of previous studies. I found that murine TDSCs isolated from tail tendon had very slow rates of proliferation, and stopped growing at early passages, making certain assays impossible to perform due to low cell numbers. For further comparison and validation tenocytes were isolated from tail tendon and murine MSCs were purchased. No studies to date have investigated the stem cell properties of murine

tenocytes, or provided a comparison with murine TDSCs. Conversely, there is a wealth of literature on the characterisation of murine MSCs, and the widely reported similarities between MSCs and TDSCs made MSCs a useful cell type for comparison of stem cell characteristics. Due to the limited availability and stockists of murine MSCs, only one biological replicate was obtained, therefore no statistical comparisons could be performed and subsequently limited the usefulness of these cells as a comparative cell type.

4.5.2 Clonogenic potential and morphology of murine tendon cells

One of the hallmarks of a stem cell, including TDSCs and MSCs, is the ability to form colonies in culture. Previous studies have shown that TDSCs in mice are able to form colonies, however these colonies are heterogeneous in nature (Bi *et al*., 2007; Zhang and Wang, 2013a). In this study I observed growth of dense heterogeneous colonies in both TDSCs and tenocytes (Fig.4.1.A), which is consistent with the previous literature for TDSCs. I also observed growth of colonies in murine MSCs, however these colonies were sparse and heterogeneous (Fig.4.1.A), which is also consistent with some of the literature (Lei *et al*., 2013), although one study found that MSCs did not form colonies (Nadri *et al*., 2007). Similar colony numbers and colony sizes were observed between tenocytes and MSCs in this study, however TDSCs demonstrated fewer colonies than the other cell types, which is possibly due to the slow growth of these cells (Fig.4.1.B-C).

The morphology of cells can also be indicative of their function and specialisation. The general consensus of the current literature on TDSCs suggests that they are rounded in shape, not fibroblastic (Zhang and Wang, 2010c; Zhang and Wang, 2013a). This is partially consistent with my findings, which have shown a rounded morphology for TDSCs, however the cells were heterogeneous and there were also some fibroblastic cells present. Tenocyte morphology was also heterogeneous, with both fibroblastic and rounded cells present (Fig.4.1.A). Considering tenocytes as tendon fibroblasts, I expected to see a typical fibroblastic spindle-like morphology, as seen in other species (Zhang and Wang, 2010b), however tenocytes with a rounded, or polygonal morphology have previously been

reported in mice (Shimada *et al*., 2014). A considerable amount of heterogeneity in the morphology of MSCs was observed in this study, with some rounded cells, some fibroblastic, as well as intermediate polygonal cells (Fig.4.1.A). This is consistent with the literature, which reports solely fibroblastic cells (Nadri *et al*., 2007; Sun *et al*., 2003; Tropel *et al*., 2004), as well as heterogeneous mixtures of fibroblastic and rounded cells (Eslaminejad *et al*., 2006; Lei *et al*., 2013; Peister *et al*., 2004).

4.5.3 Proliferation of murine tendon cells

As noted above, very slow growth of TDSCs was observed (508 hours), however tenocytes were also found to have very slow population doubling times (PDTs) (354 hours) (Fig.4.2). Previous literature on murine TDSCs is highly variable, with some studies reporting very short PDTs, ranging between 20 and 25 hours (Bi *et al*., 2007; Zhang and Wang, 2015) for cells isolated from patellar tendon. Whereas other studies have shown PDTs of 50 and 93 hours for cells isolated from the same tendon, and 100 and 167 hours for cells from Achilles tendon (Zhang *et al*., 2010; Zhang and Wang, 2013a). These differences may be due to variation in cell culture or other experimental practices, although the same group (Zhang and Wang) demonstrated very variable PDTs, therefore experimental differences seem unlikely. Possibly the strain of mice used may have an effect on the characteristics of the isolated cells, as shown in MSCs (Peister *et al*., 2004), however three of the four studies mentioned above used C57BL/6 mice, therefore this cannot account for the differences in PDT. Even the higher PDTs demonstrated by some of the studies are considerably lower than those seen in this study. I am unsure why the cells used in this study, both TDSCs and tenocytes, had such long PDTs, or in fact stopped growing completely. I followed the protocol and methods provided by previous studies, particularly Alberton and others (2014), as this is the only study published which has characterised murine TDSCs from tail tendon (Alberton *et al*., 2014). Their study demonstrated a PDT of approximately 25 hours for TDSCs, which is clearly considerably lower than that shown in this study. The reasons for this are unclear, it is possibly due to strain variation, however as Alberton and others (2014) did not state the strain of mice used in their study, I cannot confirm this. Another possible

reason is the age of the mice used, as age has been shown to reduce murine TDSC proliferation and increase PDT (Zhang and Wang, 2015). However all of the studies previously discussed including my own have used young mice of comparable age. Conversely a much shorter population doubling time was observed for MSCs compared with tendon cells in this study (Fig.4.2), which is inconsistent with the literature. Previous studies have noted doubling times of around 60 hours for murine MSCs (Baghaban Eslaminejad and Fallah, 2013; Lei *et al*., 2013), which is considerably higher than that seen in this study, although variation within strains was observed (Lei *et al*., 2013).

4.5.4 Stem cell and tenogenic marker expression of murine tendon cells

Expression of certain markers is also indicative of stem cell populations. Generally in this study increased expression of stem cell markers was observed in TDSCs compared with tenocytes and MSCs, and increased expression of tenogenic markers in tenocytes and TDSCs compared with MSCs (Fig.4.3). The pluripotency marker Nanog was poorly expressed in all cell types, evident by the low CT values, however it was most highly expressed in TDSCs (Fig.4.3). The expression of Nanog has been previously reported in murine tail TDSCs (Alberton *et al*., 2014) and MSCs (Katsara *et al*., 2011). Sca-1, a murine specific MSC marker, was expressed in similar levels by all three cell types (Fig.4.3). Sca-1 has been shown to be expressed by murine TDSCs in a number of previous studies (Asai *et al*., 2014; Bi *et al*., 2007; Mienaltowski *et al*., 2013; Zhang and Wang, 2015). The expression of Sca-1 by tenocytes was unexpected as this is a stem cell marker and the expression of Sca-1 is thought to be restricted to cells with stem cell properties. It is possible that the tenocyte population isolated in this study is a heterogeneous mixture of cells at different stages of differentiation and therefore contains some multipotent cells expressing Sca-1. As Sca-1 is a MSC marker, its expression has been widely demonstrated in these cells (Baddoo *et al*., 2003; Eslaminejad *et al*., 2006; Lei *et al*., 2013; Nadri and Soleimani, 2007; Sun *et al*., 2003), consistent with this study. CD90 and CD73 are cell surface markers traditionally associated with MSCs and used as a criterion for MSC identification (Dominici *et al*., 2006), although more recently these

markers have also been identified on other cell types, including fibroblasts (Lupatov *et al*., 2015; Tsuzaki *et al*., 2005), making them less reliable stem cell markers. In this study high expression of CD90 was observed in TDSCs and tenocytes and reduced expression in MSCs (Fig.4.3). The data for CD90 expression in TDSCs is consistent with the literature, which has shown expression of CD90 in these cells (Alberton *et al*., 2014; Bi *et al*., 2007; Mienaltowski *et al*., 2013). Although CD90 expression has not been previously demonstrated in murine tenocytes, it has in tenocytes from other species (Cadby *et al*., 2014; Tsuzaki *et al*., 2005). Interestingly, although considered a MSC marker, CD90 expression in murine MSCs was reduced when compared with TDSCs. The reasons for this are unclear, however previous studies have also reported reduced expression of CD90 in murine MSCs, when compared with other CD and MSC markers (Lei *et al*., 2013; Nadri and Soleimani, 2007). Similarly, CD73 expression was reduced in murine MSCs when compared with TDSCs, however expression in tenocytes was also reduced (Fig.4.3). CD73 expression has been poorly characterised in murine tendon cells, with only one study demonstrating CD73 expression in TDSCs (Alberton *et al*., 2014), however the study by Alberton and others (2014) is consistent with my own. I observed reduced expression of CD73 in MSCs when compared with TDSCs, which is consistent with a previous study (Baghaban Eslaminejad and Fallah, 2013), although another study observed high expression of CD73 in murine MSCs (Abdallah *et al*., 2015). Interestingly, the same study noted that the expression of CD73 decreased in MSCs which were committed to the osteogenic lineage (Abdallah *et al*., 2015). Therefore it is possible that the MSCs in this study had already begun to differentiate, which would account for the reduced expression of CD73. Scleraxis (Scx) and Mohawk (Mkx) are both transcription factors involved in tendon development and expressed by tendon cells early in development, and in the case of Scx, by mature tendon cells (Liu *et al*., 2010; Schweitzer *et al*., 2001). For this reason I expected to see increased expression of these two markers in tendon cells, particularly TDSCs, and reduced expression in MSCs, which is generally what was observed, with increased expression in TDSCs and reduced expression in MSCs, although expression in tenocytes was also low. Both markers were highly expressed in TDSCs (Fig.4.3) consistent with the literature for Scx (Alberton *et al*., 2014; Asai *et al*., 2014; Bi *et*

al., 2007; Salingcarnboriboon *et al*., 2003), although no studies to date have analysed the expression of Mkx in murine TDSCs. The expression of both markers was reduced in tenocytes when compared with TDSCs, however that is to be expected. As both markers are involved in tendon development they are most highly expressed in tendon progenitor cells, and although expression of Scx is also observed in mature tendon, the expression of these transcription factors in differentiated tendon cells would be expected to be reduced. Although the expression levels were reduced in tenocytes, there is still fairly high expression of these markers and this is consistent with the literature (Wada *et al*., 2015). Moderate expression of the two transcription factors was observed in MSCs (Fig.4.3), which may be due to the fact that MSCs are able to differentiate into tendon cells (Dai *et al*., 2015), and therefore would require the ability to express tendon developmental genes. Although not demonstrated in murine MSCs, Scx and MKX expression has been shown in MSCs from other species including human and rat (Dai *et al*., 2015; Otabe *et al*., 2015). Tenascin C (Tnc), thrombospondin-4 (Thbs4) and tenomodulin (Tnmd) are all glycoproteins found in the tendon extracellular matrix (ECM) and have therefore been proposed as tendon markers. All three markers were more highly expressed in tendon cells when compared with MSCs, as expected, with the highest expression seen in tenocytes (Fig.4.3). Tnc expression has been demonstrated in murine TDSCs (Bi *et al*., 2007) consistent with this study, however expression in tenocytes has only been observed in other species (Pauly *et al*., 2010). Thbs4 expression has not previously been demonstrated in murine TDSCs or tenocytes, however it has in TDSCs and tenocytes from rat (Jelinsky *et al*., 2010; Ni *et al*., 2013; Ning *et al*., 2015), which is consistent with this study. Tnmd was expressed at slightly lower levels than the other two tendon ECM markers, with a significant increase seen in tenocytes when compared with TDSCs (Fig.4.3). Tnmd expression has been extensively demonstrated in murine TDSCs (Alberton *et al*., 2014; Mienaltowski *et al*., 2014; Zhang and Wang, 2013a), which is consistent with this study. The expression of Tnmd in murine tenocytes has also been reported (Wada *et al*., 2015). The expression of these ECM markers in MSCs was low, particularly for Tnmd and Thbs4, however this was expected as these proteins are components of the tendon ECM. Although no studies have analysed

the expression of these markers in murine MSCs, their expression has been demonstrated in other species. Low level expression of Tnc has been demonstrated in rat MSCs (Dai *et al*., 2015), and when compared directly with expression in tendon tissue, a 200-fold decrease was observed (Burk *et al*., 2014), this is not dissimilar to my own observations which noted a fold decrease of approximately 1000. Tnmd expression has also been well characterised in MSCs of several species (Dai *et al*., 2015; Tong *et al*., 2012), including mice, where direct comparison of Tnmd expression between MSCs and TDSCs showed reduced expression in MSCs (Brown *et al*., 2015), consistent with this study. CD34 and CD45 are haematopoietic and endothelial cell markers, and are used to ensure there is no contamination of tendon cell and MSC populations with these cells. CD34 expression was fairly high in all cell types (Fig.4.3), which may be due to problems associated with the primer used, although all primers were tested for homology, efficiency and dissociation curves. Alternatively, a previous study reported an increase in CD34 expression in MSCs which had started to differentiate down the osteogenic lineage (Abdallah *et al*., 2015), therefore it is possible that the cells used in this study had already started the process of differentiation before RNA was extracted for gene expression analysis. Alternatively the cell populations isolated in this study could have been contaminated with HSCs, however frequent media changes were performed to prevent this (Soleimani and Nadri, 2009) and tendon is poorly vascularised, therefore the likelihood of contamination is small. CD45 expression was much lower, particularly for TDSCs and MSCs (Fig.4.3).

4.5.5 Multipotency of murine tendon cells

Another hallmark of a stem cell is the ability to differentiate into a range of cell types, specifically for TDSCs and MSCs: osteogenic, adipogenic and chondrogenic cells. This property was investigated using staining methods, gene expression analysis and glycosaminoglycan (GAG) assays. Tenocytes, TDSCs and MSCs all demonstrated the ability to differentiate into osteogenic cells as analysed by BCIP/NBT and alizarin red staining (Fig.4.4). BCIP/NBT staining for alkaline phosphatase can manifest in different ways, with dark brown or black precipitate,

or purple staining. The formation of dark precipitate was observed for tenocytes and purple staining for TDSCs and MSCs. Alizarin red staining, which stains calcium nodules formed during osteogenic differentiation, was observed for all three cell types. There were similar levels of staining for tenocytes and TDSCs, however much less staining observed for MSCs. My data is consistent with previous studies investigating murine TDSCs, which observed osteogenic differentiation of TDSCs, as assessed by alizarin red staining (Alberton *et al*., 2014; Bi *et al*., 2007; Mienaltowski *et al*., 2013; Salingcarnboriboon *et al*., 2003). Interestingly, I observed only small amounts of alizarin red staining for murine MSCs, much less than for TDSCs. The majority of previous literature on murine MSCs has shown strong osteogenic differentiation as determined by both alizarin red (Baddoo *et al*., 2003; Baghaban Eslaminejad and Fallah, 2013; Eslaminejad *et al*., 2006; Nadri and Soleimani, 2007) and alkaline phosphatase staining (Sun *et al*., 2003; Tropel *et al*., 2004). However several studies have highlighted the variation in stem cell properties, including differentiation potential, both between different mice strains and also within the same strain. For example, Peister and others (2004) reported strong alizarin red staining for MSCs isolated from C57BL/6J mice, but reduced staining for BALB/c (Peister *et al*., 2004). In contrast, Phinney and others (1999) found that MSCs from C57BL/6J mice did not readily expand in culture and could not be used for further analysis (Phinney *et al*., 1999). Lei and others (2013) cultured four different cell lines from one strain (the specific strain was not noted), which all exhibited different morphologies (Lei *et al*., 2013). There were significant differences in the osteogenic potential of the different cell lines, with rounded and fibroblastic cells showing no differentiation and fusiform and polygonal cells showing varying degrees of osteogenic differentiation (Lei *et al*., 2013). Although MSCs isolated from C57BL/6J mice were used for this study, which have previously shown strong osteogenic potential, it is clear that there is a great deal of variation within murine MSCs and it is likely for this reason that variable alizarin red staining was observed in this study. The gene expression data is only partially consistent with the staining pattern observed for all three cell types, but particularly for tendon cells. This is similar to the data I produced for equine TDSCs (see Chapter 3), which also saw discrepancies between the staining and gene expression data. Tenocytes and TDSCs generally

showed a decrease or no change in gene expression between the positive and negative samples (Fig.4.6). The possible reasons for this mirror those for equine TDSCs (see Chapter 3), however in brief: lack of induction of the gene; early expression of the gene which decreased by 21 days; problems associated with the primers used, although all primer efficiencies and dissociation curves were tested. There was a significant increase seen in only two genes in TDSCs, and none for tenocytes. These results are inconsistent with some of the literature which has shown positive expression of alkaline phosphatase (Mienaltowski *et al*., 2013) in murine TDSCs, although there was no comparison with a non-differentiated control. My data is consistent with Bi and others (2007) as they observed an increase in osteopontin expression in positive samples when compared with controls, as well as demonstrating no increase in alkaline phosphatase expression (Bi *et al*., 2007). The only study to quantify osteogenic gene expression found considerably higher expression of the markers than shown in this study, however, again there was no non-differentiated control (Salingcarnboriboon *et al*., 2003). The study also noted considerable variation between cell clones from one donor, with some clones showing no expression of alkaline phosphatase or osterix and others positive expression. As with MSCs, it is clear that variation is a major factor in determining the phenotype of these cells. MSC gene expression was similar when comparing the negative and positive samples for certain genes, and increased in the positive samples for others (Fig.4.6), although due to having only one biological replicate, no statistical analysis could be performed. Previous studies have demonstrated the expression of osteocalcin, osteopontin and Runx2 (Baddoo *et al*., 2003; Baghaban Eslaminejad and Fallah, 2013; Eslaminejad *et al*., 2006; Nadri and Soleimani, 2007) by MSCs, and although they were more highly expressed in the positive samples, compared with the controls, they were not quantified. In contrast, Tropel and others (2004) observed smaller increases in the expression of Col1a1, osteopontin, osteocalcin and osteonectin between positive and negative samples in murine MSCs (Tropel *et al*., 2004), which is more consistent with this study. Consistent with their staining pattern, Lei and others (2004) only observed expression of osteocalcin and osteopontin in fusiform and polygonal MSC lines, not rounded or fibroblastic (Lei *et al*., 2013).

Adipogenic differentiation, as assessed by oil red O staining which stains for oil droplets, also showed a variable staining pattern across the different cell types. No staining was observed for tenocytes, however staining was present for TDSCs and MSCs (Fig.4.4). The positive staining seen in TDSCs is consistent with other published data (Alberton *et al*., 2014; Asai *et al*., 2014; Bi *et al*., 2007; Mienaltowski *et al*., 2013; Salingcarnboriboon *et al*., 2003; Zhang and Wang, 2015). However, there was considerable variation in the quantity of staining between the different studies, with large amounts of staining observed in some studies (Alberton *et al*., 2014; Mienaltowski *et al*., 2013) and only a few droplets seen in others (Asai *et al*., 2014; Bi *et al*., 2007; Salingcarnboriboon *et al*., 2003). Adipogenic differentiation of MSCs was observed in this study, although only a few oil droplets formed. As with the previous literature on osteogenic differentiation of murine MSCs, there are many studies demonstrating the adipogenic potential of MSCs (Baddoo *et al*., 2003; Baghaban Eslaminejad and Fallah, 2013; Eslaminejad *et al*., 2006; Nadri and Soleimani, 2007; Sun *et al*., 2003; Tropel *et al*., 2004), however there are also several studies highlighting the difference between and within strains. For example, Peister and others (2004) observed adipogenic differentiation in all strains of mice, excluding DBA1, which showed no differentiation at all (Peister *et al*., 2004). In addition, Peister and others (2004) demonstrated a considerable amount of oil red O staining for BALB/c mice (Peister *et al*., 2004), whereas Nadri and others (2007) only observed formation of a few oil droplets (Nadri and Soleimani, 2007). This highlights the variance both between and within strains, which is also demonstrated by Lei and others (2013) who found that different cell lines from the same strain also demonstrate distinct differentiation patterns. They found that rounded and polygonal cell lines differentiated into adipogenic cells, however fibroblastic and fusiform cells did not (Lei *et al*., 2013). The expression of adipogenic marker genes in this study was consistent with the staining pattern for TDSCs and MSCs, but not for tenocytes. As no staining was observed in tenocytes, I expected to see no expression of adipogenic marker genes, however there were increases in all three markers in tenocytes (Fig.4.6). Consistent with the staining pattern observed, significant increases in adipogenic marker genes were seen in TDSCs. Previous studies have shown the expression of Fabp4 and Ppar-γ by murine TDSCs (Bi *et al*.,

2007; Mienaltowski *et al*., 2013; Salingcarnboriboon *et al*., 2003). In this study small increases were observed for all genes in murine MSCs in the positive samples when compared with the negative. This is consistent with the majority of the previous literature, which report increases in Ppar-γ (Baghaban Eslaminejad and Fallah, 2013; Tropel *et al*., 2004), although Lei and others (2013) only noted an increase for rounded and polygonal cells, not fibroblastic or fusiform (Lei *et al*., 2013).

Due to the large number of cells required for chondrogenic differentiation assays and the slow growth observed for TDSCs in this study I was unable to perform such assays on these cells, however I did analyse chondrogenic differentiation of tenocytes and MSCs. There was no increase in the intensity of alcian blue staining for tenocytes, however there was an increase in safranin O staining (Fig.4.4). Previous studies investigating chondrogenic differentiation of murine TDSCs observed increases in both alcian blue and safranin O staining (Alberton *et al*., 2014; Asai *et al*., 2014; Mienaltowski *et al*., 2013; Zhang and Wang, 2015). Due to the size of the negative pellets it was difficult to compare the intensity of staining for MSC pellets, however there appeared to be an increase in the intensity of safranin O staining, but not alcian blue (Fig.4.4). Previous literature on chondrogenic differentiation of murine MSCs differs to that for osteogenic and adipogenic staining, as there seems to be little or no variation between or within strains, with all studies reporting chondrogenic differentiation of murine MSCs (Baddoo *et al*., 2003; Baghaban Eslaminejad and Fallah, 2013; Eslaminejad *et al*., 2006; Lei *et al*., 2013; Peister *et al*., 2004; Sun *et al*., 2003). The expression of chondrogenic marker genes observed in this study was consistent with the staining pattern, with increases seen in all chondrogenic marker genes in MSCs and in two genes in tenocytes (Fig.4.6), although these were not significant. Previous literature on TDSCs also reported expression of aggrecan and collagen type II after chondrogenic induction (Bi *et al*., 2007; Mienaltowski *et al*., 2013), consistent with this study for tenocytes. Likewise, previous data on murine MSCs demonstrates expression of collagen type II after induction (Baghaban Eslaminejad and Fallah, 2013), although in some studies the increase in collagen type II and aggrecan expression seen between positive and negative samples was less pronounced (Eslaminejad *et al*., 2006; Tropel *et al*., 2004), which is more consistent with this

study. Despite chondrogenic staining for all cell lines, Lei and others (2013) only observed aggrecan and collagen type II gene expression in fusiform and polygonal cells, not rounded or fibroblastic (Lei *et al*., 2013), highlighting the variability within MSCs. Glycosaminoglycan (GAG) content of cell pellets after chondrogenic induction was also analysed in this study as an indicator of chondrogenic differentiation. There was a small increase in GAG content in the positive samples when compared with the negative for tenocytes, although this was not significant (Fig.4.5). There was also an increase in GAG production by MSCs after chondrogenic induction. The quantity of GAGs in MSC pellets was considerably higher than tenocytes for both positive and negative samples (Fig.4.5). No previous data is available on the ability of murine tendon cells or MSCs to form GAGs, however increased GAG production in induced cell pellets has been reported in human TDSCs (Stanco *et al*., 2014).

4.5.6 Conclusion

In this study I have isolated a population of cells in murine tendon that demonstrate many of the traditional hallmarks of a stem cell: the ability to form colonies, the expression of stem cell markers and multipotency (Dominici *et al*., 2006), although chondrogenic differentiation potential is unconfirmed. My findings are consistent with the published literature on TDSCs. Murine MSCs were also analysed as a positive control, however I found reduced differentiation potential of these cells as well as reduced expression of stem cell markers. As described above, these properties may be due to variation between and within strains, which has been demonstrated extensively in murine MSCs, particularly the variation in multipotency. A study conducted by Lei and others (2013) which investigated the properties of different cell lines obtained from one MSC donor, found that cells with differing morphologies demonstrated different properties (Lei *et al*., 2013). The MSCs used in this study were similar in morphology to the fusiform “MSC3” line used in the Lei study, and this cell line did not demonstrate adipogenic differentiation. This is inconsistent with my results, as I observed adipogenic differentiation of murine MSCs, albeit only low levels. As a subset of MSCs, TDSCs have an extremely similar phenotype to MSCs and share many properties. The

TDSCs isolated in this study had a rounded morphology, consistent with "MSC1" in the Lei study, however this cell line demonstrated adipogenic differentiation, but no osteogenic differentiation (Lei *et al*., 2013). Again, this is inconsistent with my findings and highlights the variability within and between different strains, as well as individual differences.

Many differences were observed in this study between tenocytes and TDSCs suggesting that these cells are distinct populations with differing properties. They demonstrated different cell morphologies and colony forming ability as well as significant differences in the expression of certain stem cell markers, and some differences in multipotency. TDSCs conformed to the criteria of a MSC, as specified by the ISCT (Dominici *et al*., 2006), whereas tenocytes did not. The main similarity between tenocytes and TDSCs was the expression of tenogenic markers, which was expected due to the fact that both cell populations were derived from tendon tissue. More unexpected were the differences observed between TDSCs and MSCs, however as mentioned above this may be due to strain variation, as well as the use of only one biological replicate for MSCs. No studies have previously compared murine tenocytes and TDSCs, however this has been done in other species (Zhang and Wang, 2010b) and the differences seen between the two cell types in rabbits mirrors that seen in mice in this study. A comparison of TDSCs and MSCs has previously been published in mice (Bi *et al*., 2007) and the differences in marker expression seen in the study are suggestive of considerable variability between these two cell types, which is consistent with my own findings.

The initial aim of this study was to validate the methods and assays used in Chapter 3. As I observed restricted differentiation potential of equine TDSCs I wished to ensure that these findings were not due to technical errors or issues with isolating and culturing the cells. Therefore I opted to repeat the isolation procedures and stem cell characterisation assays in mice, a species previously well characterised. I was able to reproduce previous studies in mice, and noted no restricted differentiation potential of murine TDSCs (although I was unable to confirm chondrogenic differentiation potential). This indicates that the restricted differentiation potential of equine TDSCs is a feature of this cell population and not due to technical problems with the assays. As outlined in Chapter 3, the reasons for

the restricted differentiation potential of equine TDSCs may be due to the exercise and medical history of the horses. However, the restricted differentiation potential of these cells may provide an advantage during times of injury or degeneration.

Chapter 5

Canine ligament-derived stem cell characterisation

5.1 Introduction

Following the identification of stem cells within tendon (Salingcarnboriboon *et al*., 2003), a cell population with similar properties was also identified in ligament (Seo *et al*., 2004). Seo and others (2004) isolated ligament-derived stem cells (LDSCs) from periodontal ligament and many subsequent studies have also used periodontal ligament for investigation of LDSCs, however more recently research has turned to other ligament types, including the anterior cruciate ligament (ACL). Cell populations derived from such ligaments are most likely a subset of mesenchymal stromal cells (MSCs) and share many properties with these cells. For example anterior cruciate LDSCs (ACLDSCs) express stem cell and MSC markers including Oct-4, CD90, CD44, CD73 and CD105 (Steinert *et al*., 2011; Zhang *et al*., 2011b). In addition, ACLDSCs have been shown to form colonies (Fu *et al*., 2015) and differentiate into osteogenic, adipogenic and chondrogenic cell types (Cheng *et al*., 2009; Steinert *et al*., 2011). These properties are all characteristics of MSCs (Dominici *et al*., 2006) however LDSCs differ in that they also express tenogenic markers, for example scleraxis (SCX) and tenomodulin (TNMD) (Fujii *et al*., 2008; Steinert *et al*., 2011).

LDSCs hold the potential to treat ligament injury and degeneration, and studies to date have shown promising results. The majority of these studies have focussed on periodontal ligament repair (Fujii *et al*., 2008; Yang *et al*., 2009), with only one study investigating the use of LDSCs for repair of a non-dental ligament (Jiang *et al*., 2015). LDSCs isolated from rabbit medial collateral ligament (MCL) have been shown to aid repair and function of damaged MCL (Jiang *et al*., 2015), suggesting that ACLDSCs may aid ACL repair after injury or degeneration. LDSCs from non-dental origins have only been isolated from human (Cheng *et al*., 2009; Steinert *et al*., 2011), rabbit (Jiang *et al*., 2015) and horse (Shikh Alsook *et al*., 2015) ligaments and this study will focus on the isolation and characterisation of canine LDSCs from cranial cruciate ligament (CCL) which is comparable to the human ACL (Cook *et al*., 2010). No studies to date have investigated the presence of a LDSC population in canine CCL, however ligament injuries in dogs are well documented (Witsberger *et al*., 2008). The increase in lifespan and obesity in dogs has resulted in

a rise in the number of CCL injuries (Adams *et al*., 2011; Witsberger *et al*., 2008). In addition, certain dog breeds have been found to be genetically predisposed to CCL injury, including Labrador Retrievers, Newfoundlands and Staffordshire terriers (Baird *et al*., 2014a; Whitehair *et al*., 1993; Wilke *et al*., 2006; Witsberger *et al*., 2008). To date, in dogs, there are no effective treatments dealing directly with the injured CCL in terms of regeneration and repair. The use of a canine model for investigating LDSCs is also translatable into humans, as the structure and function of the CCL is very similar to the human ACL (Cook *et al*., 2010), and non-contact injury and degeneration often occur in both ligaments in a similar manner (Cimino *et al*., 2010; Comerford *et al*., 2011; Comerford *et al*., 2005; Hasegawa *et al*., 2013; Tang *et al*., 2009), making the dog a possible model for human LDSC research.

5.2 Hypothesis

I hypothesise that a native ligament progenitor cell population resides within canine CCL which can be differentially isolated from other ligament cell types by low-density plating and/or differential adhesion to fibronectin substrates. This progenitor cell population will demonstrate all of the hallmark properties of MSCs, including clonogenicity, multipotency and stem cell marker expression, however the cell population will differ from MSCs with the increased expression of ligamentogenic markers. The progenitor cell population will differ from ligamentocytes with the latter cell type demonstrating reduced stemness when compared with progenitor cell populations.

5.3 Aims

1. To characterise canine LDSCs and ligamentocytes isolated from the CCL; including analysis of stem cell and ligamentogenic marker expression, colony-forming ability, cell proliferation and multipotency.
2. To compare the differences in phenotype between LDSCs and ligamentocytes as well as a comparison with canine MSCs as a positive control cell type.

5.4 Results

5.4.1 Ligamentocyte, LDSC and MSC morphology and colony formation

Ligament cells were isolated and seeded using three different methods: high density for isolation of ligament fibroblasts (ligamentocytes); low density for isolation of LDSCs (LDSC-L); and low density on fibronectin substrates also for isolation of LDSCs (LDSC-F). In addition, MSCs were isolated from femurs of dogs with osteoarthritic hip joints as a comparative cell type (Chapter 2, Section 2.2.1.3 and 2.2.1.4).

All ligament cell types demonstrated a similar cell morphology after passaging, with a typically fibroblastic appearance (Fig.5.1.A), however LDSC-F showed a rounded morphology upon initial plating (Fig.5.1.D) which was lost with passaging. MSCs exhibited a slightly more fusiform morphology, neither rounded nor fibroblastic.

All cell types grew in heterogeneous colonies (Fig.5.1.A), with ligamentocytes, LDSC-L and MSCs forming fairly sparse colonies, whereas LDSC-F formed dense colonies. This is further apparent when colony numbers were quantified with comparatively similar numbers between all cell types (Fig.5.1.B). Colony size was variable between the cell types as ligamentocytes had a larger average colony size than LDSC-F and MSCs which was statistically significant. LDSCs had the next largest average colony size followed by MSCs (Fig.5.1.C).

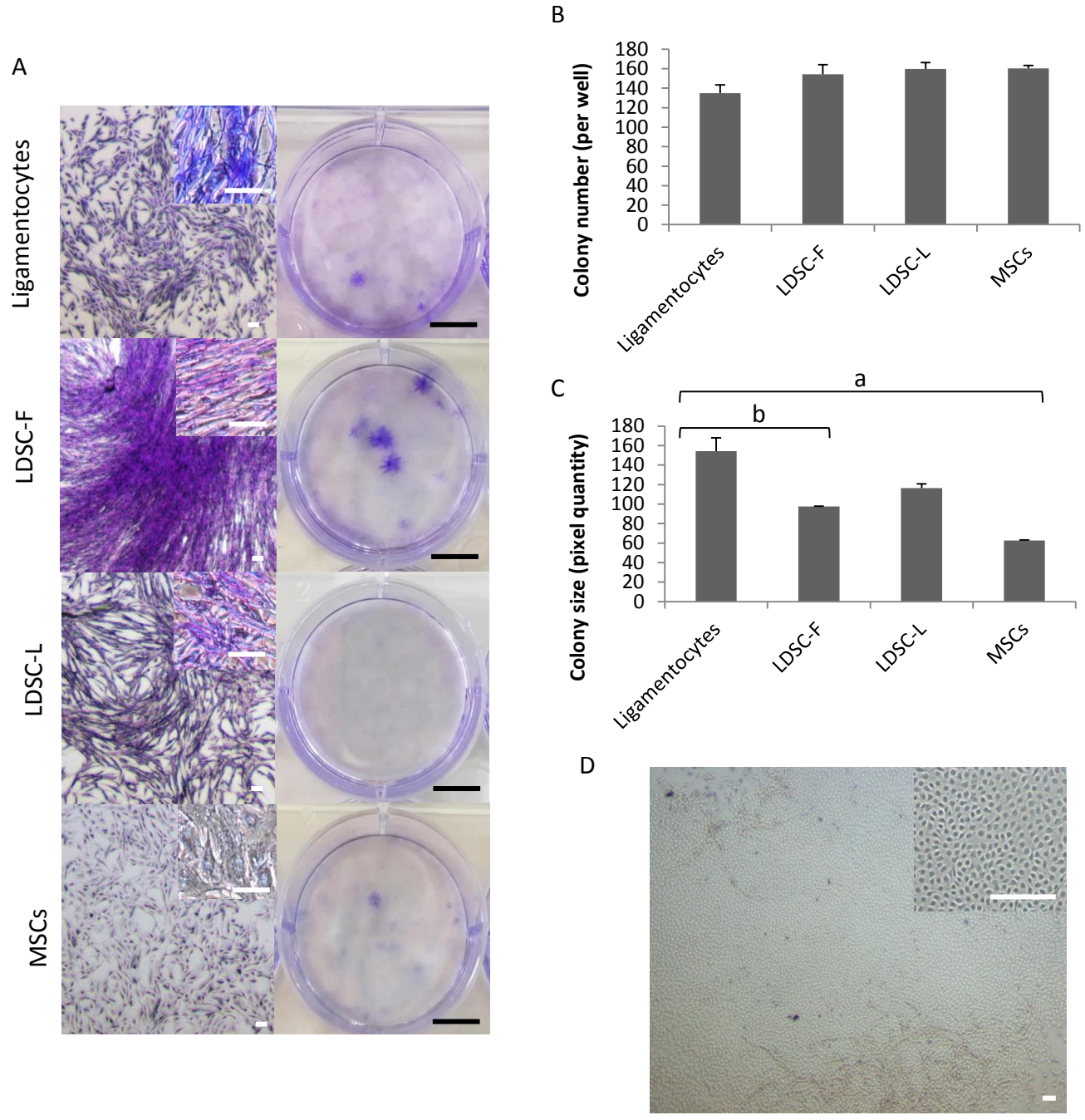


Figure 5.1. Canine ligamentocyte, ligament-derived stem cell (LDSC) and mesenchymal stem cell (MSC) morphology and colony formation at 5% oxygen. Representative images of cell morphology and colony formation are shown. Cells were grown for 7 days before staining with crystal violet and imaging for cell morphology analysis and colony formation. White bars = 100 µm, black bars = 1 cm (A). Colonies were counted (B) and measured (C) using ImageQuantTL software. n=7 biological replicates for ligamentocytes and LDSC-F, n=3 for LDSC-L and n=6 for MSCs. Error bars shown represent SEM. Pairwise comparisons were performed using a one-way ANOVA with a Tukey post-hoc test. P-values: a=<0.0001, b=0.018. Representative images shown of LDSC-F after initial plating to emphasise differences in morphology. White bars = 100 µm (D).

5.4.2 Ligamentocyte, LDSC and MSC proliferation

Population doubling times were consistent for all ligament cell types with a mean (±SEM) of 27 (±1.4), 29 (±0.8) and 35 (±8.3) hours per doubling for ligamentocytes, LDSC-F and LDSC-L respectively. MSCs had a considerably longer population doubling time of 96 (±13) hours. There was a statistically significant difference between MSCs and all ligament cell types (Fig.5.2).

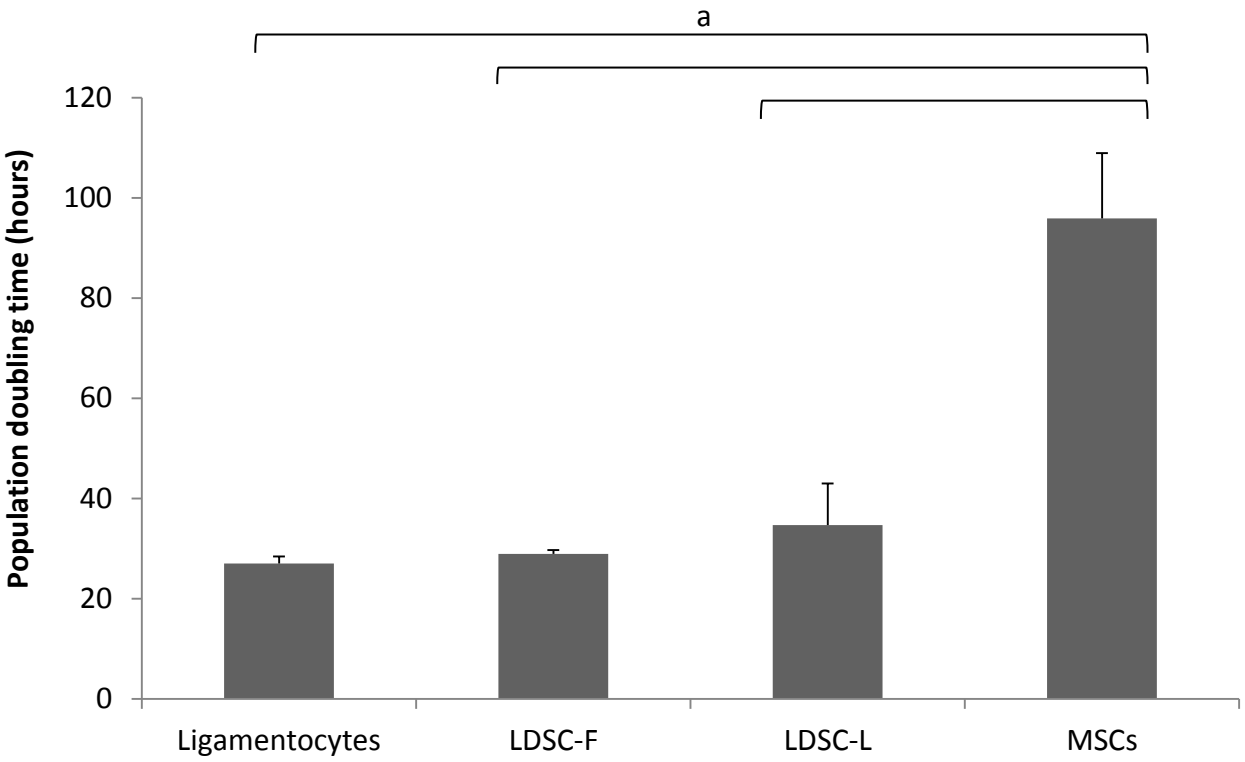


Figure 5.2. Population doubling time for canine ligamentocytes, LDSCs and MSCs at 5% oxygen. n=10 biological replicates for ligamentocytes, n=5 for LDSC-F, n=3 for LDSC-L and n=4 for MSCs. Error bars shown represent SEM. Pairwise comparisons were performed using a one-way ANOVA with a Tukey post-hoc test. P-values: a=<0.0001.

5.4.3 Ligamentocyte, LDSC and MSC marker expression

The gene expression of stem cell and tenogenic markers was assessed by qRT-PCR (Fig.5.3).

Expression of the pluripotency marker Oct4 was generally low with no significant differences between cell types, although higher levels were recorded for LDSC-F and MSCs (Fig.5.3). CD markers, markers used for identification of MSCs, were highly expressed in all cell types. CD105 was most highly expressed in LDSC-F and MSCs however there were no statistically significant differences between cell types. There was similar expression of CD44 in all cell types, whereas there were statistically significant differences in CD90 expression between cell types, with increased expression seen in ligamentocytes and LDSC-F when compared with MSCs. Likewise, there were statistically significant differences in CD73 expression between cell types, with increased expression seen in LDSC-F when compared with all other cell types (Fig.5.3). Tenogenic markers were expressed at much lower levels than CD markers, with no statistically significant differences in expression of SCX and mohawk (MKX) between cell types, although SCX and MKX were both more highly expressed in MSCs. Tenomodulin expression was significantly increased in MSCs when compared with all ligament cell types (Fig.5.3). CD34 and CD45 expression was generally low with similar levels seen in all cell types except for LDSC-L in which levels were decreased (Fig.5.3).

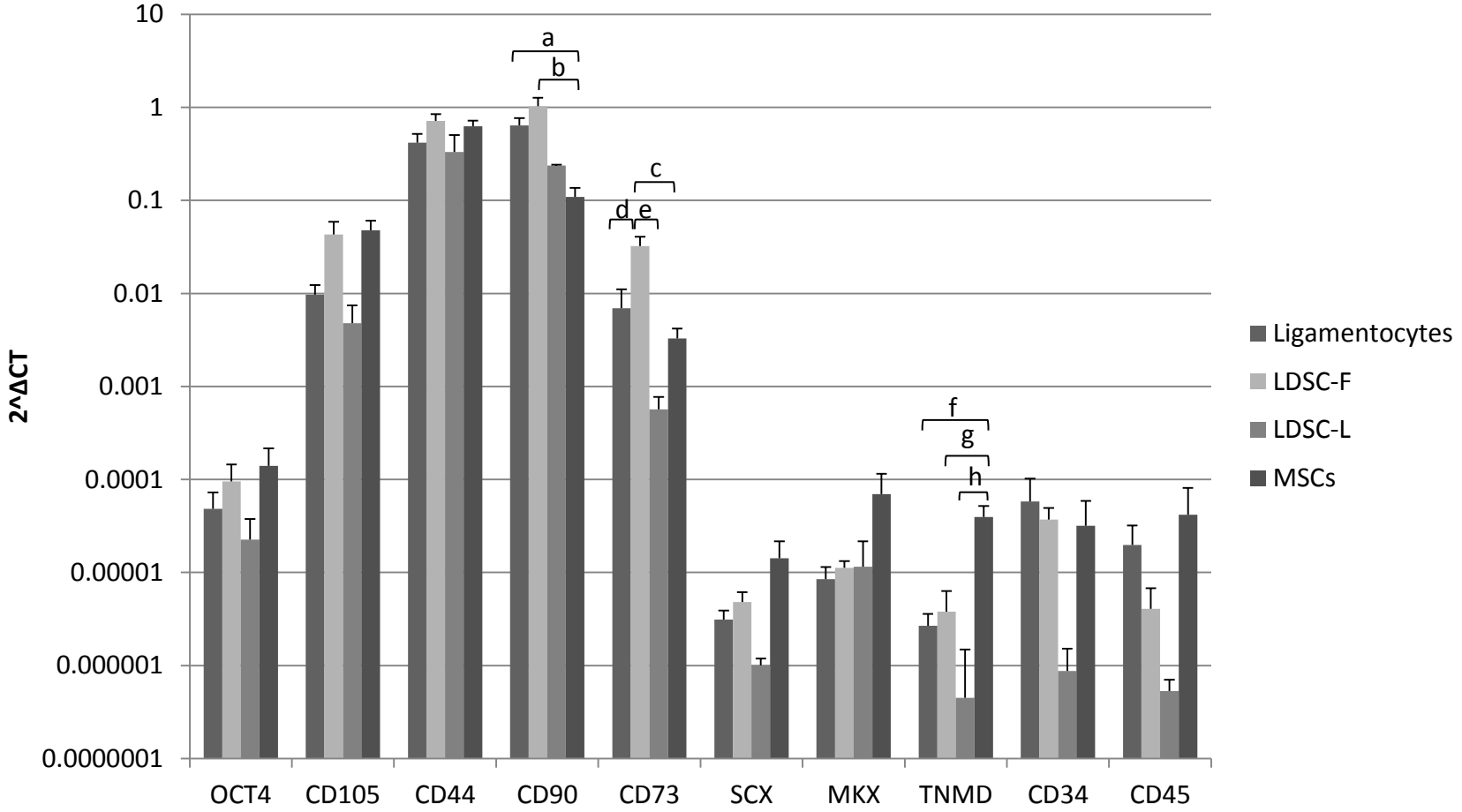


Figure 5.3. Gene expression analysis of stem cell markers in ligamentocytes, LDSCs and MSCs at 5% oxygen. Values are shown on a logarithmic scale and normalised to GAPDH. n=11 biological replicates for ligamentocytes, n=9 for LDSC-F, n=3 for LDSC-L and n=6 for MSCs. Error bars shown represent SEM. Pairwise comparisons were conducted using a one-way ANOVA with a Tukey post-hoc test after Log_{10} transformation of data. P-values: a=0.034, b=0.001, c=0.014, d=0.011, e=0.038, f=<0.0001, g=<0.0001, h=0.004.

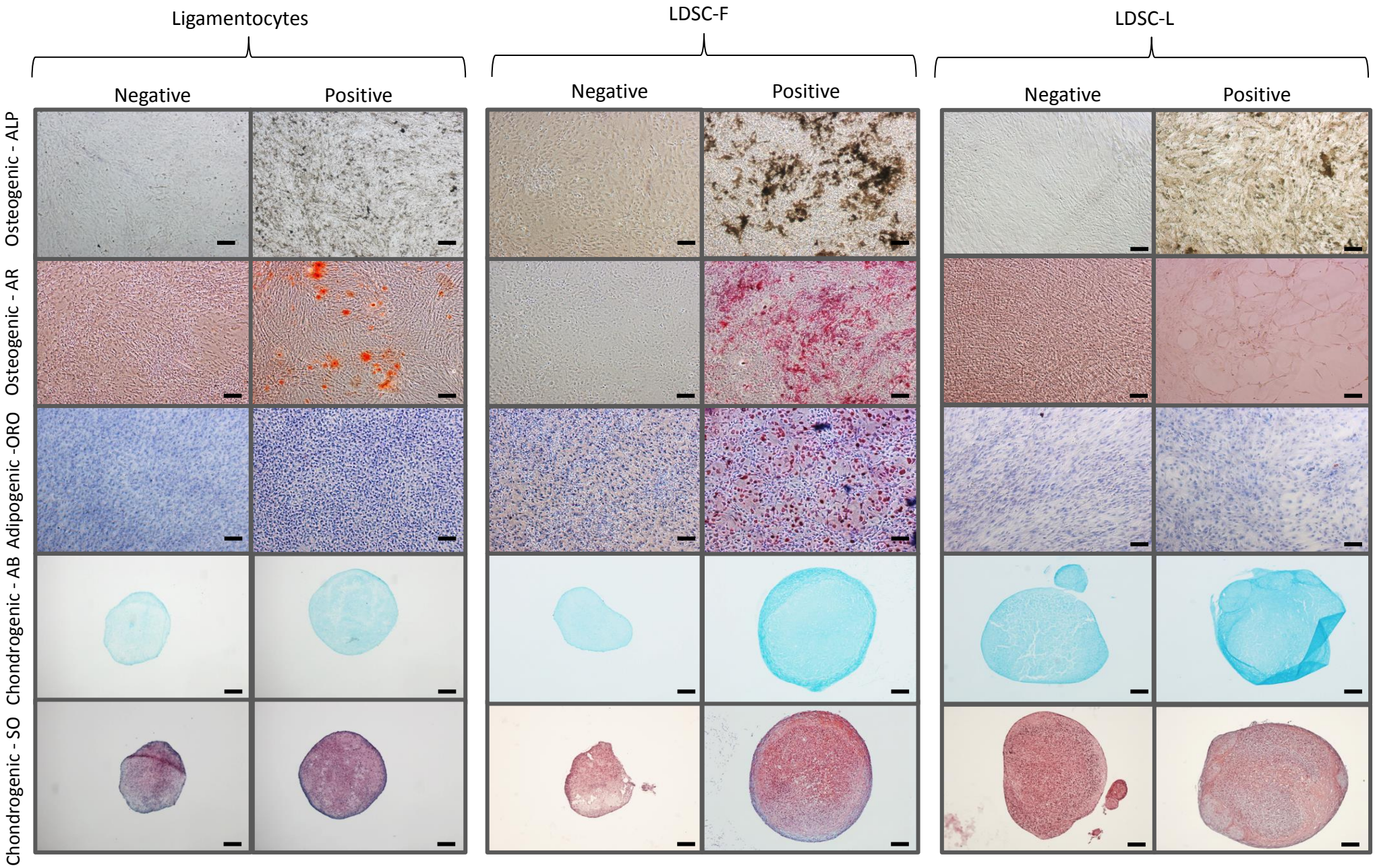
5.4.4 Ligamentocyte, LDSC and MSC tri-lineage differentiation capacity

The ability of ligamentocytes, LDSCs and MSCs to differentiate into different cell lineages was analysed by staining, glycosaminoglycan (GAG) assays and qRT-PCR for gene expression analysis.

All cell types exhibited signs of osteogenic differentiation as assessed by BCIP/NBT and/or alizarin red staining. LDSC-L demonstrated no alizarin red staining and only a small amount of BCIP/NBT staining for alkaline phosphatase, and ligamentocytes showed only low levels of both stain. Levels of both stains were considerably increased in LDSC-F and MSCs (Fig.5.4). Adipogenic differentiation, as assessed by oil red O staining was only observed in LDSC-F (Fig.5.4). Chondrogenic differentiation was varied between cell types. There was very little change in the intensity of alcian blue or safranin O staining for ligamentocytes, LDSC-L and MSCs. There was an increase in stain intensity of both stains for LDSC-F (Fig.5.4).

There was an increase in GAG content for all ligament cell types after induction of chondrogenic differentiation, however these increases were not significant when compared with controls. There was a small decrease in GAG content between samples for MSCs, however again this was not statistically significant (Fig.5.5).

Gene expression analysis of lineage specific genes showed an increase in osteogenic markers for all cell types, with statistically significant increases seen for Runx2 in ligamentocytes and LDSC-F and for OC and Runx2 in MSCs. There were also increases in adipogenic marker genes for all cell types and this was most pronounced in ligamentocytes and LDSC-F both of which demonstrated statistically significant increases in PPARγ and FABP4. There was also a significant increase in leptin gene expression in MSCs. There were increases in chondrogenic marker genes, including aggrecan and collagen type II for all ligament cell types and MSCs although the increases were small and not statistically significant (Fig.5.6).


Ligamentocytes
LDSC-F
LDSC-L
Negative
Positive
Negative
Positive
Negative
Positive
Osteogenic - ALP
Osteogenic - AR
Adipogenic -ORO
Chondrogenic - AB
Chondrogenic - SO

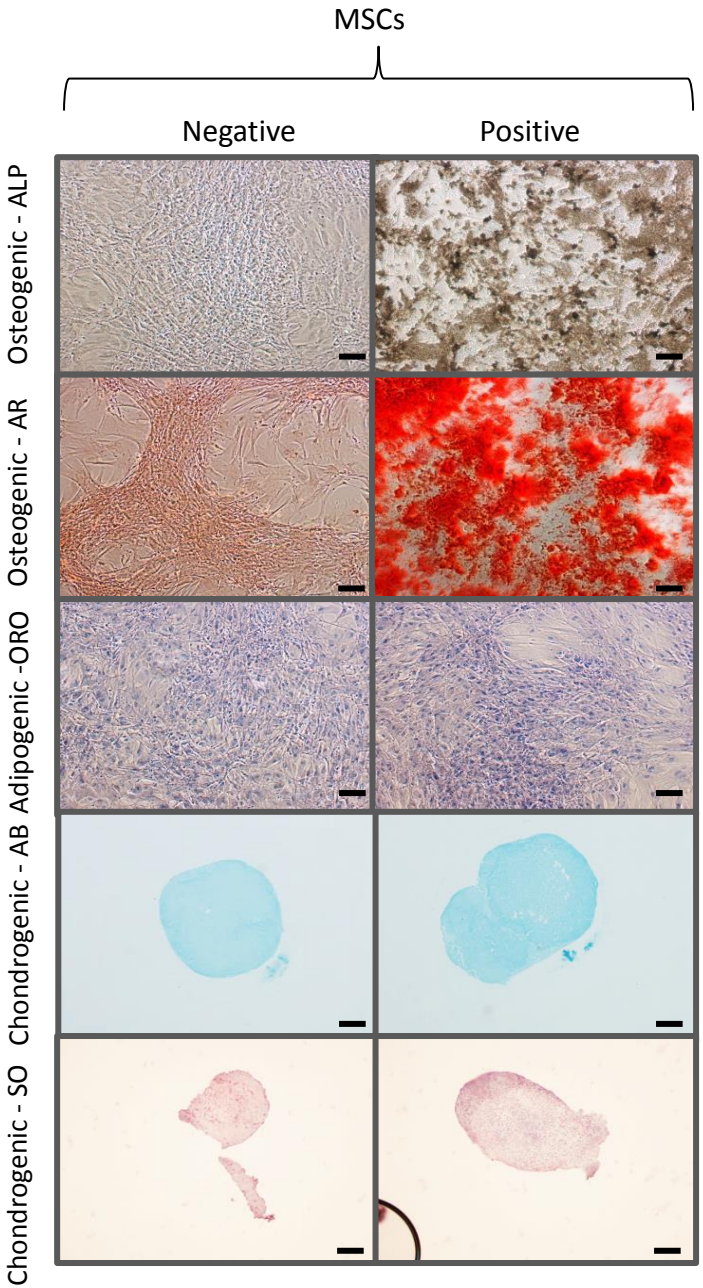


Figure 5.4. Histological analysis of tri-lineage differentiation potential of ligamentocytes, LDSCs and MSCs at 5% oxygen. Representative images are shown for each cell type after induction of osteogenic, adipogenic and chondrogenic differentiation (positive) and also for control samples (negative), after appropriate staining. Cells subjected to osteogenic differentiation media were stained for both alkaline phosphatase (ALP) activity and calcium deposits using alizarin red (AR). Cells subjected to adipogenic differentiation media were stained for oil droplet formation using oil red O (ORO), and cell pellets exposed to chondrogenic differentiation media, for GAG formation using alcian blue (AB) and safranin O (SO). Bar = 100 µm. n=8 biological replicates for ligamentocytes, n=9 for LDSC-F, n=3 for LDSC-L and n=6.

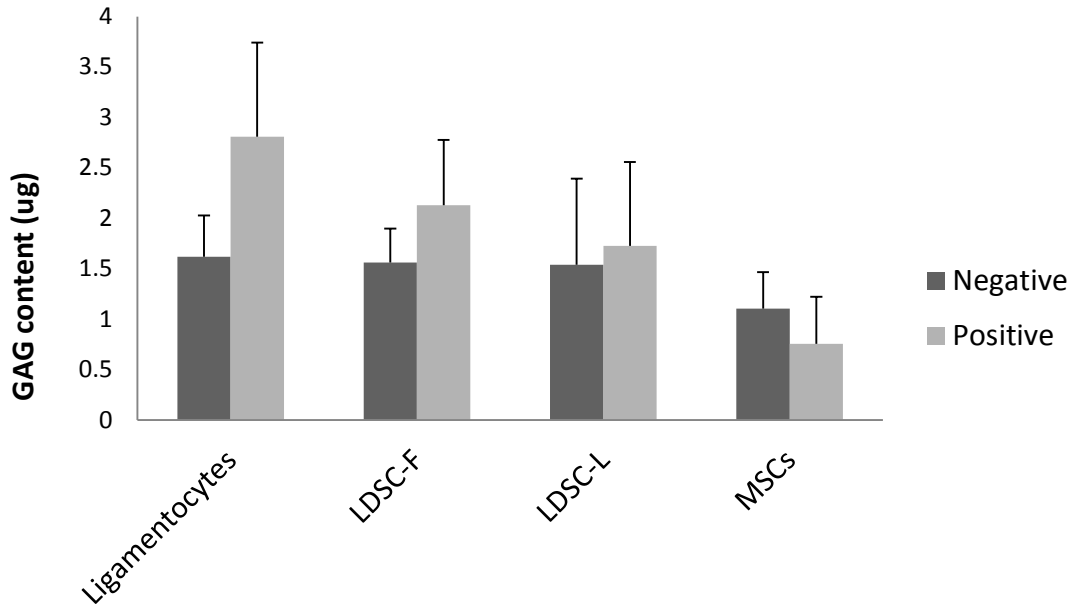
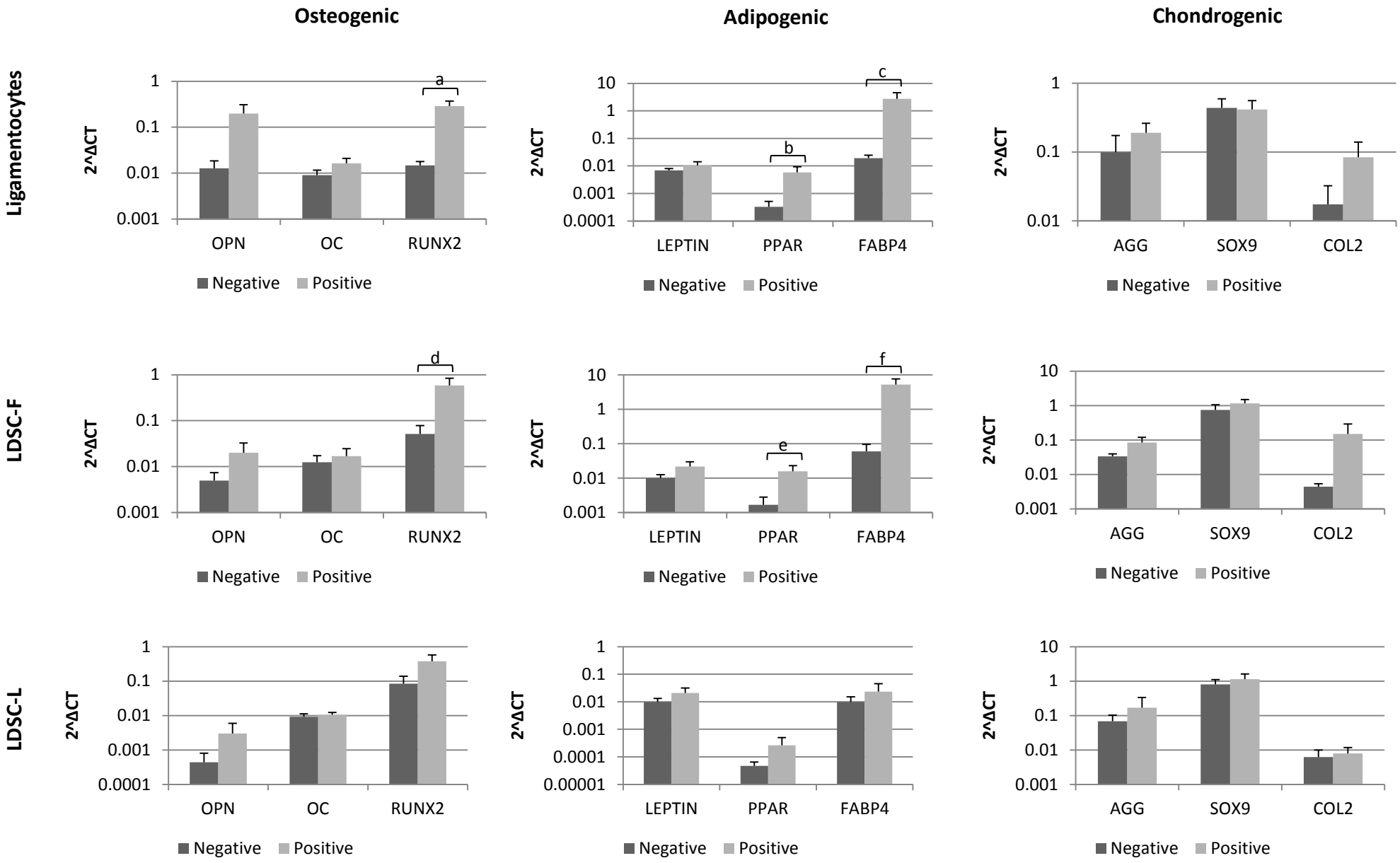


Figure 5.5. Total glycosaminoglycan (GAG) content of cell pellets with (positive) or without (negative) chondrogenic induction at 5% oxygen. n=8 biological replicates for ligamentocytes, n=9 for LDSC-F, n=3 for LDSC-L and n=6 for MSCs. Error bars shown represent SEM. Pairwise comparisons were conducted using paired Student's t-tests.


Osteogenic
Adipogenic
Chondrogenic
Ligamentocytes
LDSC-F
LDSC-L
2^ΔCT
OPN
OC
RUNX2
LEPTIN
PPAR
FABP4
AGG
SOX9
COL2
Negative
Positive
a
b
c
d
e
f

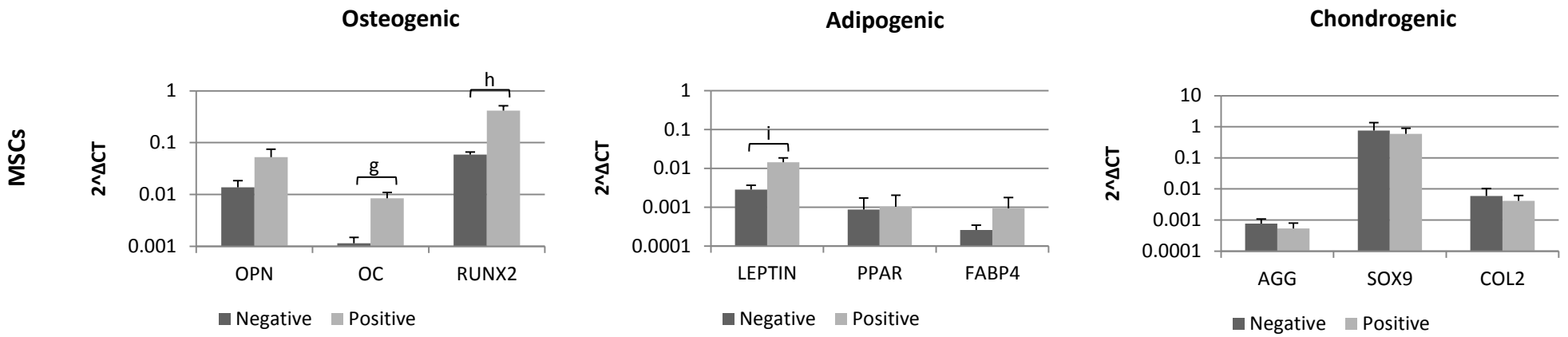


Figure 5.6. Gene expression analysis of lineage specific markers for canine ligamentocytes, LDSCs and MSCs at 5% oxygen. Values are shown on a logarithmic scale and normalised to GAPDH. n=8 biological replicates for ligamentocytes, n=9 for LDSC-F, n=3 for LDSC-L and n=6 for MSCs. Error bars shown represent SEM. Pairwise comparisons were conducted using paired Student's t-tests, after Log_{10} transformations. P-values: a=0.001, b=0.04, c=0.001, d=0.004, e=0.004, f=<0.0001, g=0.006, h=0.004, i=0.02.

5.5 Discussion

5.5.1 Isolation of canine ligament cells

The majority of research conducted into LDSCs has focused on periodontal ligament (PDL) in various species with only a few studies investigating the presence of stem cells within intra-articular ligaments such as the ACL. These studies have used a variety of methods for isolation of LDSCs including explant cultures and cell seeding after tissue digestion at a range of densities, the majority between 5000 – 10,000 cells/cm^2 (Cheng *et al*., 2010; Seo *et al*., 2004). LDSCs isolated from canine ACL tissue have not previously been reported, therefore exact isolation procedures were unknown. Based on my previous work on murine and equine TDSCs (see Chapters 3 and 4) (Williamson *et al*., 2015) I decided to use the same isolation procedures for canine LDSCs. Therefore, cells were isolated by seeding at low density on plastic substrate (LDSC-L) and onto fibronectin substrate for differential adhesion (LDSC-F) and finally at high density for isolation of ligament fibroblasts (ligamentocytes). Ligamentocytes were used as a comparative cell type to ensure the LDSC populations isolated were true stem cells and not fibroblasts demonstrating some stem cell properties and some distinct and significant differences were noted between the two cell types (Fig.5.3 and Fig.5.4). Mesenchymal stromal cells (MSCs) were also used as another comparative cell type, as LDSCs are thought to be a subset of MSCs (Huang *et al*., 2008; Segawa *et al*., 2009) I expected to see a similar phenotype for both cell types. Due to the ease of material availability and collection the MSCs were isolated from dogs undergoing total hip arthroplasty, having presented with marked osteoarthritic (OA) joints. The initial aim was not to investigate the effects of disease on MSCs, however due to the interesting results observed, namely the restricted differentiation potential of these cells, I decided to investigate MSCs from healthy canine femurs also. Unfortunately I was unable to obtain fresh healthy MSCs due to material restrictions, therefore historic samples from a previous project in my group were used. These cells proliferated extremely slowly and some biological replicates ceased growth altogether. For this reason only a small number of assays were

performed on one or two replicates and no statistical analysis could be completed, limiting the usefulness of these cells as a comparative cell type. Therefore, the data for these cells has not been included. However this work did not suggest any differences between healthy MSCs and the cells isolated from arthritic joints, most likely due to the age of these cells and the length of time stored at -80°C.

5.5.2 Clonogenic potential and morphology of canine ligament cells

One of the criteria outlined by the International Society for Cellular Therapy (ISCT) for identification of MSCs is clonogenic potential (Dominici *et al*., 2006) and heterogeneous colony formation was observed for all of the cell types in this study. LDSC-F mainly formed dense, compact colonies, whereas all of the other cell types formed larger and sparser colonies (Fig.5.1.A). Colony number was consistent across all cell types, whereas colony size was more variable with ligamentocytes forming the largest colonies and MSCs the smallest. There was a significant decrease in colony size between ligamentocytes and both LDSC-F and MSCs (Fig.5.1.B-C). My findings are consistent with the literature which has reported heterogeneous colony formation of human periodontal ligament stem cells (PDLSCs) (Hakki *et al*., 2014; Liu *et al*., 2014b; Prateeptongkum *et al*., 2015), canine PDLSCs (Lee *et al*., 2014b) as well as human ACLDSCs (Cheng *et al*., 2009; Zhang *et al*., 2011b). There are no studies to date that have investigated the clonogenic capacity of ligamentocytes. Colony formation of canine MSCs has been previously demonstrated (Rozemuller *et al*., 2010; Takemitsu *et al*., 2012) which agrees with my data. No differences in the clonogenic potential of LDSCs and MSCs was observed in this study, however previous studies have reported an increased clonogenic potential of human LDSCs when compared with MSCs (Cheng *et al*., 2010; Gay *et al*., 2007). The reason for this inconsistency may be due to species variation as both of the studies reporting a difference were conducted using human LDSCs.

The morphology of cells isolated in this study was similar across all cell types. After passaging, all cell types displayed a predominantly spindle-shaped fibroblastic morphology with MSCs demonstrating a slightly less elongated morphology than the other cells (Fig.5.1.A). This is consistent with the literature

which has also reported a fibroblastic morphology for human PDLSCs (Eleuterio *et al*., 2013; Fujii *et al*., 2008; Liu *et al*., 2014b), canine PDLSCs (Sedigh *et al*., 2010) and human ACLDSCs (Cheng *et al*., 2009; Singhatanadgit *et al*., 2009; Zhang *et al*., 2011b). In addition, previous studies have also reported a fibroblastic morphology of human PDL-derived ligamentocytes (Zhang *et al*., 2013), as well as canine MSCs (Csaki *et al*., 2007). After passaging, all cell types exhibited a fairly homogeneous morphology, however upon initial seeding growth of colonies composed of fibroblastic cells as well as colonies of rounded cobblestone-like cells for LDSC-F were observed in this study (Fig.5.1.D). These rounded cells disappeared with passaging, which may be due to changes in cell phenotype or differentiation of these cells into ligamentocytes. Alternatively, the fibroblastic shaped cells may have outgrown the rounded cells and caused a loss of this cell type. These observations are consistent with some previous studies which have also noted a rounded morphology upon initial plating and a fibroblastic morphology after passaging cells derived from human ACL remnants (Fu *et al*., 2015). In addition, other studies have reported heterogeneous cell morphology of equine and human ligament cells after passaging (Shikh Alsook *et al*., 2015; Singhatanadgit *et al*., 2009).

5.5.3 Proliferation of canine ligament cells

A similar population doubling time (PDT) was observed for all ligament cell types, with the PDT for MSCs being significantly higher (Fig.5.2). Previous studies have reported similar PDTs for human ACLDSCs, ranging between 40 and 58 hours (Cheng *et al*., 2010; Singhatanadgit *et al*., 2009; Uefuji *et al*., 2014). These values are slightly higher than those reported in this study (ranging between 27 and 35 hours for ligament cells), however not considerably. In contrast, another study reported a PDT of 120 hours for human ACLDSCs, which is approximately four to five times the PDT observed in this study. The same study found a significantly lower PDT for cells isolated from the medial collateral ligament (MCL) (Zhang *et al*., 2011b) indicating the variation between ligament types. In addition, cells isolated from equine suspensory ligaments demonstrated a PDT of 24 hours (Shikh Alsook *et al*., 2015) much similar to that seen in this study, possibly suggestive of variation between

species. Previous studies in canine MSCs have also demonstrated large PDTs (Linon *et al*., 2014) and a study comparing LDSCs and MSCs found LDSCs to proliferate faster than MSCs (Gay *et al*., 2007) which is consistent with my work.

5.5.4 Stem cell and tenogenic marker expression of canine ligament cells

Another criterion for the identification of MSCs, as outlined by the ISCT (Dominici *et al*., 2006) is the expression of certain stem cell markers. Normally this would be assessed using antibodies and flow cytometry, however I was unable to analyse marker expression using this method and therefore analysed gene expression of stem cell and tenogenic markers instead. The reason for this is due to the limited availability of canine antibodies and the lack of validation for those antibodies that are available. Rozemuller and others (2010) analysed 43 antibodies against MSC markers for cross-reactivity across a variety of species, including dogs (Rozemuller *et al*., 2010). Only a very small number of antibodies demonstrated any reactivity with canine MSCs and those that did were extremely weak. This indicates that most antibodies raised against MSC markers in humans or other species are not compatible for use in dogs and therefore any antibodies required for canine work need to be specifically designed. Unfortunately very few of these antibodies are currently available.

In this study no distinct patterns in marker gene expression were observed, however generally LDSC-F and MSCs demonstrated the highest expression of stem cell markers and MSCs the highest expression of tenogenic markers. However expression of tenogenic markers was generally low for all cell types when compared with stem cell marker expression. Oct4 expression was generally low across all cell types with the highest levels seen in LDSC-F and MSCs (Fig.5.3). This is consistent with the literature which has demonstrated low level expression of Oct4 in human ACLDSCs (Zhang *et al*., 2011b). My results are also consistent with reports that Oct4 expression is restricted to embryonic stem cells only and not somatic cells (Lengner *et al*., 2008), which would explain the low expression seen here. The CD markers were expressed in higher levels than Oct4 and were generally more highly expressed in LDSC-F. CD105 was expressed highly in LDSC-F and MSCs (Fig.5.3)

which is consistent with the literature which has demonstrated expression of CD105 in human PDLSCs (Eleuterio *et al*., 2013; Hakki *et al*., 2014; Liu *et al*., 2014b), canine PDLSCs (Wang *et al*., 2012), human ACLDSCs (Cheng *et al*., 2009; Steinert *et al*., 2011) equine suspensory ligaments (Shikh Alsook *et al*., 2015) and canine MSCs (Csaki *et al*., 2007). CD44 expression was similar between all cell types (Fig.5.3) and has previously been reported in human PDLSCs (Eleuterio *et al*., 2013; Prateeptongkum *et al*., 2015), canine PDLSCs (Lee *et al*., 2014b), human ACLDSCs (Cheng *et al*., 2009; Singhatanadgit *et al*., 2009; Zhang *et al*., 2011b), human ligamentocytes (Kowalski *et al*., 2015) and canine MSCs (Linon *et al*., 2014; Takemitsu *et al*., 2012). In this study, CD90 expression was more variable between cell types with significant differences seen between MSCs and both LDSC-F and ligamentocytes with LDSC-F showing the highest expression and MSCs the lowest (Fig.5.3). CD90 expression has previously been reported in human PDLSCs (Eleuterio *et al*., 2013; Hakki *et al*., 2014; Liu *et al*., 2014b), canine PDLSCs (Lee *et al*., 2014b), human ACLDSCs (Cheng *et al*., 2009; Steinert *et al*., 2011; Zhang *et al*., 2011b), equine suspensory ligaments (Shikh Alsook *et al*., 2015) and canine MSCs (Csaki *et al*., 2007). Similarly to CD90, CD73 expression was also variable between cell types with increased levels seen in LDSC-F, which were significant when compared with all other cell types (Fig.5.3). CD73 expression has previously been demonstrated in human PDLSCs (Eleuterio *et al*., 2013; Hakki *et al*., 2014), human ACLDSCs (Cheng *et al*., 2009; Steinert *et al*., 2011) and human MSCs from OA joints (Stiehler *et al*., 2016). Tenogenic markers that may be involved in ligament development and expressed by LDSCs and/or ligamentocytes (Brandau *et al*., 2001; Nakahara *et al*., 2013; Seo *et al*., 2004) were also analysed. All of the markers analysed were more highly expressed in MSCs when compared with ligament-derived cells. Similar levels were seen between ligament cell types with a decrease observed in LDSC-L for some of the markers. Expression levels of all three tenogenic markers were low, compared with CD markers (Fig.5.3). SCX expression has been previously reported in human PDLSCs (Fujii *et al*., 2008; Seo *et al*., 2004; Zhang *et al*., 2015) and canine PDLSCs (Wang *et al*., 2012). No studies have reported SCX expression in canine MSCs however expression has been demonstrated in MSCs isolated from rats (Dai *et al*., 2015). The expression of MKX has not previously been demonstrated in LDSCs

of any species or canine MSCs, however expression has been observed in rat and human MSCs (Dai *et al*., 2015; Otabe *et al*., 2015). SCX and MKX are transcription factors expressed by tendon and ligament cells during development as well as in mature tendon for SCX (Liu *et al*., 2010; Mendias *et al*., 2012; Nakahara *et al*., 2013; Schweitzer *et al*., 2001). Therefore, I expected to see increased levels in LDSCs compared with ligamentocytes however the differences were small and not statistically significant (Fig.5.3). I also expected to observe lower levels of expression in MSCs as these cells are not committed to the tenogenic/ligamentogenic lineage and therefore would not express tenogenic markers. This is in agreement with previous studies on tendon which have reported a reduction in SCX expression in MSCs when compared with tendon tissue (Burk *et al*., 2014). In contrast, increased expression of tenogenic markers was observed in MSCs when compared with ligament cells in this study. This may be due to the fact that the MSCs in this study were isolated from OA joints, as other cell types isolated from tissues present in OA joints have shown an increase in SCX expression when compared with cells from healthy joints (Hasegawa *et al*., 2013). TNMD expression was significantly increased in MSCs compared with all ligament cell types (Fig.5.3). TNMD expression has been demonstrated in human ACLDSCs and MSCs (Steinert *et al*., 2011), where TNMD levels were increased in MSCs compared with LDSCs, consistent with this study. CD34 and CD45 are haematopoietic and endothelial cell markers and negative expression is essential to confirm that there are no contaminating cell types. Expression was low in all cell types but particularly for LDSC-L (Fig.5.3). This is consistent with the literature which has reported low expression of CD34 and CD45 in LDSCs (Liu *et al*., 2014b; Singhatanadgit *et al*., 2009) and canine MSCs (Csaki *et al*., 2007).

In this study increased expression of stem cell markers was observed in LDSC-F and MSCs when compared with ligamentocytes, as expected. However I did not expect to see increased expression of tenogenic markers in MSCs when compared with ligament cells, although the literature has reported increased expression of some tenogenic markers in MSCs when compared with LDSCs (Steinert *et al*., 2011) which is consistent with this study. This may be due to the fact that MSCs are able to differentiate into ligamentocytes and therefore would require

the ability to express tendon/ligament developmental genes. Similar expression of tenogenic markers between the different ligament cell types was observed in this study as expected (Fig.5.3).

5.5.5 Multipotency of canine ligament cells

The final criterion for the identification of MSCs is the ability to differentiate into different cell lineages, specifically osteogenic, adipogenic and chondrogenic lineages (Dominici *et al*., 2006). Osteogenic differentiation was assessed by staining for alkaline phosphatase (ALP) activity using BCIP/NBT and calcium formation using alizarin red (AR) (Fig.5.4) as well as expression of osteogenic markers (Fig.5.6). Moderate amounts of ALP staining were observed for ligamentocytes and LDSC-L and increased amounts for LDSC-F and MSCs. Similarly, for AR staining I observed small amounts of staining for ligamentocytes, no staining for LDSC-L and increased levels for LDSC-F and MSCs. This is consistent with the literature which has shown ALP and AR positive staining in human PDLSCs (Eleuterio *et al*., 2013; Gay *et al*., 2007; Seo *et al*., 2004), canine PDLSCs (Lee *et al*., 2014b), human ACLDSCs (Cheng *et al*., 2009; Steinert *et al*., 2011) and human ligamentocytes, although reduced amounts of staining were observed when compared with LDSCs (Kowalski *et al*., 2015). Osteogenic marker gene expression was consistent with the staining described above with an increase in osteopontin (OPN), osteocalcin (OC) and Runx2 observed in all cell types, however larger increases were seen for MSCs and LDSC-F. Again this is consistent with the current literature with increases in OPN, OC and Runx2 observed in human ACLDSCs (Cheng *et al*., 2009; Zhang *et al*., 2011b), PDLSCs and ligamentocytes (Fujii *et al*., 2008). The expression of Runx2 reported in one study in ALCDSCs was considerably lower than the level reported in this study (Fu *et al*., 2015), however this may be due to the reduced differentiation period of two weeks used by Fu *et al* (2015) compared to the three weeks used in this study. No studies to date have analysed the expression of these markers in canine MSCs, however expression has been reported in other species such as humans and mice (Stiehler *et al*., 2016; Tropel *et al*., 2004). Similar expression levels between LDSC-F and MSCs were observed in this study, which is inconsistent with the literature,

which has demonstrated increased osteogenic marker expression in human MSCs when compared with ACLDSCs (Cheng *et al*., 2010; Singhatanadgit *et al*., 2009; Steinert *et al*., 2011).

Adipogenic differentiation was assessed by the staining of oil droplets using oil red O (ORO) (Fig.5.4), as well as analysis of adipogenic marker expression (Fig.5.6). Adipogenic differentiation was only observed for LDSC-F and not in any other cell type analysed. Although this was the result I expected for ligamentocytes and LDSC-F, it was unexpected for MSCs. ORO staining has previously been reported in human PDLSCs (Fujii *et al*., 2008; Liu *et al*., 2014b; Seo *et al*., 2004), canine PDLSCs (Lee *et al*., 2014b; Wang *et al*., 2012) and human ACLDSCs (Cheng *et al*., 2010; Steinert *et al*., 2011; Zhang *et al*., 2011b), consistent with this study. ORO staining has also been reported in human ACL-derived ligamentocytes (Kowalski *et al*., 2015) whereas I observed no staining in canine ligamentocytes. This may reflect differences between species or possible contamination of ligamentocyte populations with progenitor cells. As discussed previously no ORO staining for canine MSCs was seen in this study which was unexpected as the adipogenic potential of MSCs is well documented in many species, including dogs (Takemitsu *et al*., 2012). Several studies have directly compared the differentiation potential of human MSCs and ACLDSCs with some studies finding no differences in the adipogenic potential of the two cells types (Cheng *et al*., 2010; Steinert *et al*., 2011) and with some finding an increase in adipogenic potential in MSCs when compared to ACLDSCs (Singhatanadgit *et al*., 2009). The results of these studies are inconsistent with my findings and as previously discussed this may be due to the source of the MSCs for this study which were from femurs of dogs with osteoarthritic hip joints. To my knowledge no studies have investigated the effects of OA on MSC characteristics in dogs, however such studies have been conducted in humans. Two studies found that ORO staining decreased in bone marrow MSCs derived from OA joints compared with those derived from healthy controls (Murphy *et al*., 2002; Stiehler *et al*., 2016). This is consistent with my data and may indicate that the lack of adipogenic differentiation seen in canine MSCs is due to the effects of OA on the joint environment and the native cells. This may be due to changes in the composition and structure of the ECM, or alterations in growth factors and

cytokines, as well as oxidative stress, all of which are associated with OA (Loeser, 2009). Adipogenic marker gene expression was partially consistent with the pattern of ORO staining. There was an increase in all three markers for all cell types, despite the lack of ORO staining in all but LDSC-F, however the highest levels and largest increases between positive and negative samples were seen in LDSC-F. This is consistent with the literature which has shown expression of PPARγ in human PDLSCs (Fujii *et al*., 2008; Liu *et al*., 2014b; Seo *et al*., 2004) and canine MSCs (Csaki *et al*., 2007). FABP4 and PPARγ expression has also been demonstrated in human ACLDSCs (Cheng *et al*., 2010; Zhang *et al*., 2011b). I observed reduced expression of PPARγ in MSCs when compared with LDSC-F, however the literature reports the opposite with increased levels seen in human MSCs compared with ACLDSCs (Singhatanadgit *et al*., 2009). This may be due to the fact that I used MSCs from canine OA joints which demonstrated a reduction in adipogenic differentiation, as discussed previously. The expression of adipogenic marker genes in ligamentocytes, LDSC-L and MSCs does not correlate with the lack of ORO staining observed, however this may be due to discrepancies in gene and protein expression and RNA/protein degradation.

Chondrogenic differentiation was assessed by staining for proteoglycan deposition using alcian blue (AB) and safranin O (SO) (Fig.5.4), analysis of glycosaminoglycan (GAG) content (Fig.5.5) and chondrogenic marker genes (Fig.5.6). For ligamentocytes, LDSC-L and MSCs there were small increases in stain intensity indicative of limited chondrogenic differentiation potential. There were larger increases seen in stain intensity for LDSC-F suggestive of a greater chondrogenic differentiation potential than the other cell types. This is consistent with previous studies that have reported SO staining in human PDLSCs (Zhou *et al*., 2014) and AB and SO staining in human ACLDSCs (Cheng *et al*., 2009; Kowalski *et al*., 2015; Uefuji *et al*., 2014). Chondrogenic staining has also been demonstrated for ligamentocytes derived from human ACL (Kowalski *et al*., 2015) as well as canine MSCs (Csaki *et al*., 2007). Analysis of GAG content of cell pellets after chondrogenic induction in different cell types showed increases in GAG content in all ligament cell types, however due to large variation between biological replicates these differences were not significant. A decrease in GAG content was observed in the

positive samples compared with the negative samples for MSCs which was unexpected. Several studies have analysed the formation of GAGs after chondrogenic differentiation in human ligament cells and MSCs. One study observed similar levels of GAG formation in human ACLDSCs and ligamentocytes (Kowalski *et al*., 2015), as reported in this study. Another study reported GAG formation in ACLDSCs and MSCs with increased levels produced by MSCs (Steinert *et al*., 2011), inconsistent with my data. The reason for this is most likely due to the OA source of the MSCs used in this study. A previous study using human MSCs found a decrease in GAG formation in MSCs from OA joints compared with healthy controls (Murphy *et al*., 2002) which correlates with my findings. Gene expression of chondrogenic markers was weak for all cell types, with little difference observed between positive and negative samples. This is inconsistent with the literature, which has generally demonstrated greater induction of chondrogenic markers than reported in this study. Collagen type II (COL2) and SOX9 expression has been reported in human PDLSCs (Gay *et al*., 2007; Zhou *et al*., 2014) and COL2, SOX9 and aggrecan (AGG) expression in human ACLDSCs (Cheng *et al*., 2010; Cheng *et al*., 2009; Steinert *et al*., 2011; Zhang *et al*., 2011b). Although some studies have also reported lower expression of chondrogenic markers in human ACLDSCs with reduced expression levels of AGG, SOX9 and COL2 (Fu *et al*., 2015; Singhatanadgit *et al*., 2009) as demonstrated by my data. I observed the same expression levels between positive and negative samples for canine MSCs. This is inconsistent with the previous literature which has demonstrated COL2 and SOX9 expression in canine MSCs (Csaki *et al*., 2007), however as mentioned previously this is most likely due to the OA source of MSCs in this study. One study observed only moderate staining of chondrogenic pellets for COL2 in MSCs from OA joints (Murphy *et al*., 2002) which may explain the lack of chondrogenic marker expression in this study.

5.5.6 Conclusion

I have successfully isolated a population of cells from canine cranial cruciate ligament (CCL) which demonstrate many of the hallmark properties of stem cells (Dominici *et al*., 2006). They are able to form colonies, express a variety of stem cell

markers, as well as possessing the ability to differentiate into osteogenic, adipogenic and chondrogenic cell types. To date no other studies have isolated such cells from canine CCL, however LDSCs have been isolated from human ACL, and these cells demonstrate the same properties as the cells isolated in this study (Cheng *et al*., 2010; Steinert *et al*., 2011; Zhang *et al*., 2011b).

The LDSCs I have characterised were isolated by differential adhesion to fibronectin substrates. Although no previous studies on LDSCs have used this method for cell isolation it has been used previously for isolation of cartilage stem cells from bovine articular cartilage (Dowthwaite *et al*., 2004), as well as human endothelial progenitor cells (Bueno-Betí *et al*., 2013), indicating the potential use of this method for isolation of a range of progenitor cells. I also used this method for the isolation of equine tendon-derived stem cells (TDSCs) (see Chapter 3) alongside the more traditional low-density plating method. I initially attempted isolation of LDSCs using the low-density plating method as this method has been successfully employed to isolate TDSCs in a number of studies and I have also used this method to successfully isolate murine TDSCs. Unfortunately this method did not yield ligament cells with the desired phenotype, demonstrating reduced stem cell and tenogenic marker expression and a restricted differentiation potential. Even though this method has been successfully employed to isolate stem cells from tendon tissue in a number of species (Bi *et al*., 2007) and despite the similarities between tendon and ligament, it might not be an appropriate method for isolation of LDSCs. This may be due to species or tissue differences (Kharaz *et al*., 2016) or the cellular environment. Previous studies that have isolated LDSCs have used higher seeding densities than those used in this study and this may be due to the variation in phenotype seen at lower densities, which have been observed here. Due to the restricted phenotype of cells isolated by low-density plating, cells were also seeded at slightly higher densities on to fibronectin substrates to promote differential adhesion. This method was more successful and I was able to isolate a population of cells with stem cell properties, as previously described. Ligamentocytes (ligament fibroblasts) were also isolated as a comparative cell type. There are few studies that have investigated the stem cell properties of ligamentocytes or compared these cells with LDSCs, however I noted a number of differences between ligamentocytes

and LDSC-F. Namely, differences in colony formation, stem cell marker expression and multipotency. Conversely very few differences were seen between ligamentocytes and LDSC-L suggesting that LDSC-L are not stem cells. This is concurrent with the literature which has demonstrated similar characteristics between human ligamentocytes and LDSC-L (Fujii *et al*., 2008). The MSCs isolated in this study did not demonstrate the expected characteristics as demonstrated in previous studies for canine MSCs (Csaki *et al*., 2007; Takemitsu *et al*., 2012). The main difference being the restricted differentiation potential with a lack of adipogenic differentiation seen in MSCs isolated in this study. I also observed several differences between MSCs and LDSC-F. As LDSCs are a subset of MSCs I expected to see many similarities between these cell types, however I observed differences in colony formation, cell proliferation, stem cell and tenogenic marker expression and multipotency. A small number of studies have directly compared the characteristics of human LDSCs and MSCs and generally observed similar characteristics between cell types with MSCs demonstrating reduced colony formation but increased multipotency when compared with LDSCs (Cheng *et al*., 2010; Gay *et al*., 2007). As described previously, the differences seen here are most likely due to the source of the MSCs used in this study which were from femurs of dogs with osteoarthritic hip joints. The effects of OA on cells within the joint is well-documented with MSCs showing changes in stem cell marker expression as well as reduced multipotency (Chua *et al*., 2014; Grogan *et al*., 2009; Murphy *et al*., 2002), as seen here.

The cell population I have isolated from canine CCL could hold therapeutic potential for the treatment of ligament injuries. Many studies have investigated the use of PDLSCs for repair of periodontal ligament with many successful outcomes (Fujii *et al*., 2008; Liu *et al*., 2008; Yang *et al*., 2009), however no studies have yet investigated the use of ACLDSCs to repair ACL injuries. One study has assessed the ability of LDSCs isolated from rabbit medial collateral ligament (MCL) to promote ligament repair (Jiang *et al*., 2015). The study found that LDSCs promoted ligament repair and remodelling (Jiang *et al*., 2015) and highlights the potential use of LDSCs for the repair of injured and degenerated ligament. The cells isolated in this study could potentially be used in a similar manner. The majority of animal tissue

engineering studies using stem cells involve extracting the cells from the tissue of euthanized animals, culturing them, seeding them on to scaffolds or matrices before implanting them into induced injury sites of other animals (Chen *et al*., 2014; Jiang *et al*., 2015; Lui *et al*., 2014; Xu *et al*., 2014). This technique is useful and provides an insight into the repair potential of certain cell types for particular injuries, however this approach is not translatable in a clinical setting. Clinically this is not a feasible approach as it would involve surgery to extract LDSCs and culture of these cells over an uncertain time period, before further surgery to re-implant the cells. For this reason MSCs are commonly used to treat tendon and ligament injuries as they are easier to extract from bone marrow and adipose tissue and many studies have demonstrated successful tissue repair with such cells (Carvalho *et al*., 2013; Conze *et al*., 2014; Linon *et al*., 2014; Smith *et al*., 2013). These studies injected the MSCs into the lesion, which avoided invasive surgical procedures and could provide a potential route for the administration of LDSCs into injury sites. Regardless of the success of using MSCs for tendon and ligament repair, several studies have shown the inferiority of such cells when compared with native cells isolated from the tissue of interest (Huang *et al*., 2008; Pietschmann *et al*., 2013; Tan *et al*., 2012b). Therefore, the use of LDSCs for ligament repair would be preferable over MSCs. The use of allogeneic LDSCs, and injection of these cells into the lesion could provide a potential treatment strategy for ligament injuries in animals and humans, however there are many issues associated with the use of allogeneic cells, for example the requirement for immunosuppression (Lane *et al*., 2014).

Alternatively, a less invasive and high-risk method could be employed which would involve knowledge of the extracellular environment of LDSCs. If the extracellular environment of the LDSCs, or stem cell niche, could be characterised and factors which promote stem cell viability and beneficial differentiation identified, then those factors could form the basis of a treatment strategy. These factors could be introduced artificially to the lesion where they would promote native stem cell proliferation and viability as well as differentiation into ligamentocytes and ECM production. This approach has been used successfully to modulate the niche of various stem cell types in order to treat a range of

conditions (Cianfarani *et al*., 2006; Olnes *et al*., 2012). Therefore analysis of the LDSC niche and the factors that promote LDSC viability will form the basis of future work in this area. In addition, future work would include further analysis of LDSC-F populations, with particular emphasis on the differences in phenotype of the different cell morphologies seen upon initial plating compared with later passages. Identification of markers specific to LDSCs would also be beneficial for LDSC characterisation and analysis, as well as for clinical implementation of this research.

Chapter 6

Proteomic analysis of the canine ligament-derived stem cell niche

6.1 Introduction

The stem cell niche is the environment in which stem cells reside and consists of a number of different cellular and molecular factors (Lane *et al*., 2014). One example is the protein composition of the extracellular matrix (ECM) which has been shown to be integral for stem cell survival and function in a number of different cell populations. For example, neural stem cells are regulated by a number of factors including the ECM protein tenascin C (Garcion *et al*., 2004; Garwood *et al*., 2004). Tenascin C is highly expressed in the mammalian central nervous system and tenascin C-deficient mice have altered numbers of neural stem cells (Garcion *et al*., 2004) as well as increased migration and reduced proliferation of neural precursors (Garcion *et al*., 2001). The lack of tenascin C expression by neural stem cells also promotes early differentiation (Garwood *et al*., 2004). The haematopoietic stem cell (HSC) niche is also regulated by ECM composition. Fibronectin and collagen have both been found to regulate HSCs (Yokota *et al*., 1998). Fibronectin and laminin promoted growth and colony formation of HSCs (Sagar *et al*., 2006; Yokota *et al*., 1998) whereas collagen type I caused a decrease in HSC expansion unless present in combination with other ECM proteins (Çelebi *et al*., 2011), indicating the importance of ECM protein interactions in stem cell regulation. Tendon-derived stem cells (TDSCs) are also regulated by the ECM, with the genetic inactivation of fibromodulin and biglycan causing increased clonogenicity and proliferation, but a decrease in the ability of TDSCs to differentiate into tenocytes, with decreased expression of tendon markers such as scleraxis and collagen. Erroneous differentiation of TDSCs was promoted with both ossification and chondrogenesis being observed (Bi *et al*., 2007). The role of ECM proteins in stem cell regulation in these tissues, particularly tendon, is suggestive of the importance of the LDSC niche in LDSC regulation.

The study of the entire complement of proteins produced by a cell or tissue, otherwise known as the proteome, is known as proteomics (Wilkins *et al*., 1996). This technique is now widely used to investigate the proteomes of many tissue types, including musculoskeletal tissues such as cartilage, tendon and

ligament (Kharaz *et al*., 2016; Little *et al*., 2014; Peffers *et al*., 2014a; Peffers *et al*., 2014b; Wilson *et al*., 2010). Proteomics is able to identify proteins present, as well as protein interactions, modifications and structure (de Hoog and Mann, 2004).

Mass spectrometry (MS) is a commonly utilised method for proteomic analysis and this technique firstly involves digestion and separation of the proteins to aid analysis. Digestion is normally achieved using a protease such as trypsin to produce peptides with specific molecular weights, defined by the peptide sequence. Peptide separation can be achieved using gel electrophoresis, or for high throughput experiments, liquid chromatography (LC) can be used (de Hoog and Mann, 2004). Proteins were digested using trypsin and separated using LC in this study due to the complexity of the protein sample. Mass spectrometry involves three parts: ion source, mass analyser and detector (Aebersold and Mann, 2003). Peptides are ionised using either electrospray ionisation (ESI) or matrix-assisted laser desorption/ionisation (MALDI) (Aebersold and Mann, 2003). ESI is preferred for complex samples and is easily coupled to LC, therefore this method was used for this study. After ionisation peptides of a particular mass are passed through to a mass analyser for fragmentation. There are four main types of mass analyser: ion trap, quadrupole, time-of-flight (TOF) and Fourier transform ion cyclotron (FT-MS) (Aebersold and Mann, 2003). Each mass analyser is designed for a particular purpose and has advantages and disadvantages, therefore it is common to use multiple mass analysers in tandem. This study utilised tandem MS using quadrupole and ion trap mass analysers. Peptides are then fragmented by either collision induced dissociation (CID) or higher energy collisional dissociation (HCD) in the second mass analyser (Aebersold and Mann, 2003). Finally, the detector allows visualisation of mass spectra for further analysis (de Hoog and Mann, 2004).

Protein identification is achieved by converting the raw MS spectra into lists of peptides and proteins using software such as PEAKS or MASCOT and then comparing the profile of peptides produced from the sample in question with species-specific theoretical peptide library databases such as UniProt or RefSeq (Matthiesen and Jensen, 2008). Proteins can then be quantified using either absolute or relative quantitation. Relative quantitation has been used in this study

with two sample groups being compared. There is also a choice of whether to use label-free or label-based methods, with both techniques providing certain advantages and disadvantages. In this study I have used a stable heavy isotope metabolic labelling method based on the 'stable isotope labelling with amino acids in cell culture' (SILAC) technique. Label-based proteomic analysis provides increased quantitative accuracy when compared with label-free methods (Bantscheff *et al*., 2007; Chen *et al*., 2015d; Collier *et al*., 2010; Liu *et al*., 2013; Merl *et al*., 2012). In addition, metabolic labelling methods enable analysis of protein synthesis and turnover (Doherty *et al*., 2009; Doherty *et al*., 2005; Schwanhäusser *et al*., 2009). Proteomic analysis of a cell or tissue provides only a snapshot of that cell or tissue's proteome at a single point in time. The proteome is constantly changing as proteins are synthesised and degraded reflecting the developmental, physiological and pathological status of the cell or tissue (Claydon and Beynon, 2012; Pratt *et al*., 2002). Therefore, the investigation of proteome dynamics is integral in order to fully understand the function and role of proteins within their environment.

6.2 Hypothesis

I hypothesise that LDSCs isolated from canine cranial cruciate ligament (CCL) reside within a specific niche within ligament tissue and this is reflected in the specific ECM composition of the LDSC environment and which differs to the ligamentocyte environment. There will be differences in the rate of synthesis and turnover of specific ECM components, with increased synthesis and turnover of non-collagenous proteins compared with collagenous proteins. There will also be differences in protein synthesis and turnover rates between LDSCs and ligamentocytes.

6.3 Aims

1. To characterise the LDSC niche in canine CCL in terms of ECM composition and compare the extracellular environments of LDSCs and ligamentocytes.
2. To investigate the rate of protein synthesis and degradation of ECM components in the LDSC and ligamentocyte niche.

6.4 Results

6.4.1 Optimisation of cellular and extracellular matrix extraction methods

Three different cellular extraction methods were tested (see Chapter 2, Section 2.2.3.3): EDTA, trypsin and detergent for their ability to remove cells and leave the ECM intact. Cells were subjected to each extraction method and light microscopy was used to detect any remaining cellular matter. EDTA was unable to remove cells (Fig.6.1.A) and therefore was not included in further experiments. Trypsin removed the majority of cells with a few remaining (Fig.6.1.B) whereas detergent appeared to remove all cells leaving behind possible ECM remnants (Fig.6.1.C).

Next, the quantity and complement of ECM proteins extracted using a urea extraction buffer and a sample buffer in combination with the cellular extraction methods previously discussed were analysed, as well as the ability of canine ligament cells to incorporate heavy labelled amino acid isotopes during protein synthesis (Fig.6.2). The use of trypsin to remove cells resulted in an increased number of ECM proteins identified when compared with detergent for which there was little protein present (Fig.6.2.A). There was also increased incorporation of ^{14}C-labelled amino acids in the trypsin samples compared with the detergent (Fig.6.2.B), most likely due to the increased number and quantity of proteins in the trypsin samples. The urea extraction buffer yielded fewer proteins than the sample buffer (Fig.6.2.A), however incorporation of ^{14}C-labelled amino acids appeared to be similar between extraction buffers (Fig.6.2.B). Due to the potential incompatibility of the sample buffer with downstream processing the urea extraction buffer was used for subsequent ECM extractions and trypsin for cellular removal.

Validation of this method using mass spectrometry was then performed, however only 135 protein hits were recorded, therefore another extraction technique was tested using Rapigest. After removal of cells with trypsin ECM proteins were extracted using a Rapigest buffer which yielded 603 protein hits (data not shown).

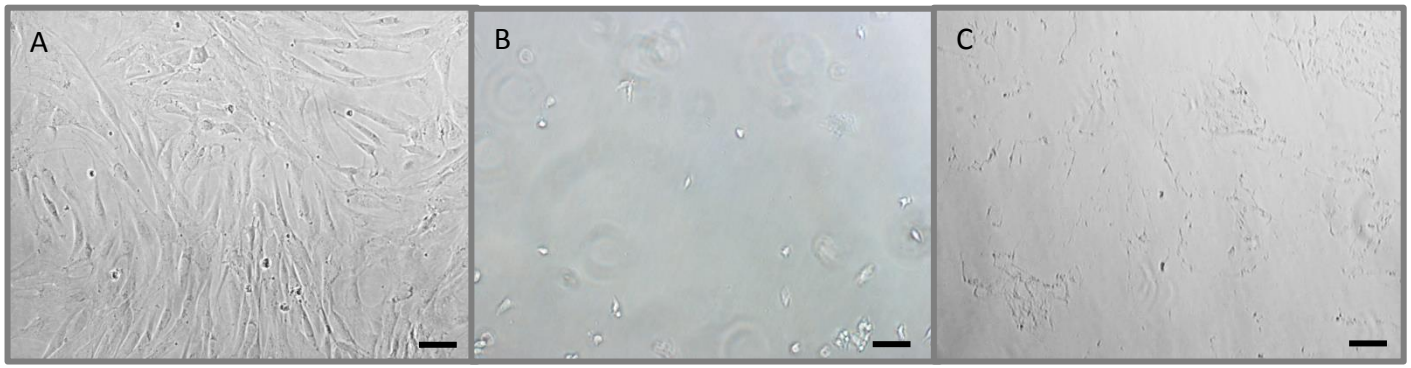


Figure 6.1. Comparison of cellular extraction methods. LDSCs grown in monolayer were subjected to varying cellular extraction methods for ECM denuding: EDTA (A); trypsin (B); and detergent (C). Scale bars = 100 µm.

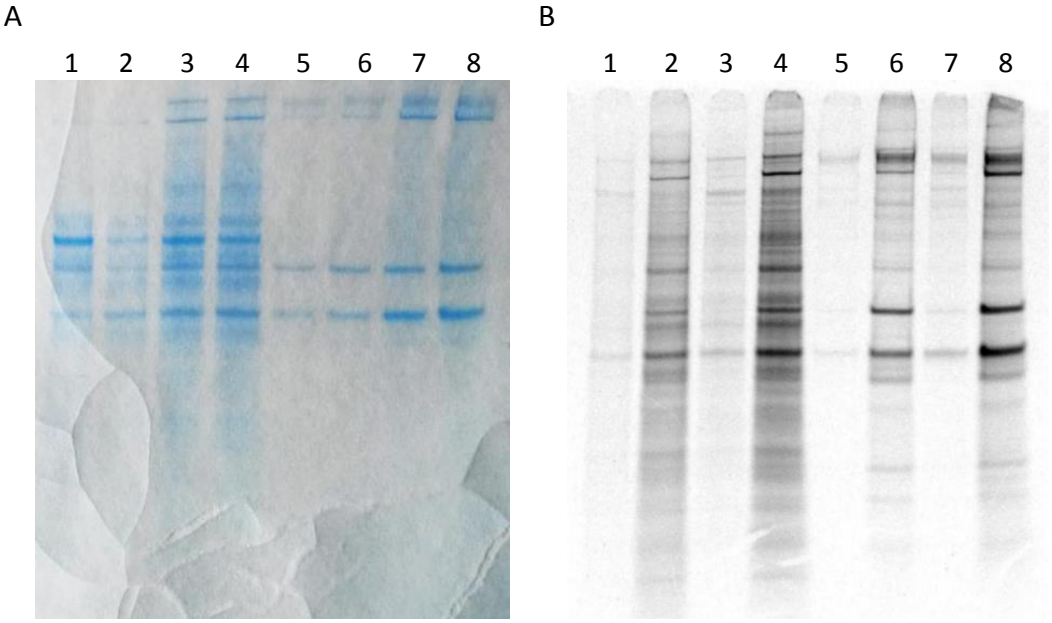
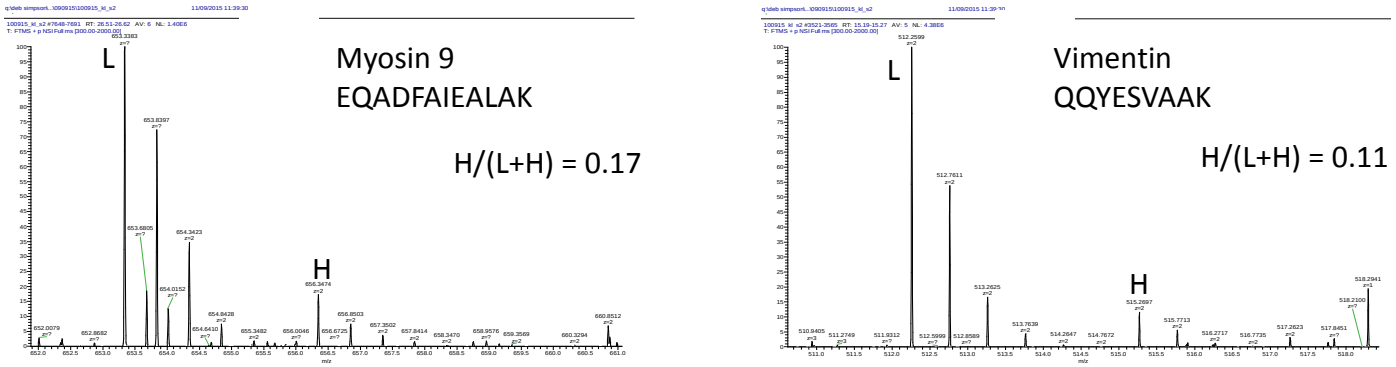


Figure 6.2. Comparison of extracellular matrix and cellular extraction methods. LDSCs grown in monolayer were labelled with either [^{14}C]L-proline (pro) for labelling of collagenous proteins, or [^{14}C]L-lysine (lys) and [^{14}C]L-arginine (arg) for labelling of non-collagenous proteins. Cells were then subjected to varying cellular/ECM extraction method combinations: 1 – Pro – trypsin/urea buffer; 2 – Lys/arg – trypsin/urea buffer; 3 – Pro – trypsin/sample buffer; 4 – Lys/arg – trypsin/sample buffer; 5 – Pro – detergent/urea buffer; 6 – Lys/arg – detergent/urea buffer; 7 – Pro – detergent/sample buffer; 8 – Lys/arg – detergent/sample buffer. ECM protein extracts were then separated by electrophoresis and stained with GelCode (A). The gels were then dried and analysed using a phosphorimager to detect incorporation of ^{14}C-labelled amino acids into ECM proteins (B).

6.4.2 Optimisation of heavy lysine labelling for proteomic analysis

After determining that heavy labelled amino acid isotopes could be incorporated into canine ligament cells I next determined that such incorporation could be detected by mass spectrometry. Using the urea extraction buffer method [^{13}C]L-lysine labelling was only observed for cellular proteins and incorporation was low (Fig.6.3.A). However, after changing to the Rapigest extraction method and increasing initial cell numbers incorporation of [^{13}C]L-lysine into ECM proteins as well as cellular proteins was observed (Fig.6.3.B).

A



B

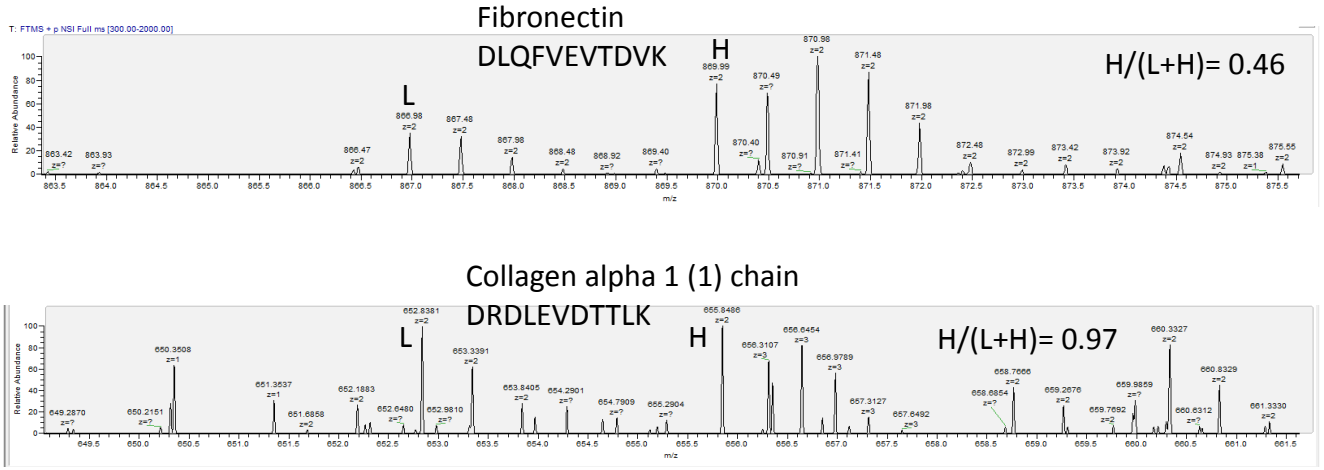


Figure 6.3. Extracted ion chromatograms of representative proteins from ^{13}C-lysine labelled canine ligament cell ECM extracts demonstrating label incorporation. L = light isoform, H = heavy isoform. H/(L+H) = proportion of peptide labelled with heavy isoform.

6.4.3 Identification and label-free quantification of ECM proteins in the LDSC and ligamentocyte niche

ECM samples extracted from LDSC and ligamentocyte cultures using a Rapigest extraction buffer were analysed using LC-MS/MS (see Chapter 2, Section 2.2.3.7).

A total of 83 extracellular matrix related proteins were identified by PEAKS software (Zhang *et al*., 2012) and the Matrisome Project database (Naba *et al*., 2016) as being produced by ligament cells (both LDSCs and ligamentocytes). The list of proteins is provided in Table 6.1 and the proportion of proteins belonging to each ECM category is shown in Figure 6.4. The Matrisome Project categorises proteins into ECM collagens (10 proteins), ECM proteoglycans (6 proteins), ECM glycoproteins (17 proteins), ECM affiliated proteins (14 proteins), ECM regulators (29 proteins) and secreted factors (7 proteins). A STRING protein interaction map shows strong interactions between collagens with weaker clusters forming for some regulatory proteins and glycoproteins (Fig.6.5).

A comparison between ECM proteins produced by LDSCs and ligamentocytes identified only small differences with 8 proteins unique to LDSCs and 8 proteins unique to ligamentocytes (Fig.6.6). Those were predominately glycoproteins and regulatory proteins unique to ligamentocytes and collagens and proteoglycans unique to LDSCs (Table 6.2).

Label-free quantitative analysis was performed using PEAKS software and the Matrisome Project database to determine differential abundance of ECM proteins between LDSCs and ligamentocytes, however there were no differentially abundant ECM proteins between the two cell types.

ECM collagens	Collagen type I, alpha 1 Collagen type I, alpha 2 Collagen type II, alpha 1 Collagen type III, alpha 1 Collagen type V, alpha 1 Collagen type V, alpha 2 Collagen type VI, alpha 1 Collagen type VI, alpha 2 Collagen type VI, alpha 3 Collagen type XII, alpha 1
ECM proteoglycans	Biglycan Decorin Lumican Mimecan Proteoglycan 4 Versican
ECM glycoproteins	EGF-like repeats and discoidin I-like domains 3 Elastin Elastin microfibril interfacer 1 Fibulin 1 Fibrinogen alpha chain Fibrinogen beta chain Fibrinogen gamma chain Fibronectin Latent transforming growth factor beta binding protein 2 Milk fat globule-EGF factor 8 protein Periostin Transforming growth factor beta I Thrombospondin 1 Thrombospondin 4 Tenascin C Tsukushi small leucine rich proteoglycan homolog Vitronectin
ECM affiliated proteins	Annexin A1 Annexin A2 Annexin A4 Annexin A5 Annexin A6 Annexin A7 Annexin A8 Annexin A11 Chondroitin sulphate proteoglycan 4

	Hemopexin Lectin, galactoside-binding, soluble 1 Lectin, galactoside-binding, soluble 3 Lectin, mannose-binding, 1 Plexin A3
ECM regulatory proteins	Alpha-2-macroglobulin Alpha-1-microglobulin/bikunin precursor Cystatin B Cathepsin B Cathepsin D Cathepsin K Cathepsin Z Coagulation factor II (thrombin) Coagulation factor IX HtrA serine peptidase 1 HtrA serine peptidase 3 Inter-alpha (globulin) inhibitor H1 Inter-alpha (globulin) inhibitor H2 Inter-alpha (globulin) inhibitor H3 Inter-alpha (globulin) inhibitor H4 Prolyl-hydroxylase, alpha polypeptide I Prolyl-hydroxylase, alpha polypeptide II Plasminogen Procollagen-lysine, 2-oxogluterate 5-dioxygenase 1 Procollagen-lysine, 2-oxogluterate 5-dioxygenase 3 Anionic trypsin Serpin peptidase inhibitor A7 Serpin peptidase inhibitor C1 Serpin peptidase inhibitor D1 Serpin peptidase inhibitor E1 Serpin peptidase inhibitor E2 Serpin peptidase inhibitor F1 Serpin peptidase inhibitor H1 TIMP metallopeptidase inhibitor 1
Secreted factors	Cytokine receptor-like factor 1 S100 calcium binding protein A4 S100 calcium binding protein A6 S100 calcium binding protein A10 S100 calcium binding protein A11 Wingless-type MMTV integration site family, member 5A Wingless-type MMTV integration site family, member 5B

Table 6.1. Identification of ECM proteins produced by canine ligament cells. All ECM proteins produced by ligament cells as determined by PEAKS software and the Matrisome Project database.

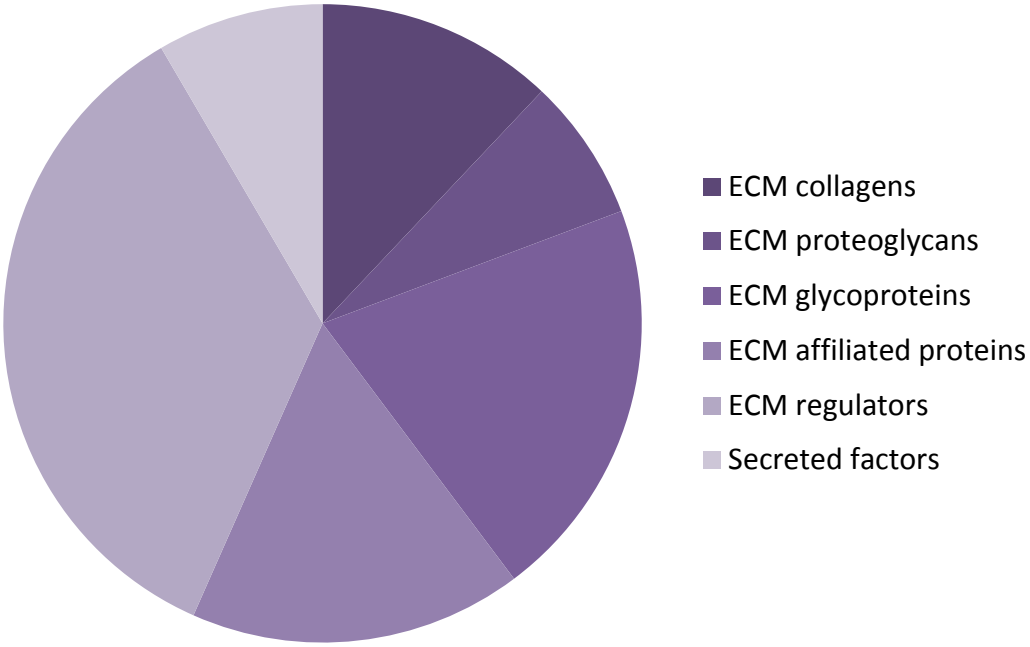


Figure 6.4. Identification of ECM proteins produced by canine ligament cells. All ECM proteins produced by ligament cells as determined by PEAKS software and the Matrisome Project database. The proportion of protein identifications belonging to each ECM category is depicted in a pie chart.

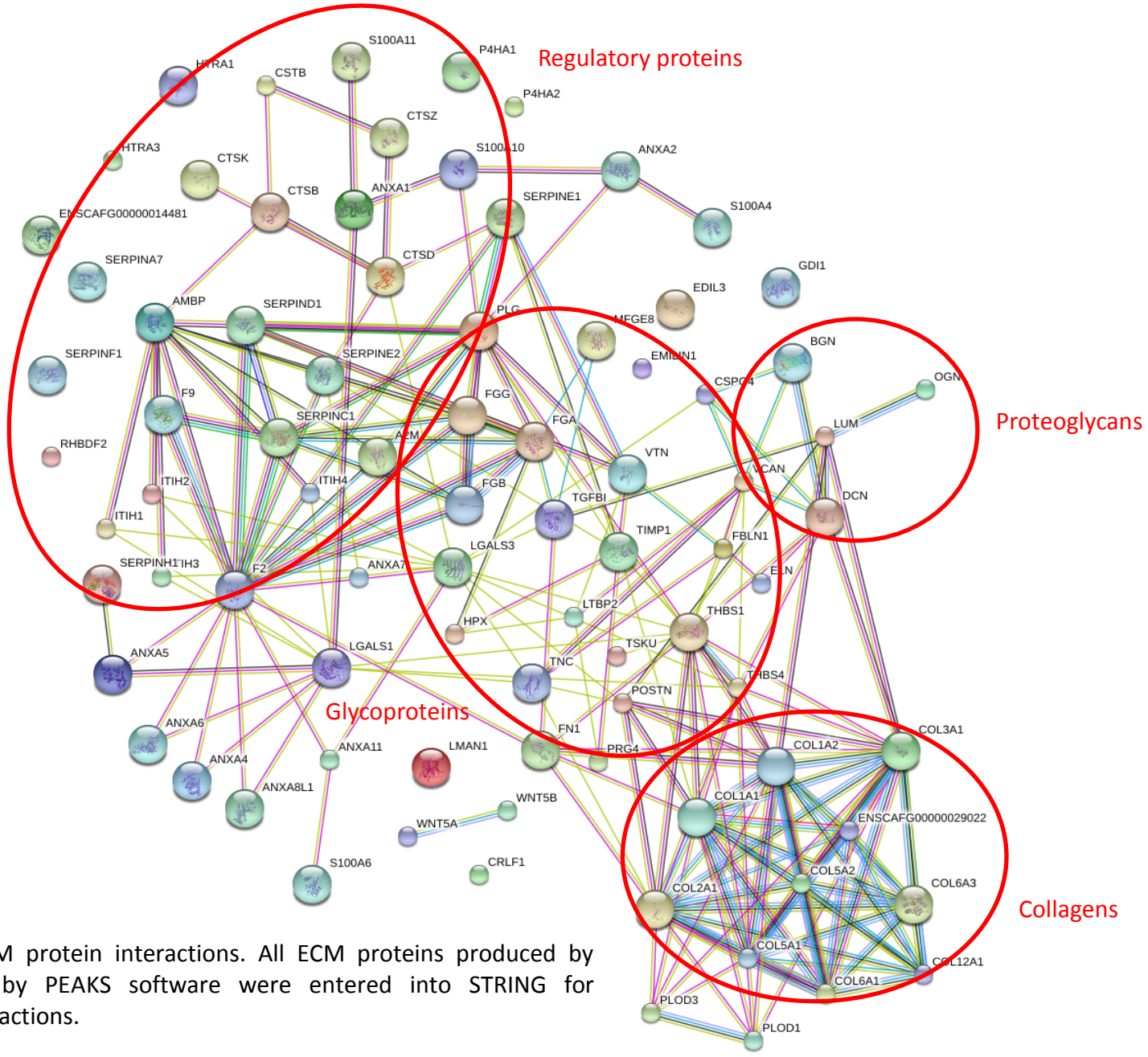


Figure 6.5. Canine ligament ECM protein interactions. All ECM proteins produced by ligament cells as determined by PEAKS software were entered into STRING for visualisation of ECM protein interactions.

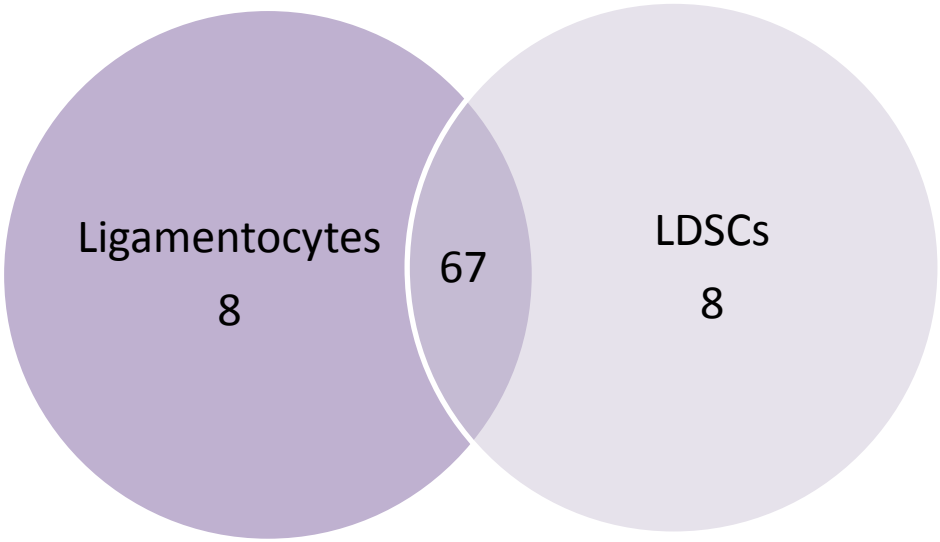


Figure 6.6. Comparison of ECM proteins produced by ligamentocytes and LDSCs as determined by PEAKS software and the Matrisome Project database.

A

ECM glycoproteins	Fibronectin Periostin Thrombospondin 4
ECM regulatory proteins	Cathepsin K Inter-alpha (globulin) inhibitor H1 Procollagen-lysine, 2-oxogluterate 5-dioxygenase 3 Serpin peptidase inhibitor D1
Secreted factors	Wingless-type MMTV integration site family, member 5B

B

ECM collagens	Collagen type II, alpha 1 Collagen type V, alpha 1 Collagen type V, alpha 2 Collagen type XII, alpha 1
ECM proteoglycans	Decorin Versican
ECM glycoproteins	Latent transforming growth factor beta binding protein 2
ECM regulatory proteins	Cathepsin Z

Table 6.2. Comparison of ECM proteins produced by ligamentocytes and LDSCs as determined by PEAKS software and the Matrisome Project database. Proteins apparently unique to ligamentocytes and LDSCs are shown in (A) and (B) respectively.

6.4.4 Metabolic labelling of ECM proteins in the LDSC and ligamentocyte niche – analysis of protein synthesis and turnover

ECM samples extracted from LDSC and ligamentocyte cells using a Rapigest extraction buffer were analysed for protein synthesis (heavy peptide content) and turnover (total peptide content) using LC-MS/MS after heavy isotope metabolic labelling (see Chapter 2, Section 2.2.3.6-2.2.3.7) (Fig.6.7).

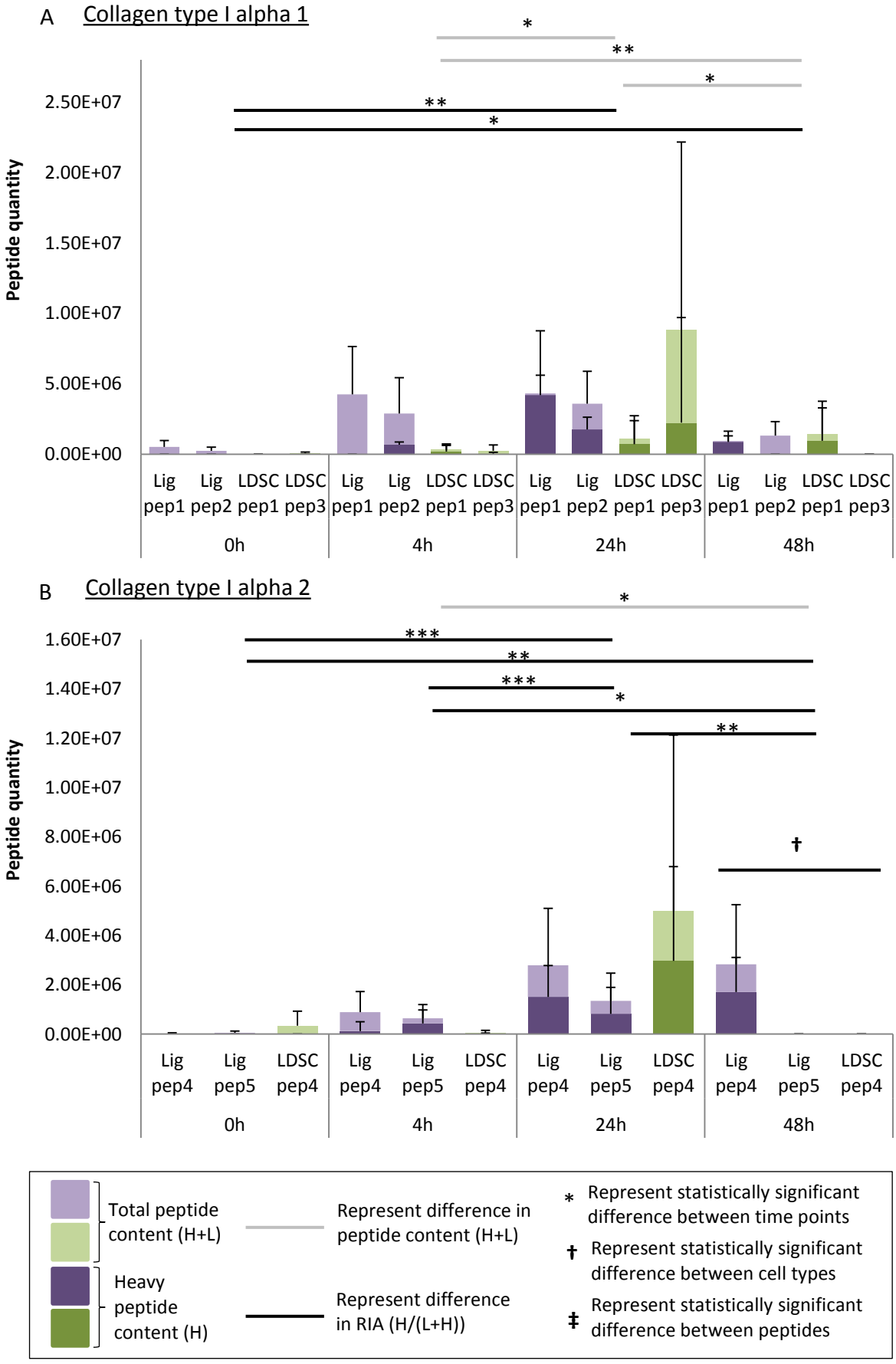
Collagens: There was new synthesis of collagen type I alpha 1 and 2, collagen type III alpha 1 and collagen type VI alpha 1, 2 and 3 (Fig.6.7.A-F). The general trend for new protein synthesis consisted of an initial increase in collagen synthesis over the first 24 hours, with a decrease in synthesis seen at 48 hours. This is consistent with the protein turnover data which suggested an increase in total protein quantity (new protein synthesis plus pre-existing protein) over the first 24 hours and a decline at 48 hours. There were some exceptions to this trend, for example collagen type VI alpha 1 and 3 with new protein synthesis continuing to increase over 48 hours.

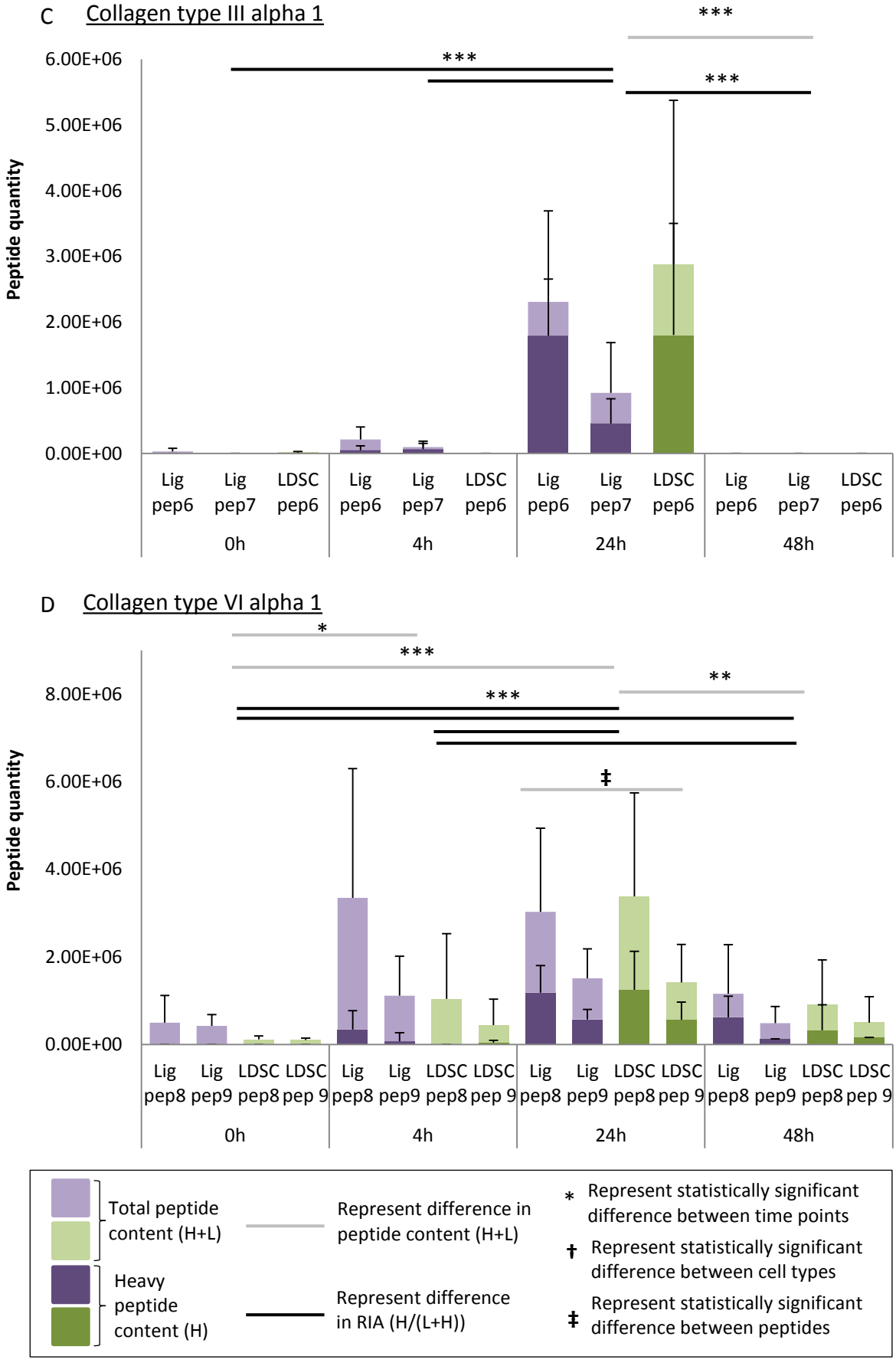
Proteoglycans: New synthesis of decorin, lumican and Tsukushi was observed (Fig.6.7.G-I). New protein synthesis for proteoglycans generally increased over 48 hours. Consistent with this, total protein quantity also continued to increase over 48 hours.

Glycoproteins: There was new synthesis of transforming growth factor β1 (TGF β1), tenascin C, fibronectin and elastin (Fig.6.7.J-M). Similar to proteoglycans, synthesis of new glycoproteins generally continued to increase over 48 hours. The total protein pool varied between glycoproteins, with some proteins demonstrating an increase in total protein quantity over time (such as tenascin C and Tsukushi), consistent with the synthesis of new protein. In contrast, some proteins showed an initial increase in total protein quantity over 24 hours followed by a decrease at 48 hours (such as fibronectin and elastin), indicative of increased protein degradation between 24 and 48 hours.

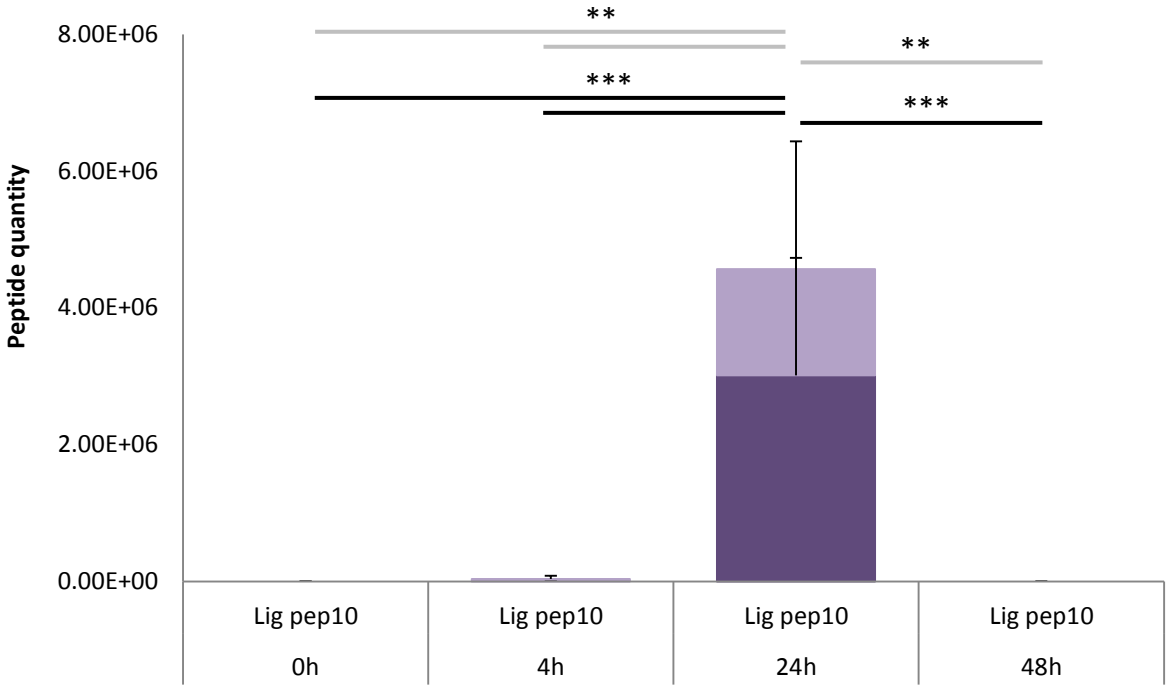
ECM regulatory proteins: New synthesis of HtrA serine peptidases 1 and 3, cathepsins B and D and serpin peptidase inhibitors E2 and H1 were observed (Fig.6.7.N-S). Synthesis of new regulatory proteins generally continued to increase

over 48 hours, with some proteins starting to plateau between 24 and 48 hours. The total protein content for ECM regulatory proteins increased over 24 hours and then started to decrease by 48 hours, indicative of increased protein degradation between 24 and 48 hours. The only exception to this was serpin peptidase inhibitor E2, which showed a continued increase in total protein content over 48 hours.

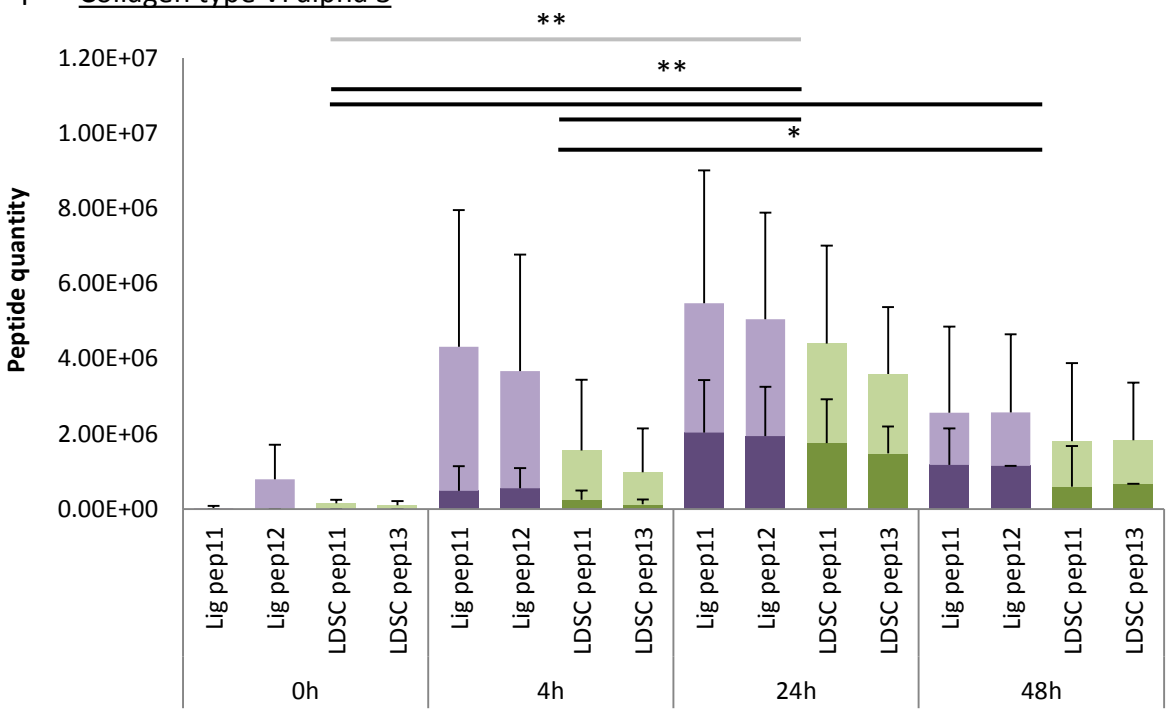

A Collagen type I alpha 1
Peptide quantity
2.50E+07
2.00E+07
1.50E+07
1.00E+07
5.00E+06
0.00E+00
Lig pep1
Lig pep2
LDSC pep1
LDSC pep3
0h
4h
24h
48h
B Collagen type I alpha 2
1.60E+07
1.40E+07
1.20E+07
1.00E+07
8.00E+06
6.00E+06
4.00E+06
2.00E+06
0.00E+00
Lig pep4
Lig pep5
LDSC pep4
Total peptide content (H+L)
Heavy peptide content (H)
Represent difference in peptide content (H+L)
Represent difference in RIA (H/(L+H))
* Represent statistically significant difference between time points
† Represent statistically significant difference between cell types
‡ Represent statistically significant difference between peptides

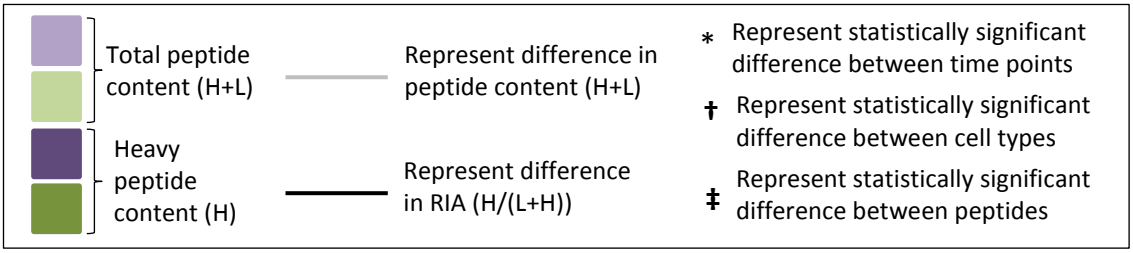

C Collagen type III alpha 1
Peptide quantity
6.00E+06
5.00E+06
4.00E+06
3.00E+06
2.00E+06
1.00E+06
0.00E+00
***
***
***
Lig pep6
Lig pep7
LDSC pep6
0h
4h
24h
48h
D Collagen type VI alpha 1
Peptide quantity
8.00E+06
6.00E+06
4.00E+06
2.00E+06
0.00E+00
*
***
***
**
‡
Lig pep8
Lig pep9
LDSC pep8
LDSC pep 9
Total peptide content (H+L)
Heavy peptide content (H)
Represent difference in peptide content (H+L)
Represent difference in RIA (H/(L+H))
* Represent statistically significant difference between time points
† Represent statistically significant difference between cell types
‡ Represent statistically significant difference between peptides

E Collagen type VI alpha 2

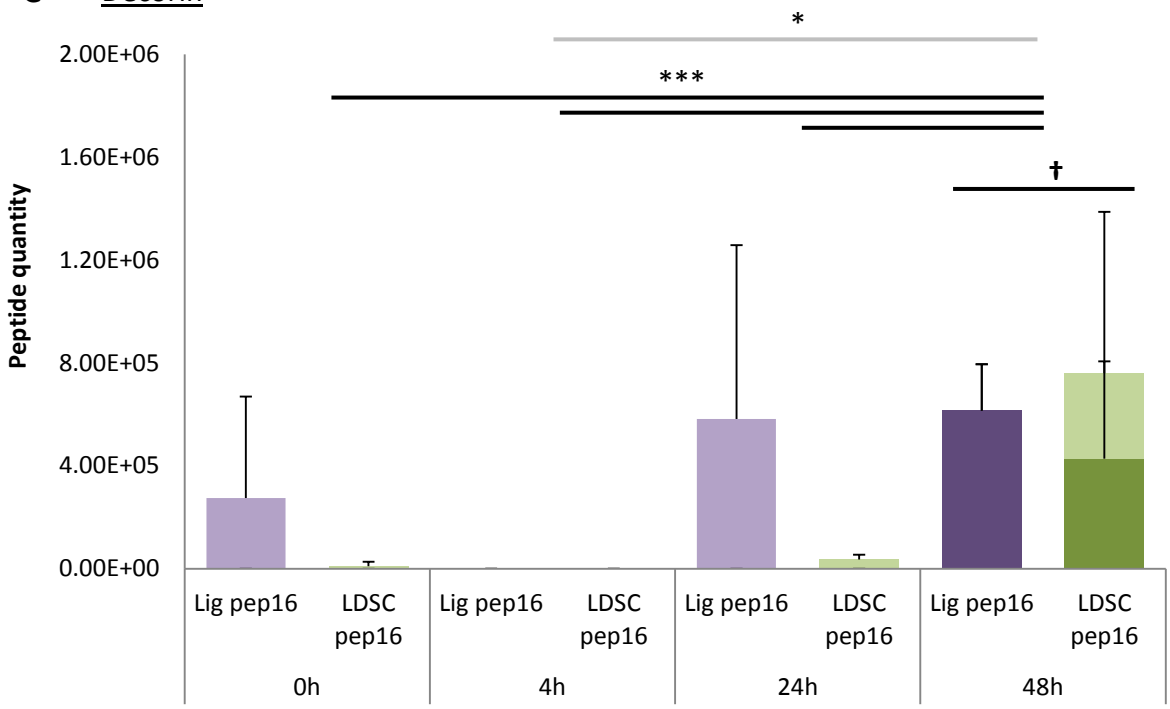


F Collagen type VI alpha 3

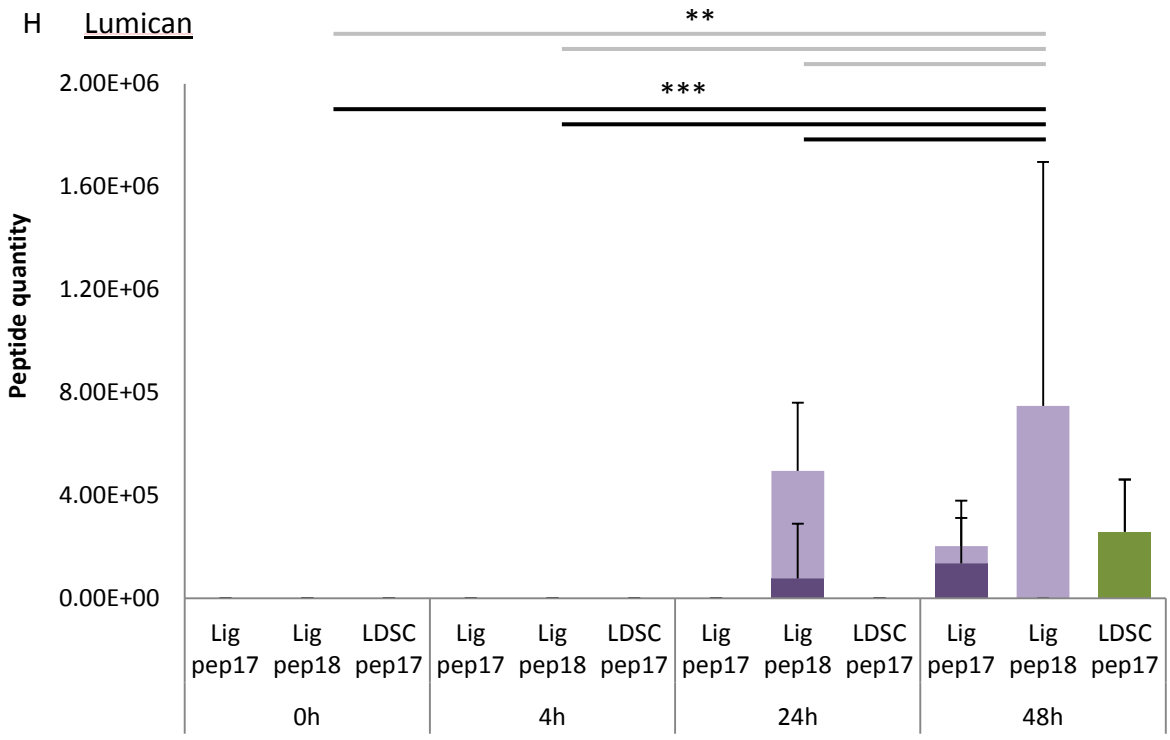


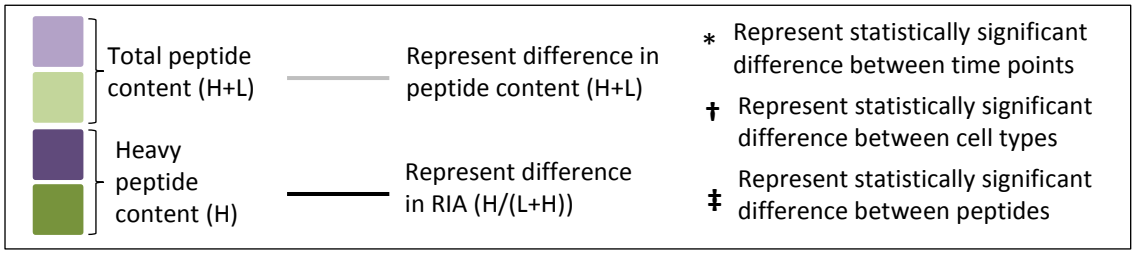


G Decorin

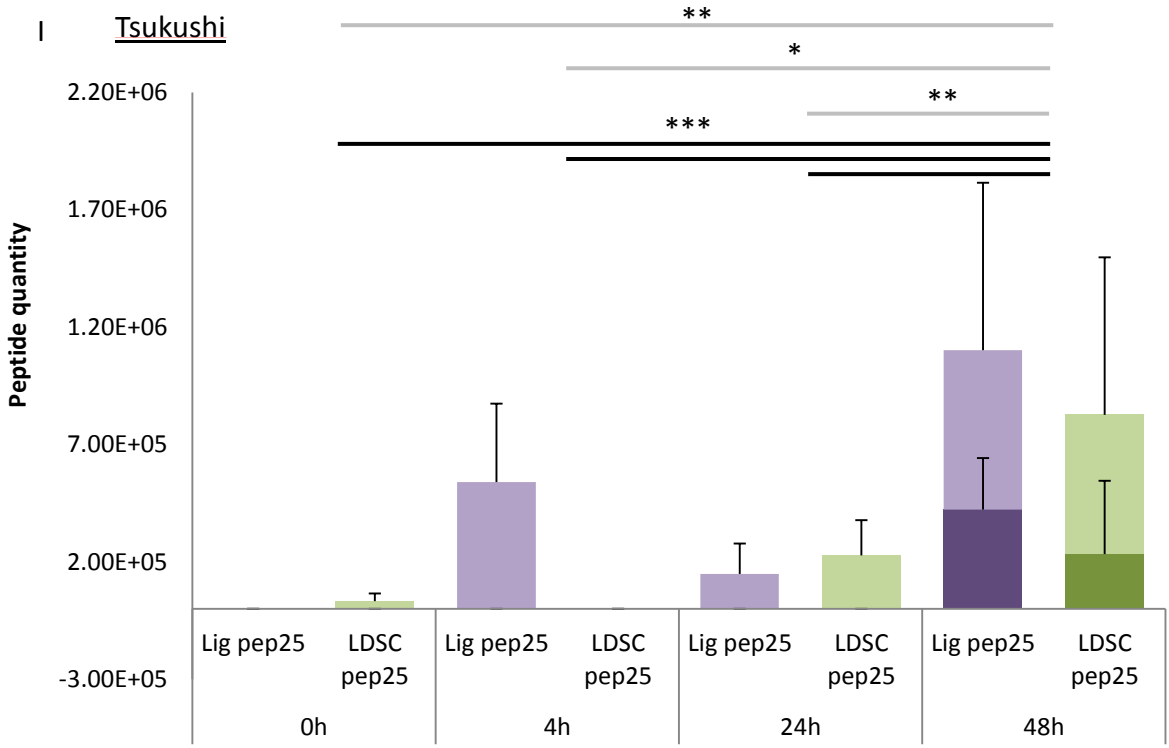


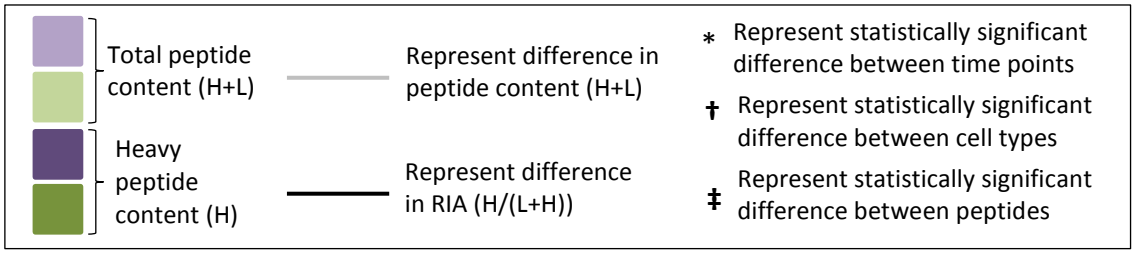
H Lumican



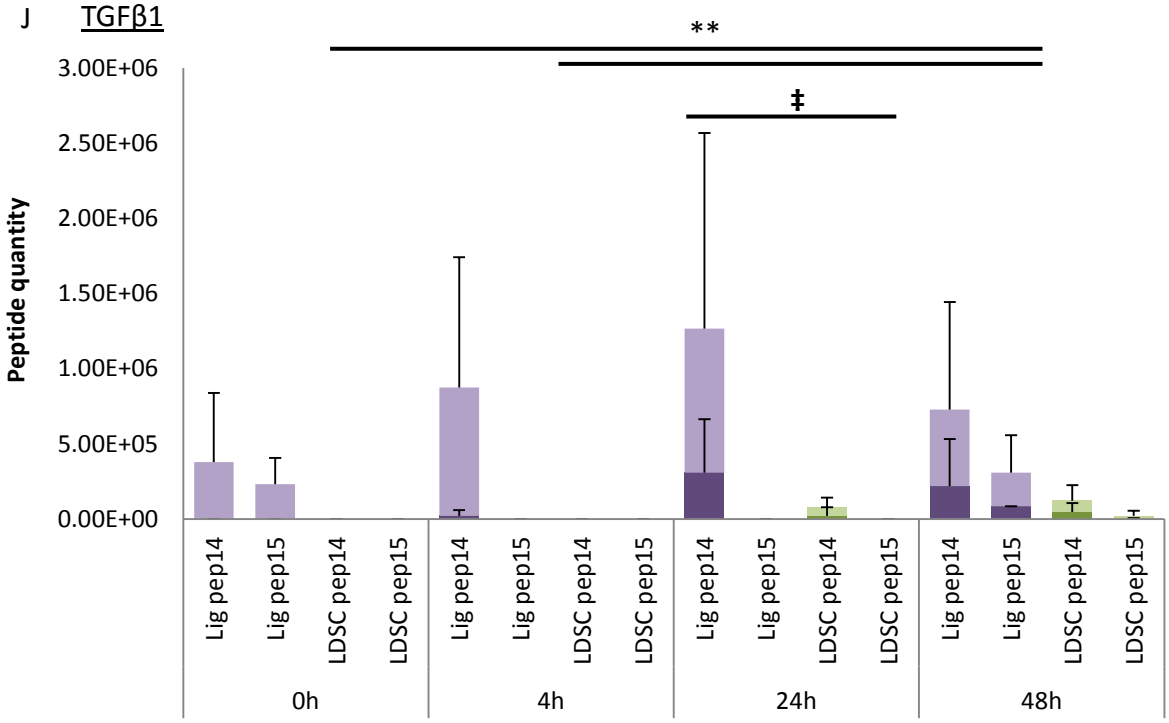


I Tsukushi

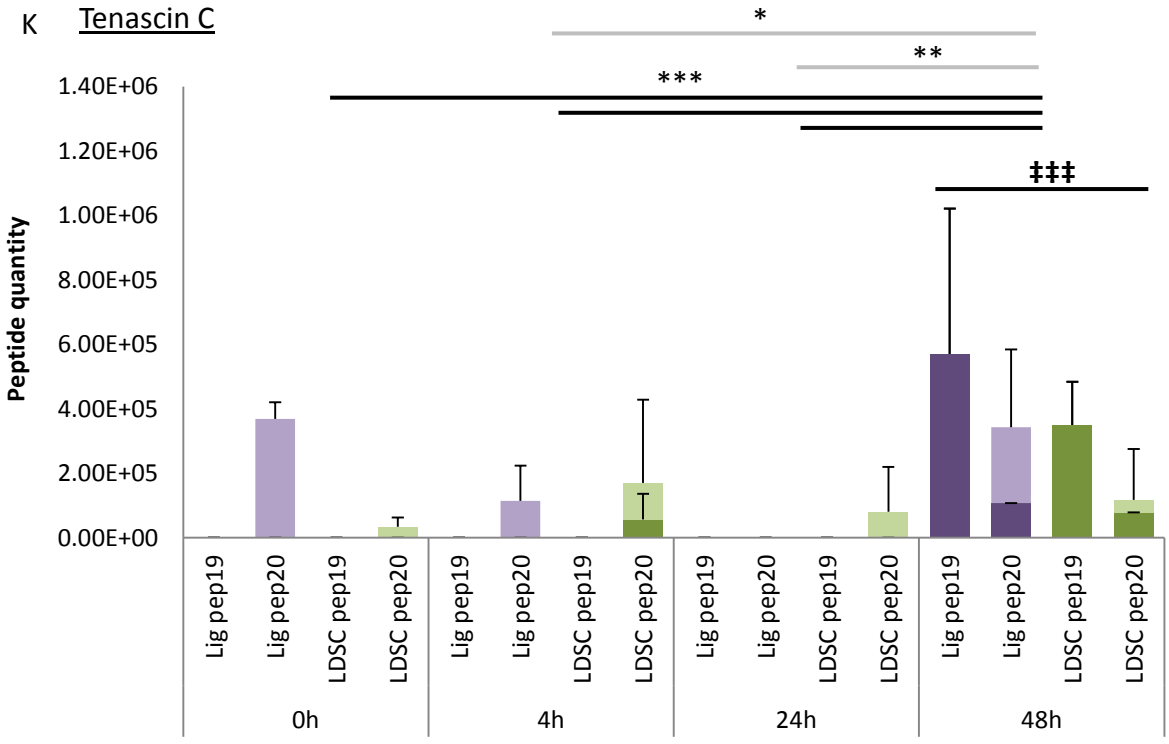

**
*
**
***
Peptide quantity
2.20E+06
1.70E+06
1.20E+06
7.00E+05
2.00E+05
-3.00E+05
Lig pep25
LDSC pep25
Lig pep25
LDSC pep25
Lig pep25
LDSC pep25
Lig pep25
LDSC pep25
0h
4h
24h
48h

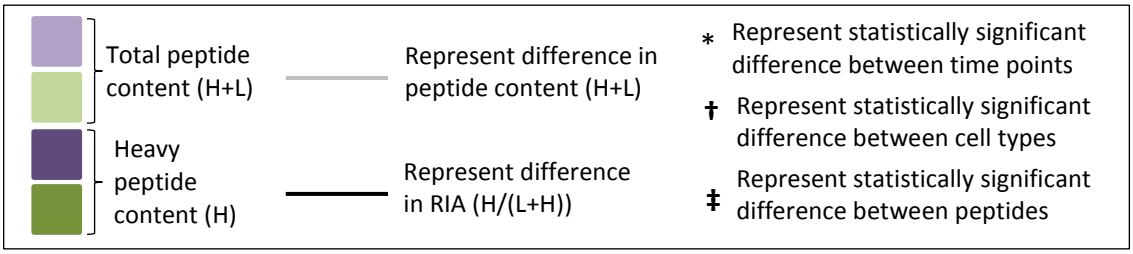

Total peptide content (H+L)
Heavy peptide content (H)
Represent difference in peptide content (H+L)
Represent difference in RIA (H/(L+H))
* Represent statistically significant difference between time points
† Represent statistically significant difference between cell types
‡ Represent statistically significant difference between peptides

J TGFβ1

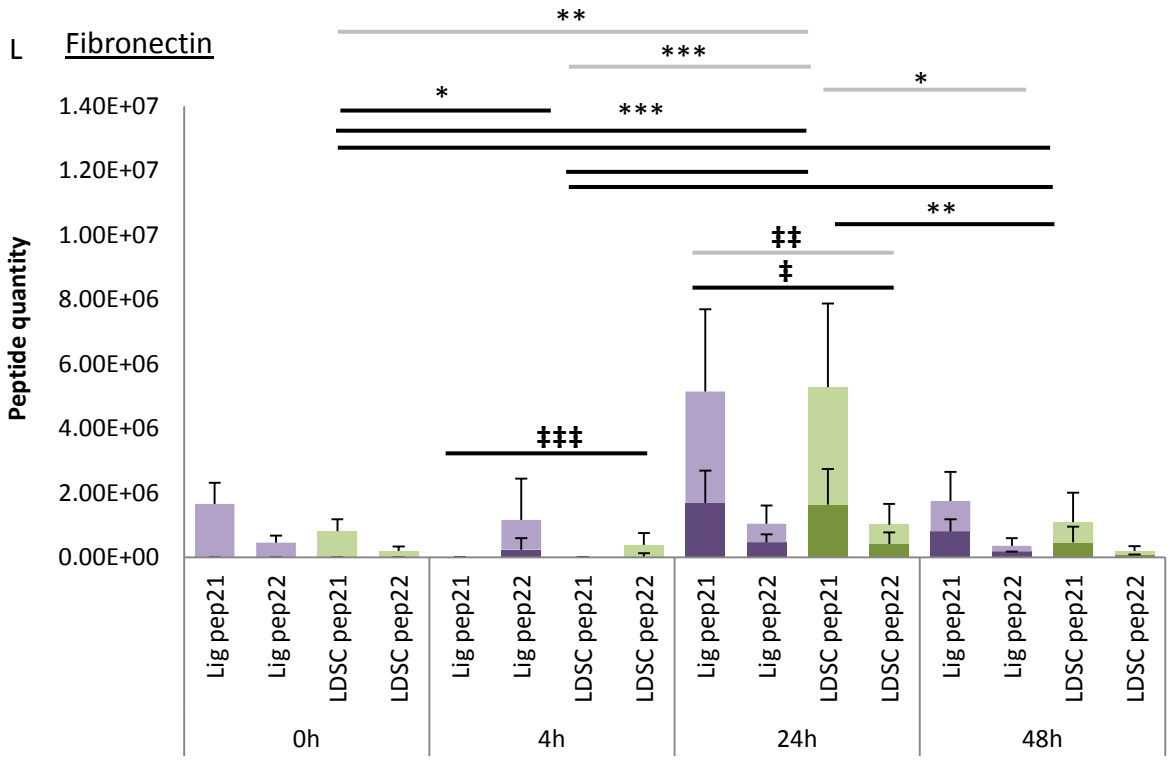


K Tenascin C

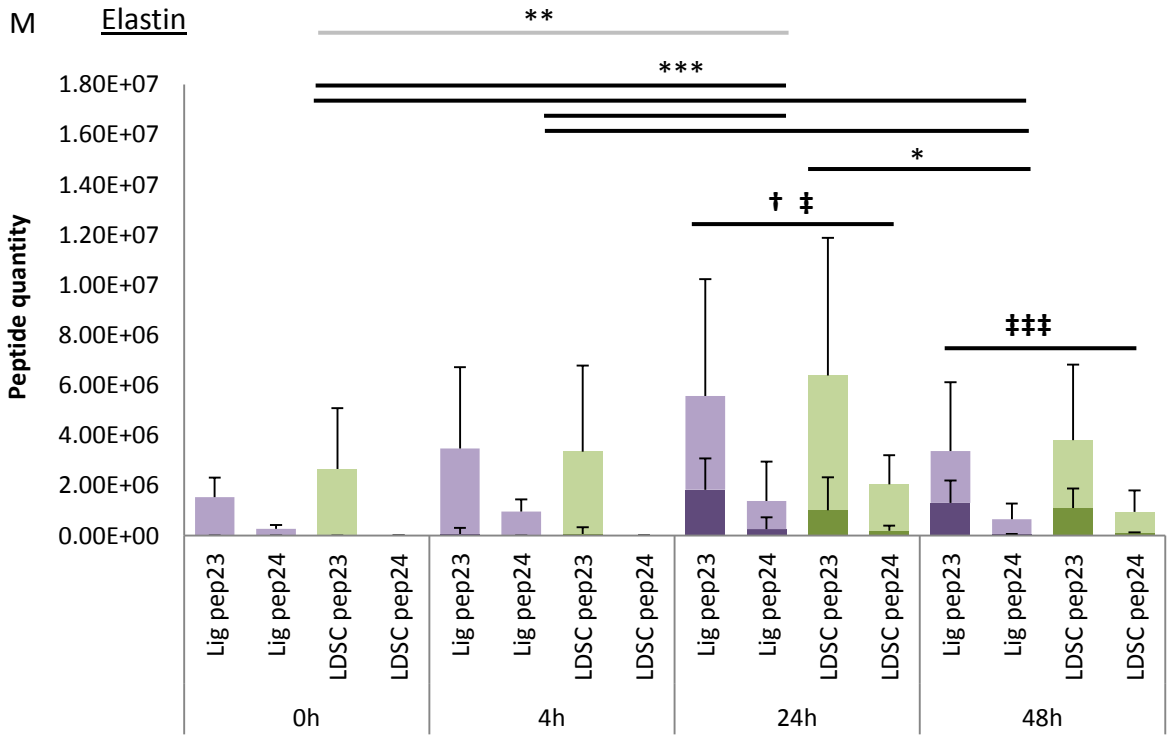


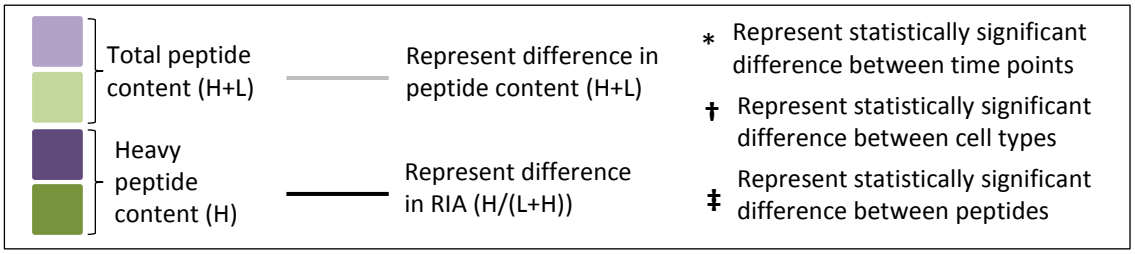


L Fibronectin

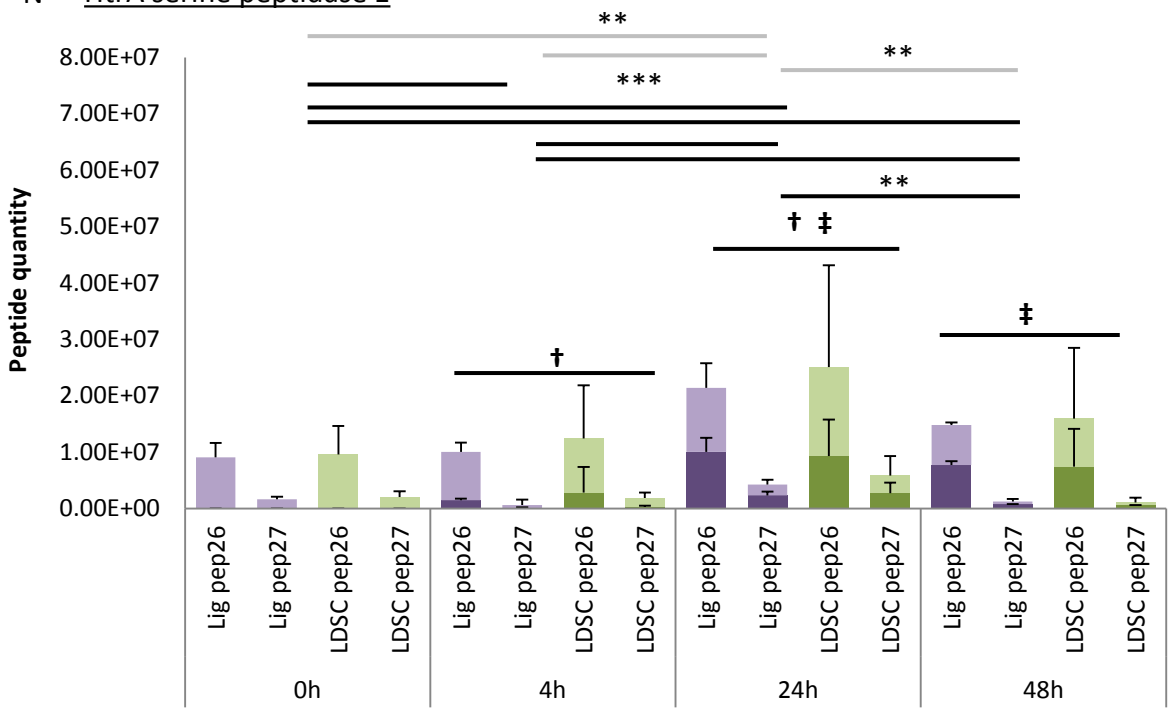


M Elastin

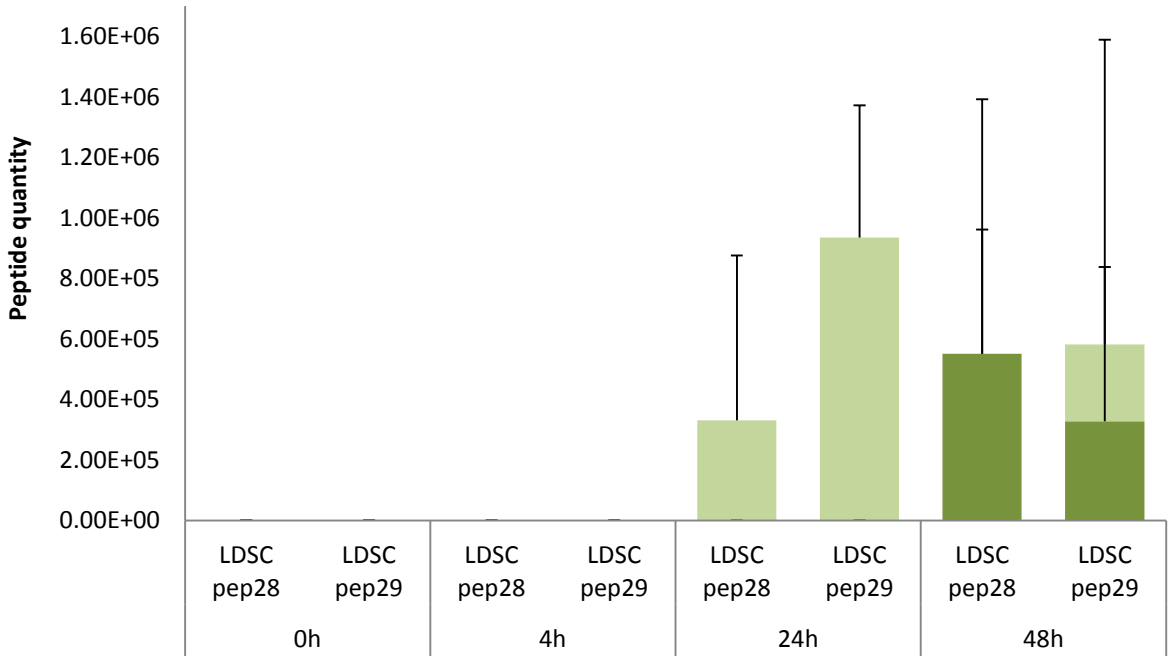


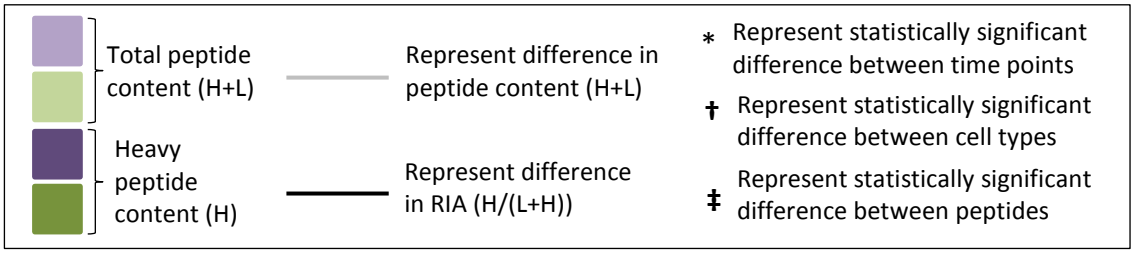


N HtrA serine peptidase 1

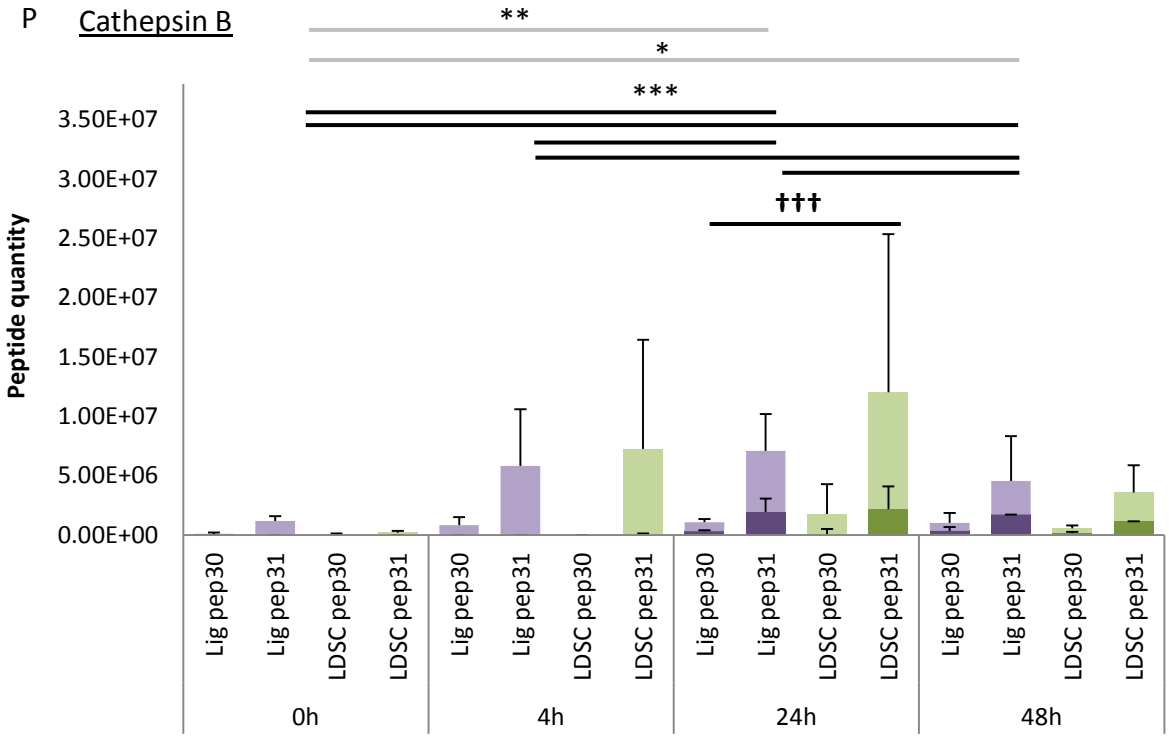


O HtrA serine peptidase 3

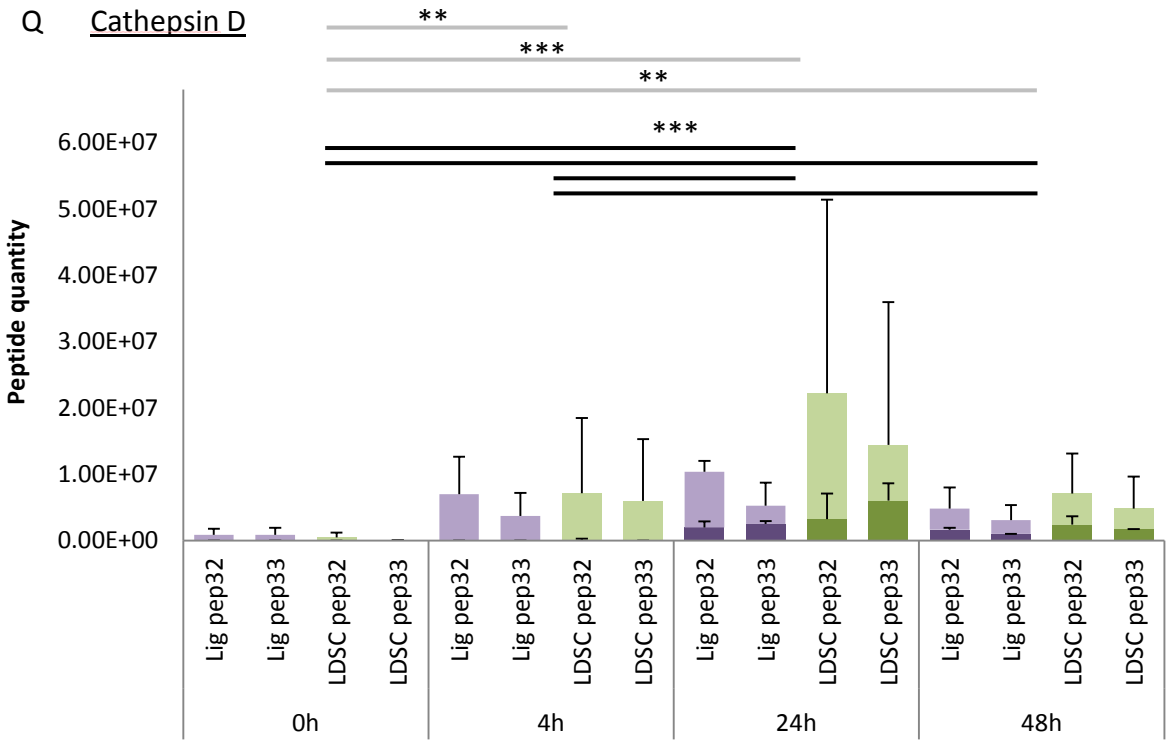




P Cathepsin B



Q Cathepsin D



Total peptide content (H+L)

Heavy peptide content (H)

Represent difference in peptide content (H+L)

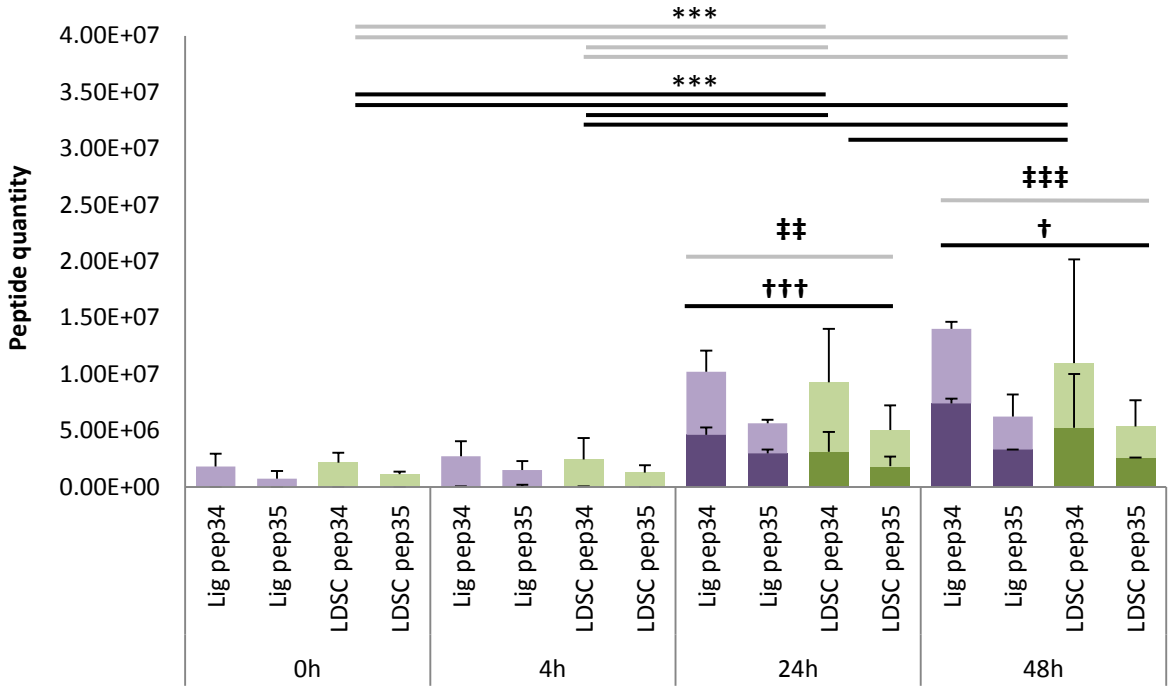
Represent difference in RIA (H/(L+H))

* Represent statistically significant difference between time points

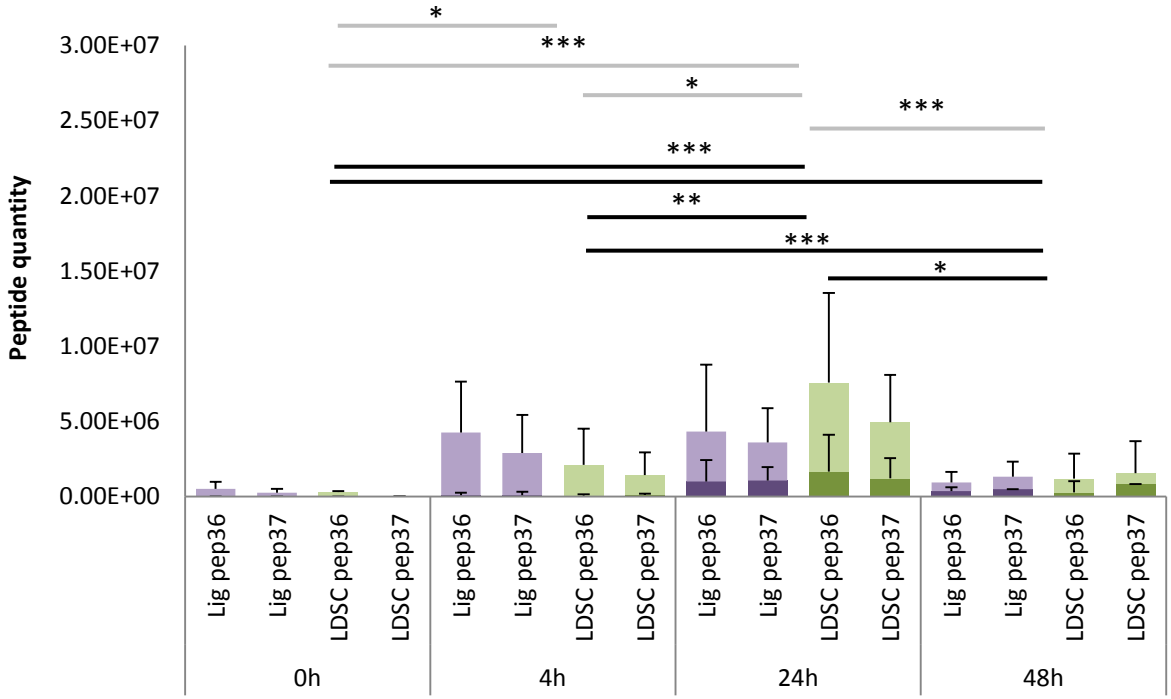
† Represent statistically significant difference between cell types

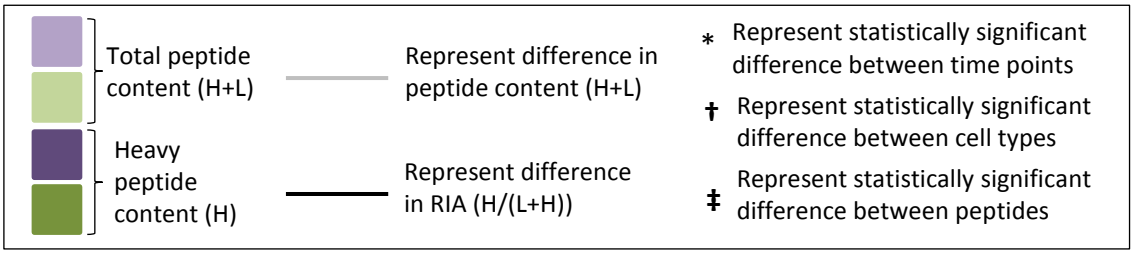
‡ Represent statistically significant difference between peptides

R Serpin peptidase inhibitor E2



S Serpin peptidase inhibitor H1





T

Peptide number	Protein	Peptide
1	Collagen type I alpha 1	GFSGLDGAK
2		GLTGSPGSPGPDGK
3		GFPGADGVAGPK
4	Collagen type I alpha 2	GFPGAPGNVGPAGK
5		GYPGNIGPVGAVGAPGPHGPVGPTGK
6	Collagen type III alpha 1	GEAGSPGIPGPK
7		GPVGPSGPPGK
8	Collagen type VI alpha 1	LLLFSDGNSQGATAAAIEK
9		DTTPLSVLCGPDIQVVSVGIK
10	Collagen type VI alpha 2	GPQGALGEPGK
11	Collagen type VI alpha 3	VPQIAFVITGGK
12		IEDGVPQHLVLFLGGK
13		SDDEVDDSAAELK
14	TGFβ1	LTLLAPLNSVFK
15		DVLATNGVIHFIDELLIPDSAK
16	Decorin	ISPGAFTPLLK
17	Lumican	NNQIDHIDEK
18		ILGPLSYSK
19	Tenascin C	VATYLPAPEGLK
20		LSWTADEGVFDSFVLK
21	Fibronectin	DLQFVEVTDVK
22		YEKPGSPPR
23	Elastin	FPGVGVLPGVPTGTGVK
24		AGYPTGTGVGTQAAAAAAK
25	TSKU	ELQSLQVLDLSGNPK

Peptide number	Protein	Peptide
26	HtrA serine peptidase 1	VTAGISFAIPSDK
27		VKVELK
28	HtrA serine peptidase 3	GGIQDGDIIVK
29		TITPSLVEELK
30	Cathepsin B	HYGCSSYSVSDNEK
31		EQWPNCPTIK
32	Cathepsin D	VSTLPDVTLK
33		LSSEDYTLK
34	Serpin peptidase inhibitor E2	ALGITEMFDPSK
35		DIVTVANAVFVK
36	Serpin peptidase inhibitor H1	HLAGLGLTEAIDK
37		SAGLAFSLYQAMAK

Figure 6.7. Metabolic labelling of canine ligamentocytes and LDSCs. Cells were labelled with ^{13}C lysine for 4, 24 and 48 hours before the ECM was harvested and LC-MS/MS performed. ECM proteins labelled with ^{13}C lysine were identified using MASCOT and extracted ion chromatograms were analysed using Xcalibur. Heavy (H) and light (L) peaks were identified for each labelled peptide and the area under the peak recorded. Total peptide content (H+L) was plotted on bar charts along with heavy peptide content (H) against time for each peptide (A-S). Purple bars represent ligamentocytes and green bars represent LDSCs, light bars represent total peptide content and dark bars represent heavy peptide content. A two-way ANOVA with repeated measures was used to analyse total peptide content (H+L) and relative isotope abundance (H/(L+H)) for differences between time points, cell types and peptides. Grey bars represent statistically significant differences in total peptide content (H+L) and black bars represent statistically significant differences in relative isotope abundance (H/(L+H)) . * represent statistically significant differences between time points, † represent statistically significant differences between cell types and ‡ represent statistically significant differences between peptides. * = $p<0.05$, ** = $p<0.01$ and *** = $p<0.001$. Lig = ligamentocytes, LDSC = ligament-derived stem cell, pep = peptide. Peptide sequences are provided in the table in part T.

6.5 Discussion

6.5.1 Optimisation of cellular and extracellular matrix extraction methods

In order to ensure sufficient protein quantity for mass spectrometry ECM protein extraction techniques were optimised (Fig.6.2). Several methods for removal of cells prior to ECM extraction were also tested (Fig.6.1). Cellular extraction was necessary as the presence of cellular proteins during proteomic analysis would have overwhelmed the ECM proteins, which may only be present in small quantities. I tested cellular and ECM extraction techniques previously published or previously used within my group.

EDTA and trypsin have been successfully used for the removal of cells prior to ECM extraction (Choi *et al*., 2011), however EDTA was unable to remove cells in this study. This may be due to different concentrations used as Choi and others (2011) did not state the concentration of EDTA used. Trypsin was used successfully to remove cells in this study, consistent with Choi and others (2011), although it proved difficult to completely remove all cells using this method. I also tested a method published by Soteriou and others (2013) which used a detergent to remove cells (Soteriou *et al*., 2013). This method appeared effective, with the absence of cells, however this method also removed a lot of the ECM deposited by the cells and so I opted to continue with trypsin for cell removal.

For the extraction of ECM a urea extraction buffer was tested, which had been previously used within my group as well as a sample buffer published by Soteriou and others (2013). The sample buffer successfully extracted increased numbers of identifiable proteins and a wider complement of proteins compared with the urea buffer, however there was similar incorporation of ^{14}C-labelled amino acids into proteins using both extraction methods. Due to the potential incompatibility of the sample buffer with protein assays and further downstream processing I opted to continue with the urea buffer for further validation. Unfortunately the combination of trypsin and the urea buffer resulted in low protein hits when LC-MS/MS was performed, therefore a Rapigest method,

previously used within my group (Peffers *et al*., 2015b), replaced the urea buffer for all further experiments.

6.5.2 Identification and label-free quantification of ECM proteins in the LDSC and ligamentocyte niche

I have demonstrated the production of a number of collagens, proteoglycans and glycoproteins, as well as numerous ECM related proteins by canine ligament cells as determined by mass spectrometry (Table 6.1, Fig.6.4-5). The ECM related proteins produced by ligament cells in this study are consistent with previous literature looking at the proteome of whole canine CCL tissue (Kharaz *et al*., 2016). Particularly, Kharaz and others (2016) observed the presence of collagen type I, V, VI, decorin, biglycan, lumican, tenascin C, fibulin 1 and thrombospondin 1, consistent with this study. Similarly, a study investigating the proteome of human spinal ligaments (ligamentum flavum) observed the presence of the proteins mentioned above as well as collagen type II, III, mimecan, versican, proteoglycan 4, fibrinogen, fibronectin, periostin, thrombospondin 4, vitronectin, latent transforming growth factor beta binding protein 2, as well as ECM related proteins including annexins and plasminogen, consistent with this study (Sato *et al*., 2016). Similar proteins were observed in human anterior cruciate ligaments (ACL) with all of the proteins listed above as well as collagen type XII, elastin, TGFβ1 and the ECM related proteins hemopexin and inter-alpha (globulin) inhibitor H1 (Little *et al*., 2014). Generally, fewer proteins were observed in this study than the studies discussed above, however this is most likely due to the fact that I extracted ECM protein from cells grown in culture as opposed to extracting protein from whole ligament tissue. Protein assays and gel electrophoresis I have previously conducted (data not shown) demonstrate that increased matrisomal protein can be extracted from tissue compared with cells in culture. No studies to date have investigated the ECM proteome of intra-articular ligament cells or LDSCs. However several studies have demonstrated gene expression of certain ECM components by human ACL and periodontal LDSCs including collagen type I, III, tenascin C, fibronectin and

thrombospondin 4 (Cheng *et al*., 2010; Huang *et al*., 2008; Lee *et al*., 2015b), consistent with this study.

Few differences were observed in this study between ECM proteins produced by ligamentocytes and LDSCs (Fig.6.6, Table 6.2) with several glycoproteins and regulatory proteins unique to ligamentocytes and a number of collagens and proteoglycans unique to LDSCs. I expected to see greater differences in ECM production and the niche between the two cell types. The phenotypic differences between the two cell types seen in this study (see Chapter 5) are suggestive of two distinct cell populations with differing genotypes and phenotypes and therefore a difference in the cells' requirements and environment would be expected. In addition, the requirement for a specific niche has been demonstrated for multiple stem cell populations (Bi *et al*., 2007; Calvi *et al*., 2003; Garcion *et al*., 2004), therefore I hypothesised that LDSCs would also require a specific environment in which to survive and function, separate to that of native fibroblasts. To my knowledge no study has compared the ECM proteome/niche between stem cells and fibroblasts from the same tissue, therefore I am unable to compare with my own data. It is possible that the small differences in ECM production between LDSCs and ligamentocytes observed in this study are instrumental in maintaining the cells' differing phenotypes. For example, collagen type V and decorin (ECM proteins I found to be unique to LDSCs) have been shown to be present in the bone marrow niche. When bone marrow-derived mesenchymal stromal cells are grown on a matrix containing these ECM components their replication and clonogenicity is promoted and differentiation potential is regulated (Chen *et al*., 2007). Interestingly, some of the proteins found to be unique to ligamentocytes in this study have previously been shown to play key roles in niches for other stem cell populations. Fibronectin has been shown to promote expansion of both muscle satellite cells (Bentzinger *et al*., 2013) and HSCs (Malara *et al*., 2014), and periostin indirectly supports HSC survival and growth (Tanaka *et al*., 2016). It is an unexpected finding in this study for ECM components known to influence stem cells to be uniquely produced by ligamentocytes. It is possible that there is a certain amount of redundancy in stem cell niches, and the absence of specific ECM components may have little effect on stem cell survival and function. However the

knockdown of fibronectin caused impaired satellite cell replication which was restored with over-expression of fibronectin (Bentzinger *et al*., 2013) suggesting this ECM component at least is not redundant. Alternatively, there may be low level production of fibronectin, periostin, thrombospondin 4, cathepsin K, inter-alpha (globulin) inhibitor H1, procollagen-lysine, 2-oxogluterate 5-dioxygenase 3, serpin peptidase inhibitor D1 and wingless-type MMTV integration site family, member 5B by LDSCs, however due to the selected parameters for PEAKS software these proteins were excluded from the output. It is clear that further investigation into the role of these ECM proteins in LDSC survival and function is needed.

Label-free quantitative analysis of LDSC and ligamentocyte ECM proteins resulted in no differentially abundant ECM proteins between the two cell types therefore no data is shown. This was unexpected due to the reasons discussed previously, however the lack of differentially abundant proteins between cell types may be due to high variability between biological replicates within each cell type which would reduce peptide significance scores, or possibly that the cell types share a similar proteome and niche.

6.5.3 Metabolic labelling of ECM proteins in the LDSC and ligamentocyte niche

The SILAC proteomic method involves culturing cells in the presence of amino acids labelled with a light or heavy isotope. Newly synthesised proteins will incorporate the light (L) or heavy (H) isotope and this incorporation can be measured and quantified, providing a comparison between different samples or treatments, as well as analysis of protein dynamics (Chen *et al*., 2015d; Hoedt *et al*., 2014). I have utilised a variation of the SILAC method in this study to investigate which proteins are being actively synthesised by LDSCs and ligamentocytes and at what rate they are being synthesised. I analysed new protein synthesis by measuring the quantity of heavy isotope that had been incorporated into newly synthesised ECM proteins. I investigated both total heavy isotope incorporation values as well as calculating the proportion of the total protein pool that had been labelled with heavy isotope. This technique also provides information on protein turnover which is dependent on both protein synthesis and degradation. By

analysing the total protein pool over time I was able to investigate protein pool expansion and reduction for individual ECM proteins.

The data has been presented as bar charts demonstrating both total peptide content as well as heavy peptide content (new peptide synthesis), however I was unable to show relative isotope abundance (RIA) in this format (Fig.6.7). RIA represents the proportion of the total protein pool that is labelled with heavy isotope (calculated as H/(L+H)). Although RIA is not shown directly it is important to keep in mind the proportion of the total protein pool that has been labelled with heavy isotope. For many of the proteins the total amount of protein decreased at 48 hours compared with 24 hours with the quantity of protein labelled with heavy isotope also decreasing. However the RIA actually increased for many of these proteins: 9 proteins out of a total of 19, with the proportion of the total pool labelled with heavy isotope increasing (such as fibronectin) which is indicative of continued new protein synthesis, albeit with concurrent degradation of existing protein. Therefore, it is important to consider RIA when assessing this data and for this reason I have performed statistical analysis on RIA data rather than absolute heavy labelled values. The reason for the reduced protein quantities at 48 hours is unknown. It could possibly be due to cell death as the cells are cultured in serum-free media for the labelling period, however there appeared to be little or no loss of cells after 48 hours. Alternatively, the media could have been completely depleted of lysine prior to 48 hours, which would result in reduced protein synthesis. A suggestion for future work would be to measure the quantity of both ^{12}C- and ^{13}C-labelled lysine in the media extracts from the samples using nuclear magnetic resonance (NMR) (Hattori *et al*., 2013) to determine if lysine had been depleted from the media. However, the reasons outlined above are unlikely to be the cause of the reduced protein levels at 48 hours as some ECM proteins (as well as some intracellular proteins – data not shown) demonstrated increased levels of both total protein and protein labelled with heavy isotope at this time point (such as decorin and lumican), indicating that the cells had not died or exhausted the supply of lysine.

New synthesis and turnover of a number of ECM proteins was observed in this study, including collagens, proteoglycans and glycoproteins (Fig.6.7). The

expression of these proteins by ligament tissue has previously been demonstrated (see Section 6.5.2). However, little previous research has investigated turnover of ECM proteins in any tissue with the exception of collagen, as discussed below. I also observed synthesis and turnover of some ECM regulatory proteins. The presence of some of these proteins has been demonstrated in periodontal ligament, such as HtrA serine peptidase 1, cathepsin B and serpin peptidase inhibitor H1 (Goseki *et al*., 1996; Li and Zhang, 2015; Yoshimatsu *et al*., 2008), however to date there are no reports of HtrA serine peptidase 3, cathepsin D and serpin peptidase inhibitor E2 expression in any ligament tissue. There is also little research on protein dynamics of ECM regulatory proteins.

I observed a general trend for collagens of an initial increase in new protein synthesis up to 24 hours followed by a decrease between 24 and 48 hours. Similarly, the total protein content increased up to 24 hours followed by a decrease. At 24 hours a variable RIA was observed for different collagens with as much as 93% of collagen type I alpha 1 being turned over in a 24 hour period and 46% of collagen type VI alpha 3. Several previous studies have utilised methods based on the incorporation of stable isotope into newly synthesised proteins for analysis of protein turnover. Human tendon and ligament demonstrated collagen turnover rates of 0.045%/h and 0.04%/h respectively after intravenous infusion of heavy labelled proline into subjects prior to surgery (Babraj *et al*., 2005). The turnover levels reported by Babraj and others (2005) are considerably lower than those reported here, however they still demonstrate a high turnover of collagen in these tissues. The discrepancies between Babraj and others (2005) and this study may be due to the use of healthy tissue in this study and the use of ruptured ligament tissue by Babraj and others (2005), however a comparison of healthy and ruptured tendon showed increased collagen turnover in ruptured tendon (Riley *et al*., 2002). Alternatively, species differences may play a role as smaller mammals have been shown to exhibit increased protein turnover (Nagy *et al*., 1999), or the differences in turnover may be due to the use of tissue by Babraj and others (2005) and the use of *in vitro* cultured cells in this study. Another study analysing protein turnover using intravenous infusion of heavy labelled proline into subjects found an increase in incorporation after strenuous exercise with a peak incorporation of 0.08%/h

(Miller *et al*., 2005). Intraperitoneal infusion of heavy labelled proline enabled analysis of collagen turnover in murine periodontal ligament with a half-life of 2.4-6.5 days depending on the section of ligament (Perera and Tonge, 1981). Other methods have been utilised to analyse collagen turnover; one study analysed collagen propeptides and collagen degradation products using microdialysis which suggested a high turnover of collagen in human tendon (Langberg *et al*., 1999). However, some studies analysing collagen turnover have reported much greater half-lives, for example, analysis of aspartic racemization and collagen degradation markers in equine tendon revealed a collagen half-life of 34-198 years depending on the tendon type (Thorpe *et al*., 2010). In addition, analysis of human tendon core tissue turnover using the ^{14}C bomb-pulse method revealed that the tendon core is formed during development and the tissue is not renewed after this time (Heinemeier *et al*., 2013). It is possible that the increased quantity of collagen synthesised in this study and others is degraded quickly before incorporation into tissue which would account for the discrepancy in collagen turnover observed between these studies and Heinemeier and others (2013). This degradation could occur extracellularly in the matrix or intracellularly. Although cellular matter was removed before extraction of ECM proteins in this study there were still some cells remaining and the identification of intracellular proteins by LC-MS/MS confirmed this (data not shown). It is possible that the new collagen synthesis observed during this study may, in part, be intracellular procollagen. Secretory proteins including ECM proteins have been shown to have short cellular half-lives (Schwanhausser *et al*., 2011) and therefore procollagen may be degraded rapidly intracellularly and not contribute to new mature collagen synthesis, or procollagen may be secreted from the cells but not incorporated into longer-lived collagen fibrils. Alternatively, it is possible that turnover occurs in the peripheral region of the tendon and not in the core. The decline in total and labelled peptide seen at 48 hours in this study is due to both reduced synthesis and increased degradation. The reduction in heavy isotope incorporation is suggestive of reduced protein synthesis, and the reduction in light isotope labelled protein is indicative of protein degradation. The cause of these changes in collagen turnover are unknown, however the reduction in collagen synthesis may be due to changes in collagen regulatory enzymes and the increase in

collagen degradation is most likely due to upregulation of collagen degrading enzymes (discussed further below).

For proteoglycans there were a limited number of peptides which had been labelled with heavy isotope therefore the conclusions I can draw for these proteins are with less confidence than the other protein categories such as collagens. The trend for proteoglycan turnover generally consisted of a continual increase in both total peptide content as well as heavy labelled peptide content. The data for proteoglycans was considerably more variable and there were instances for all proteoglycans where there were increased light labelled peptides at later time points compared with earlier time points. As all available lysine was of the heavy isotope during the labelling period cells should not be able to synthesise proteins containing the light isotope as lysine is an essential amino acid. Due to the limited number of labelled peptides mentioned above and the low quantity of protein in general (compared with other protein categories) my confidence in these proteins is reduced. Previous reports have demonstrated high turnover of proteoglycans in tendon which is comparable with this study, although I did not observe synthesis or turnover of biglycan, aggrecan or versican which is in contrast to previous studies in bovine tendon and ligament (Ilic *et al*., 2005; Rees *et al*., 2000; Samiric *et al*., 2004a, b; Winter *et al*., 2000). This may be due to tissue or species differences, or differences in methodology and analysis of protein turnover. Alternatively this may be due to the low quantities of proteoglycans in canine CCL tissue observed here. The parameters used for protein identification and quantification may have gated out certain proteoglycans which are present in only small quantities, therefore I was unable to analyse their turnover. Samiric and others (2004) found a half-life of greater than 20 days for small proteoglycans and 2 days for large proteoglycans (Samiric *et al*., 2004b) in bovine tendon. These values are generally lower than those observed for collagen turnover in tendon tissue (Thorpe *et al*., 2010), however I observed similar levels of new protein synthesis for collagens and proteoglycans, although levels of degradation varied.

Synthesis of new glycoproteins followed a similar pattern to proteoglycans with a continual increase over time, however total protein content was variable with a continual increase for some proteins (such as tenascin C and Tsukushi), and a

decrease after 48 hours for other proteins (such as fibronectin and elastin) indicative of increased protein degradation. This variability could reflect differences in regulation of these proteins and differences in function. Previous studies in equine tendon demonstrated a relatively slow rate of turnover for non-collagenous proteins (both proteoglycans and glycoproteins) with a half-life of 2-3.5 years depending on the tendon type, however these values were still considerably shorter than those for collagen (Thorpe *et al*., 2010). There is very little previous research available on the dynamics of glycoprotein turnover and metabolism.

ECM regulatory protein synthesis continually increased over time, whereas total protein content increased until 24 hours and then decreased between 24 and 48 hours, indicative of increased protein degradation, except for serpin peptidase inhibitor E2 which continued to increase over time. Little is known about the turnover of these ECM regulatory proteins, however due to their regulatory nature and the requirement for fast responsiveness to changing cellular demands it would be expected that their turnover would be high (Schwanhausser *et al*., 2011), as seen in this study.

There are complex interactions between ECM core and regulatory proteins which may be reflected in their expression and turnover profiles. Collagen is transcriptionally regulated by TGFβ1, with TGFβ1 causing an increase in collagen type I, III and VI gene expression (Heckmann *et al*., 1992; Klein *et al*., 2002; Verrecchia and Mauviel, 2004). I observed an initial increase in collagen synthesis followed by a decrease after 24 hours, although I observed a continual steady increase in TGFβ1 synthesis the total protein content of TGFβ1 also declined after 24 hours. This may suggest that the decline in collagen synthesis was due to a reduction in TGFβ1 content. The reduction in TGFβ1 may be due to the increased production of decorin, tsukushi and/or HtrA serine peptidases which have been reported to bind to and inhibit TGFβ1, thereby reducing collagen synthesis (Fullár *et al*., 2016; Niimori *et al*., 2012; Niimori *et al*., 2014; Oka *et al*., 2004; Tocharus *et al*., 2004; Wang *et al*., 2016). In addition, lumican also reduces collagen fibril formation (Matheson *et al*., 2005; Neame *et al*., 2000; Rada *et al*., 1993) and a continual increase in synthesis of this proteoglycan was observed. Interestingly a decrease in total serpin peptidase inhibitor H1 content, also known as heat shock protein 47

and collagen binding protein 1, after 24 hours was observed. Serpin peptidase inhibitor H1 is vital for collagen maturation and folding and is positively regulated by TGFβ1 (Ishida *et al*., 2006; Ito and Nagata, 2016; Razzaque and Ahmed, 2002). The reduction in TGFβ1 may have reduced the production of serpin peptidase inhibitor H1, as the synthesis of this protein starts to plateau after 24 hours, thereby further reducing collagen synthesis. The decrease in total collagen content seen in this study is due to both reduced synthesis and increased degradation of collagen. The increase in degradation is likely due to an increase in collagen degrading enzymes such as HtrA serine peptidase 1, cathepsins and serpins, which I have shown to be produced by canine CCL cells. HtrA serine peptidase 1, the synthesis of which I have shown to increase over time, has previously been found to degrade collagen type I, III and VI (Murwantoko *et al*., 2004; Polur *et al*., 2010). Cathepsins B and D are also able to degrade collagen, including types I and III (Creemers *et al*., 1998; Li *et al*., 2016a; Scott and Pearson, 1981; Turk *et al*., 2012). Despite their ability to degrade collagen, it is unlikely that cathepsins played a significant role in the degradation of collagen in this study as total protein content of these enzymes decreased after 24 hours. Cathepsin B also activates plasmin (Guo *et al*., 2002) which has been shown to activate collagenases (Berman, 1993; Mazzieri *et al*., 1997) and may contribute to the degradation of collagen seen here, however no matrix metalloproteinase (MMP) collagenases were identified in this study, which are the main family of enzymes responsible for collagen degradation. Different collagen types also interact with each other and may contribute to regulation of collagen synthesis, maturation and stabilisation (Harumiya *et al*., 2002; Liu *et al*., 1997). A continual increase in tenascin C expression was observed over time which may have been stimulated by the earlier increase in TGFβ1 as TGFβ1 has been shown to increase tenascin C expression (Estany *et al*., 2014; Islam *et al*., 2014; Mercado-Gómez *et al*., 2014). Tenascin C has been shown to interfere with fibronectin fibrillogenesis (To and Midwood, 2010), which can cause a reduction in fibronectin function. As mature fibronectin is required for collagen assembly (Sottile and Hocking, 2002), the reduced total protein levels of fibronectin observed here may have contributed to the reduction in collagen observed after 24 hours. Fibronectin is stimulated by TGFβ1 (Quan *et al*., 2005), therefore the continual

increase in synthesis of TGFβ1 may have contributed to the continual increase in synthesis of fibronectin (despite reduced levels of total protein content for both of these proteins after 24 hours). The reason for the decrease in total fibronectin content after 24 hours despite continued synthesis is due to degradation. Fibronectin is degraded by a number of enzymes including HtrA serine peptidase 1 and cathepsins (Buck *et al*., 1992; Grau *et al*., 2006; Jiang *et al*., 2012; Richter and Hörmann, 1983) and these enzymes may contribute to the degradation of fibronectin observed here. Continued synthesis of elastin was observed over time which may again have been stimulated by TGFβ1 (Oleggini *et al*., 2007). The total protein content of elastin however decreased after 24 hours which was due to increased degradation, possibly by HtrA serine peptidase 1 and/or cathepsins as these enzymes have previously been shown to degrade elastin (Jones *et al*., 2011; Turk *et al*., 2012). Serpin peptidase inhibitor E2, also known as protease nexin, inhibits collagenases both directly and indirectly thereby preventing degradation of collagen as well as fibronectin (Bouton *et al*., 2012; Francois *et al*., 2014; Santoro *et al*., 2015). Serpin peptidase inhibitor E2 also increases collagen synthesis via TGFβ1 signalling (Li *et al*., 2016b). Therefore, the continued increase in synthesis and total protein content of serpin peptidase inhibitor E2 over time would be expected to correlate to an increase in collagen content over time (through both increased synthesis and reduced degradation), however this was not the case. It is possible that other proteins and factors are required for serpin peptidase inhibitor E2 to function that were not present here, or alternatively that other proteins and factors required for collagen synthesis were not present, and/or other degradative enzymes unaffected by serpin peptidase inhibitor E2 were present. The total protein content of serpin peptidase inhibitor E2 was relatively high therefore it is unlikely that this protein was not present in a high enough quantity to affect function, however this is a possibility. A summary of the potential interactions between the metabolically labelled ECM core and regulatory proteins in this study is shown in Figure 6.8.

Very few differences in protein turnover were observed between ligamentocytes and LDSCs. There were no differences in total protein content between cell types however there were a few statistically significant differences

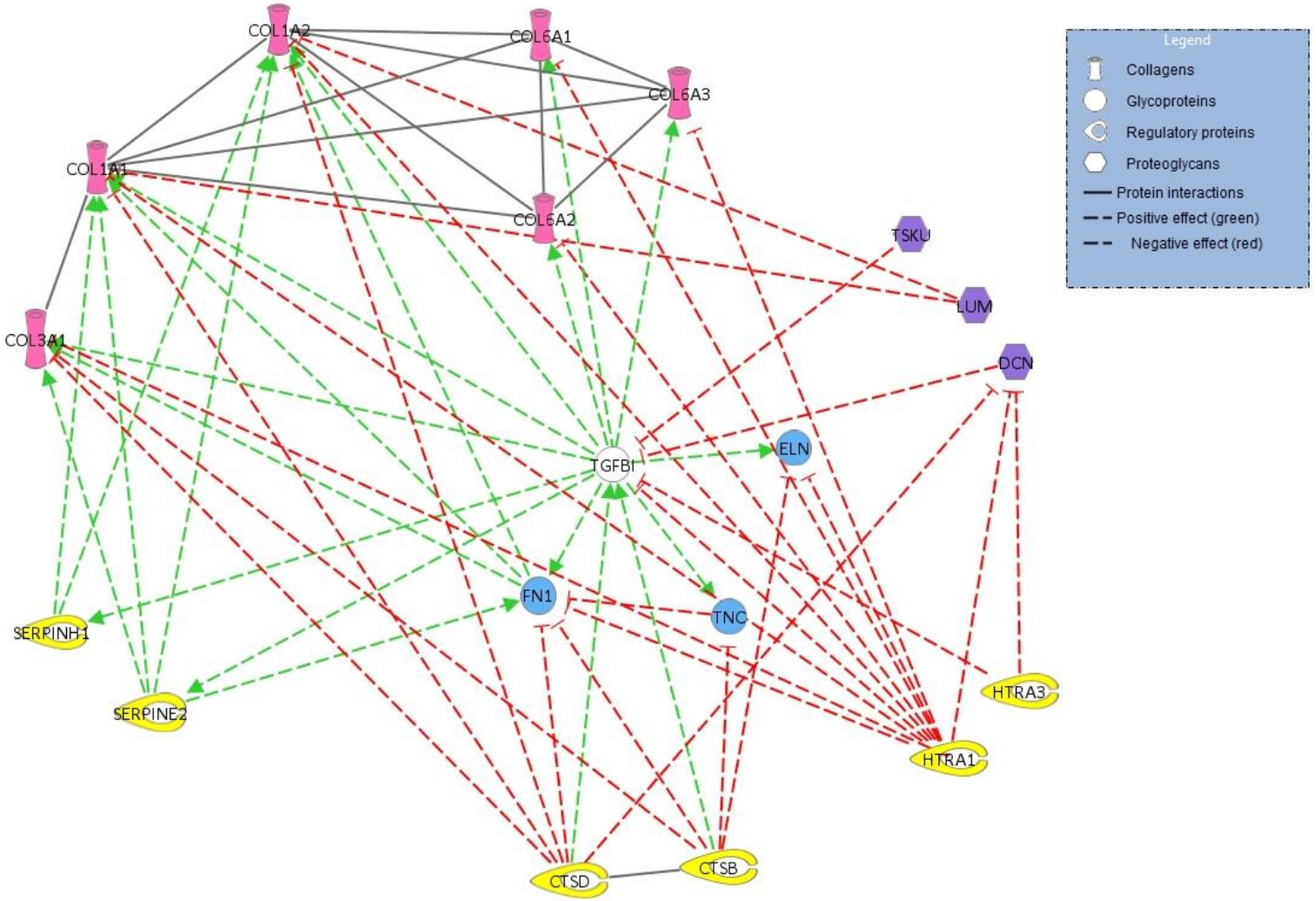


Figure 6.8. Protein interaction map highlighting the potential positive and negative interactions/effects between the core and regulatory ECM proteins metabolically labelled in this study. The map was produced using Ingenuity Pathway Analysis (IPA) (Ingenuity systems. www.ingenuity.com).

between RIA with all differences being an increase in RIA in ligamentocytes. This indicates that there was an increase in new protein synthesis and turnover in the niche of ligamentocytes compared with LDSCs. This would suggest that LDSCs require a more stable environment than ligamentocytes, however it would be expected that due to the dynamic needs of stem cells and the requirement for proliferation and differentiation at times of need that stem cell niches would be dynamic with increased turnover of niche components (Gattazzo *et al*., 2014). In contrast, in the absence of injury or challenge many stem cell populations tend to be dormant and quiescent and are only activated during times of repair (Foudi *et al*., 2009; Sottocornola and Lo Celso, 2012; Wilson *et al*., 2008). This may be the case in healthy canine CCL with LDSCs in a state of quiescence. It would be valuable to compare the expression and turnover of the LDSC niche for LDSCs isolated from ruptured or diseased ligament.

6.5.4 Conclusion

I have characterised the ECM composition of the canine CCL cell niche in terms of protein expression and turnover. The expression of ECM proteins by canine CCL cells was, as expected, similar to that described in previous reports in a variety of ligaments from a number of difference species. The identification of ECM proteins using PEAKS software yielded a number of proteins unique to either ligamentocytes or LDSCs. For example, fibronectin was found by PEAKS to be unique to ligamentocytes, however as can be seen from the metabolic labelling data, fibronectin has been metabolically labelled with heavy isotope in both cell types. It is clear that there are many differences between different software and databases in terms of sensitivity. It is possible that fibronectin is produced by both cell types, however it is produced in a lower quantity by LDSCs which was not detected by PEAKS (based on the parameters used) but was detected by MASCOT which may have an increased sensitivity and decreased threshold. However, this is inconsistent with the metabolic labelling data, which found no significant difference in fibronectin expression between cell types, although fibronectin expression was slightly reduced for LDSCs compared with ligamentocytes at three out of four time

points. This highlights the potential unreliability of using PEAKS software for such analysis and enforces the necessity of validating any data produced using mass spectrometry. This is often achieved using Western blotting, unfortunately due to time and cost restraints as well as the lack of available validated canine antibodies I was unable to confirm the unique protein identifications observed in this study.

I have, for the first time, developed a proteomics-based method enabling analysis of the dynamics of newly synthesised ECM proteins *in vitro*, including collagens, proteoglycans and glycoproteins. Using this method I observed new synthesis of a number of different ECM proteins by canine CCL cells with varying synthesis and turnover profiles. My data demonstrated a decline in collagen synthesis after 24 hours and a continual increase in non-collagenous synthesis over time, although there was variability between proteins. This may suggest an important role for non-collagenous proteins in the ligament cell niche and further investigation of the role of such proteins in the ligament cell niche is required. It is clear that the study of protein dynamics is of vital importance for a number of reasons. Firstly for protein identification, for example, if only proteins present at 48 hours had been investigated then this would not have given an accurate representation of the ECM niche of ligament cells. Several proteins were produced early on and had been degraded by 48 hours with very low or no protein present at this time, such as collagen type III alpha 1 and serpin peptidase inhibitor H1. As well as the role of protein dynamics in protein identification, the study of protein turnover is necessary to fully understand the function and regulation of proteins. It also enables a comparison of the dynamics of different proteins and highlights potential protein interactions and roles based on protein synthesis and degradation profiles. The study of protein dynamics is of great importance for future research not only in ligament but for many different fields.

Chapter 7

General Discussion

7.1 General discussion

Tendon and ligament are prone to age- and injury-related degeneration (Cimino *et al*., 2010; Maffulli *et al*., 2003). Tendon and ligament are unable to heal effectively after injury (Frank, 2004; Maffulli *et al*., 2003) and current treatment strategies have variable success rates (Dawson *et al*., 2016; Mayor, 2012; Murawski *et al*., 2014). The identification of stem cell populations in tendon and ligament (Bi *et al*., 2007; Seo *et al*., 2004) holds potential for new therapeutic strategies to treat tendon and ligament injuries.

The aim of this study was to characterise stem cell populations in equine superficial digital flexor tendon (SDFT) and canine cranial cruciate ligament (CCL), as well as to characterise the stem cell niche of these stem cell populations. Equine SDFT and canine CCL are both prone to injury and degeneration (Ely *et al*., 2009; Wilke *et al*., 2006; Witsberger *et al*., 2008) and therefore effective treatment options are currently being sought. The study of tendon and ligament in these species also provides a suitable translatable model for human research due to the similarities in these tissues between species (Innes and Clegg, 2010). There is limited data available on tendon-derived stem cell (TDSC) populations in equine SDFT (Lovati *et al*., 2011), however, to date, there is no data available on ligament-derived stem cells (LDSCs) isolated from canine CCL. Therefore characterisation of these cell populations is vital in order to assess their suitability for tendon and ligament repair strategies. Characterisation of stem cell populations in tendon and ligament involved the use of a number of assays to analyse the stemness of the cells based on the criteria outlined by the International Society for Cellular Therapy (ISCT) (Dominici *et al*., 2006). These included clonogenic assays, gene expression analysis and tri-lineage differentiation assays. The stem cell niche has been shown to be vital for survival and function of a number of stem cell populations, including tendon (Bi *et al*., 2007; Calvi *et al*., 2003; Garcion *et al*., 2004). Therefore characterisation of the stem cell niche is also necessary to understand the regulation of stem cell populations. In addition, modulation of the stem cell niche can induce changes in both stem cell phenotype and function and holds therapeutic potential (Lane *et al*., 2014).

TDSC populations from equine SDFT were isolated along with tenocytes (tendon fibroblasts) as a comparative cell type (see Chapter 3). The TDSC population isolated in this study demonstrated clonogenicity, stem cell marker expression and multipotency, all hallmarks of mesenchymal stromal cells (MSCs) (Dominici *et al*., 2006). However, limited multipotency of TDSC populations was observed, with an inability of these cells to differentiate down the adipogenic lineage. In addition, I observed very few differences between TDSCs and tenocytes. There were also no differences in extracellular matrix (ECM) composition and production between cell types. These results were not consistent with previous reports in equine SDFT (Lovati *et al*., 2011) and suggested a heterogeneous population of cells at different stages of differentiation. Optimisation of TDSC isolation procedures are required to ensure a purer population of TDSCs. The reduced multipotency of the TDSCs isolated in this study may provide a therapeutic benefit during injury and with further characterisation may form the basis for future treatment strategies. The use of TDSCs isolated from tendons of various species have successfully aided tendon repair after injury (Ni *et al*., 2012; Yin *et al*., 2010; Zhang *et al*., 2011a), indicating that TDSCs present in equine SDFT may be targets for future tendon repair strategies.

In order to ensure the reduced multipotency observed in equine TDSCs was not due to experimental or technical differences, TDSCs from murine tail tendons were also isolated, along with tenocytes as a comparative cell type (see Chapter 4). I observed clonogenicity, the expression of stem cell markers and multipotency of murine TDSC populations. Interestingly, I observed increased multipotency of murine TDSCs compared with equine TDSCs, with murine TDSCs able to differentiate down the adipogenic lineage as well as osteogenic and chondrogenic lineages. These results are consistent with the literature (Alberton *et al*., 2014; Bi *et al*., 2007; Salingcarnboriboon *et al*., 2003). In addition, several differences between murine TDSCs and tenocytes were observed, including clonogenicity, stem cell marker expression and multipotency, suggestive of two distinct cell populations within murine tail tendon. These results indicated that the restricted multipotency of equine TDSCs is a feature of this cell population rather than due to experimental differences.

LDSCs from canine CCL were isolated as well as ligamentocytes (ligament fibroblasts) as a comparative cell type (see Chapter 5). LDSCs demonstrated clonogenicity, stem cell marker expression and multipotency, consistent with previous reports in other species (Fu *et al*., 2015; Steinert *et al*., 2011). Differences in clonogenicity, stem cell marker expression and multipotency were observed between LDSCs and ligamentocytes, suggestive of two distinct cell populations within canine CCL. LDSCs isolated from canine CCL may provide a basis for future treatment strategies for CCL injuries. Studies conducted using LDSCs isolated from rabbit medial collateral ligament have shown promising results in terms of ligament repair (Jiang *et al*., 2015), suggestive of the therapeutic potential of canine LDSCs.

The canine CCL niche was also characterised for LDSCs and ligamentocytes in terms of ECM composition (see Chapter 6). Mass spectrometry was used to identify the complement of ECM proteins present in the LDSC niche as well as analysing protein synthesis and turnover using a label-based proteomics method. Many ECM proteins were identified in the niche of LDSCs and ligamentocytes including collagens, glycoproteins, proteoglycans, ECM affiliated proteins, ECM regulatory proteins as well as secreted proteins. The proteins identified within canine CCL were consistent with the literature (Kharaz *et al*., 2016; Little *et al*., 2014; Sato *et al*., 2016). There were a small number of proteins which were apparently unique to either LDSCs or ligamentocytes, with several glycoproteins and regulatory proteins unique to ligamentocytes and a number of collagens and proteoglycans unique to LDSCs. Confirmation of the role of these proteins in the LDSC and ligamentocyte niche is necessary to understand the regulation and maintenance of these cell populations. Analysis of protein synthesis and turnover yielded some interesting data with characteristic patterns emerging for different ECM protein categories, for example collagens showed an initial increase in content and synthesis which declined with time, however the content and synthesis of proteoglycans and some glycoproteins continued to increase over time. These results may suggest that non-collagenous proteins play an important role in the maintenance of canine CCL cell populations. There were very few differences in protein synthesis and turnover between cell types, however these differences all reflected increased synthesis of proteins by ligamentocytes. This suggests that ligamentocytes require a more

dynamic niche compared with LDSCs which require an environment which is more stable. Although initially this seems counter-intuitive, it is possible that LDSCs are in a state of dormancy in healthy tissue and are only activated during times of homeostasis and repair, as seen in other stem cell populations (Foudi *et al*., 2009; Sottocornola and Lo Celso, 2012; Wilson *et al*., 2008). The role of these newly synthesised proteins in the maintenance and regulation of CCL cell populations needs to be elucidated. Their active synthesis is suggestive of their importance in the CCL niche, however the exact role and function of these proteins is an area for future research. The modulation of some of these proteins may improve LDSC and ligamentocyte survival and function and thereby be of potential therapeutic benefit.

7.2 Suggestions for future work

Suggestions for future work on tendon-derived stem cells include: optimisation of equine TDSC isolation methods; identification of TDSC-specific markers; characterisation of pure cell populations; tissue engineering/stem cell therapy approaches to tendon injury utilising equine TDSCs; comparison of TDSC phenotype between healthy and injured tissue.

Suggestions for future work on ligament-derived stem cells include: identification of LDSC-specific markers; tissue engineering/stem cell therapy approaches to ligament injury utilising canine LDSCs; comparison of LDSC phenotype between healthy and injured tissue; investigation of the regulation of different LDSC subpopulations, including the role of epigenetics.

Suggestions for future work on the ligament-derived stem cell niche include: validation of protein identifications; investigation of the role of proteins unique to either cell type; investigation of the role of proteins with significantly different synthesis or turnover rates between cell types; determination of candidates for ligament cell niche modulation therapies; comparison of ligament cell niche between healthy and injured tissue.

Chapter 8

Appendices

8.1 Appendix A – Joint-first author paper published in Journal of Orthopaedic Research

8.2 Appendix B – Selection of best housekeeping genes for qPCR and analysis of primer efficiencies

All equine primers used in this study were kindly donated by Dr. Kate Williamson (University of Liverpool), who had previously designed the primers using the same software described in Chapter 2, Section 2.2.2.5. All primers were tested for efficiency and the best housekeeping genes had also previously been determined, therefore this data is not included.

8.2.1 Determination of best housekeeping genes

Analysis of murine housekeeping genes using the geNorm gene stability algorithm (Vandesompele *et al*., 2002) revealed that GAPDH and B2M possessed equal stability (Fig.8.1-8.2), therefore GAPDH was selected for use in future experiments.

Analysis of caninehousekeeping genes using the geNorm gene stability algorithm (Vandesompele *et al*., 2002) revealed that GAPDH and RPS13 possessed equal stability (Fig.8.3-8.4), therefore GAPDH was selected for use in future experiments.

```
Step 1:

stability values M:

   RPS13     ACTB    RPL13a     GAPDH       B2M      X18S
1.489960 1.754691 1.823530 1.855685 1.972452 2.824383
average stability M:   1.95345017415506

variable with lowest stability (largest M value):     X18S

Pairwise variation, (5/6):     0.449623653312269

################################################################

Step 2:

stability values M:

   RPS13     ACTB     GAPDH       B2M    RPL13a
1.314197 1.422728 1.518825 1.601194 1.732975
average stability M:   1.51798388199079

variable with lowest stability (largest M value):     RPL13a

Pairwise variation, (4/5):     0.319142565151587

################################################################

Step 3:

stability values M:

   GAPDH       B2M      ACTB     RPS13
1.342864 1.347084 1.399408 1.409270
average stability M:   1.37465640743623

variable with lowest stability (largest M value):     RPS13

Pairwise variation, (3/4):     0.295359097556527

################################################################
```

```
 Step 4:

stability values M:

     B2M    GAPDH     ACTB
1.216605 1.363272 1.440251
average stability M:   1.3400426868367

variable with lowest stability (largest M value):    ACTB

Pairwise variation, (2/3):    0.443614637219158

##################################################################

Step 5:

stability values M:

     B2M    GAPDH
1.139627 1.139627
average stability M:   1.13962697647197

>
> ranks <- HK$ranking
>
> ranks
       1        1        3        4        5        6
   "B2M"  "GAPDH"   "ACTB"  "RPS13" "RPL13a"   "X18S"
```

Figure 8.1. R script used to determine most stable murine housekeeping gene.

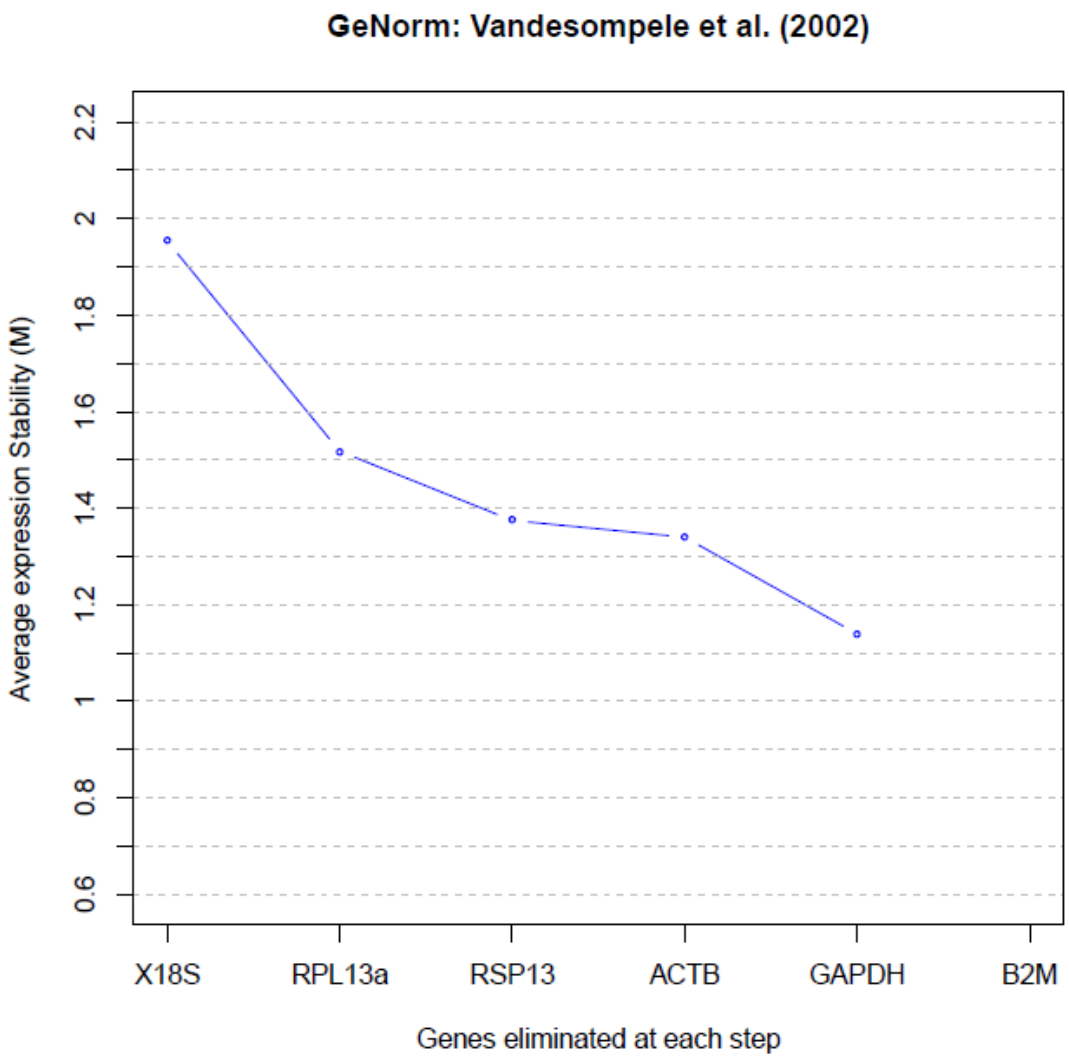


Figure 8.2. Graphical representation of R script highlighting the most stable murine housekeeping gene.

```
Step 1:

stability values M:

   RPS13    GAPDH      B2M     ACTB
1.736057 2.044986 2.630631 2.650524
average stability M:   2.26554970320895

variable with lowest stability (largest M value):    ACTB

Pairwise variation, (3/4):    0.607435751668605

##################################################################

Step 2:

stability values M:

   RPS13    GAPDH      B2M
1.507763 1.820493 2.313470
average stability M:   1.88057551391956

variable with lowest stability (largest M value):    B2M

Pairwise variation, (2/3):    0.759568484198066

##################################################################

Step 3:

stability values M:

   GAPDH    RPS13
1.014786 1.014786
average stability M:   1.01478555088537

>
> ranks <- HK$ranking
>
> ranks
      1       1       3       4
"GAPDH" "RPS13"   "B2M"  "ACTB"
```

Figure 8.3. R script used to determine most stable canine housekeeping gene.

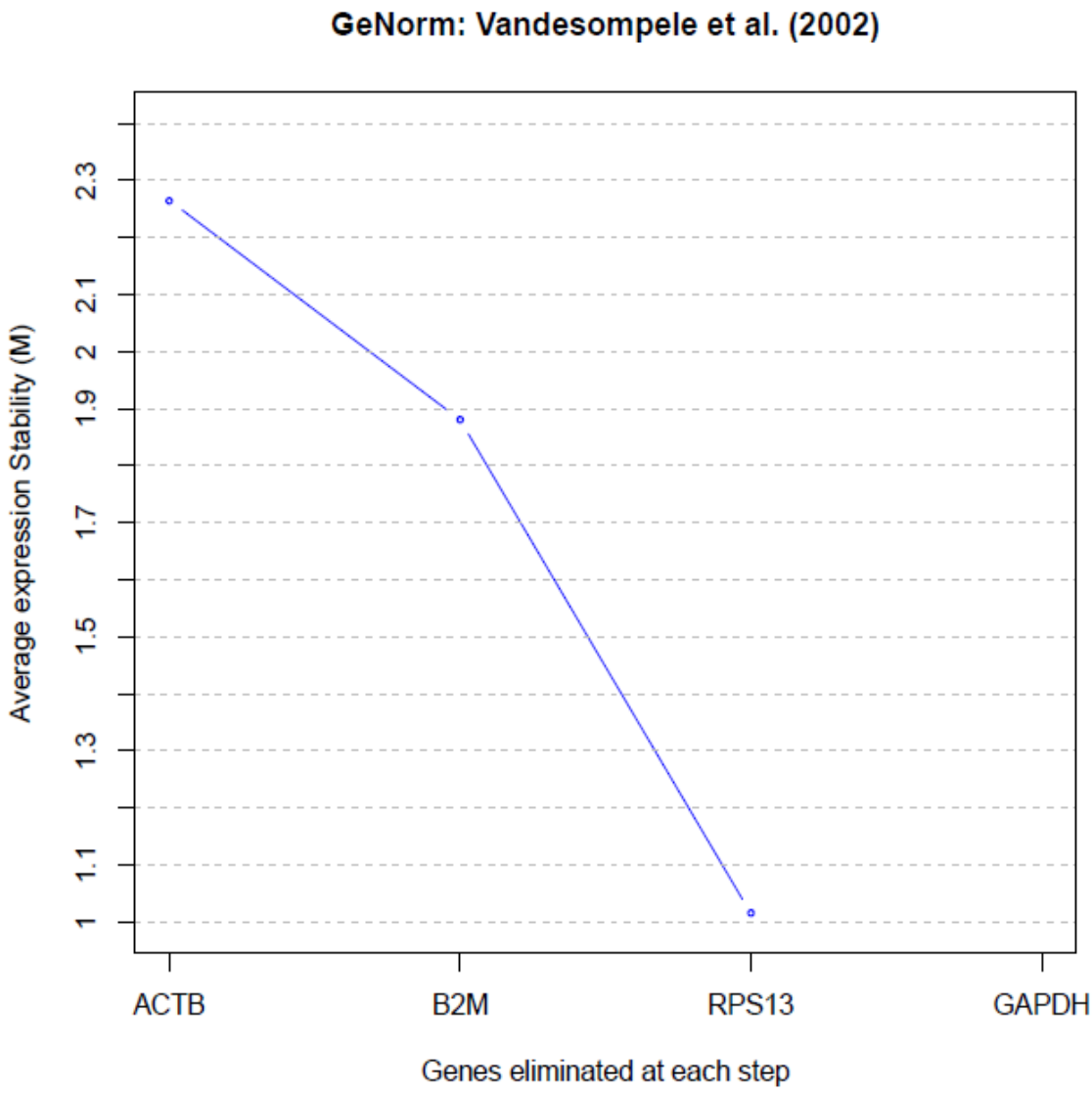


Figure 8.4. Graphical representation of R script highlighting the most stable canine housekeeping gene.

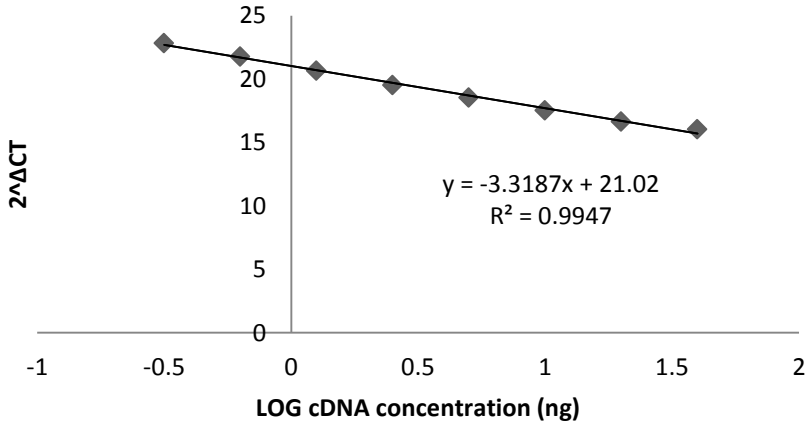
8.2.2 Assessment of primer efficiencies

All primers were tested for efficiency before use in experiments, including all housekeeping primers used. Serial dilution of cDNA was performed ranging from 0.3-40 ng, which encompassed the cDNA concentration normally used: 10 ng. qPCR was then conducted as described in Chapter 2, Section 2.2.2.5. Primer efficiencies were then plotted and calculated and dissociation curves assessed. Representative images are shown below (Fig.8.5). Primers with efficiencies between 90-110% were deemed to be acceptable and were used for future experiments.

A

Template amount (ng)	Log template amount (ng)	CT Target (GAPDH)
40	1.6	16.0375
20	1.3	16.641
10	1	17.5375
5	0.7	18.5315
2.5	0.4	19.5325
1.25	0.1	20.669
0.63	-0.2	21.7715
0.31	-0.5	22.835
Slope efficiency		-3.3187
Efficiency		1.001348901
% efficiency		100.13

B



C

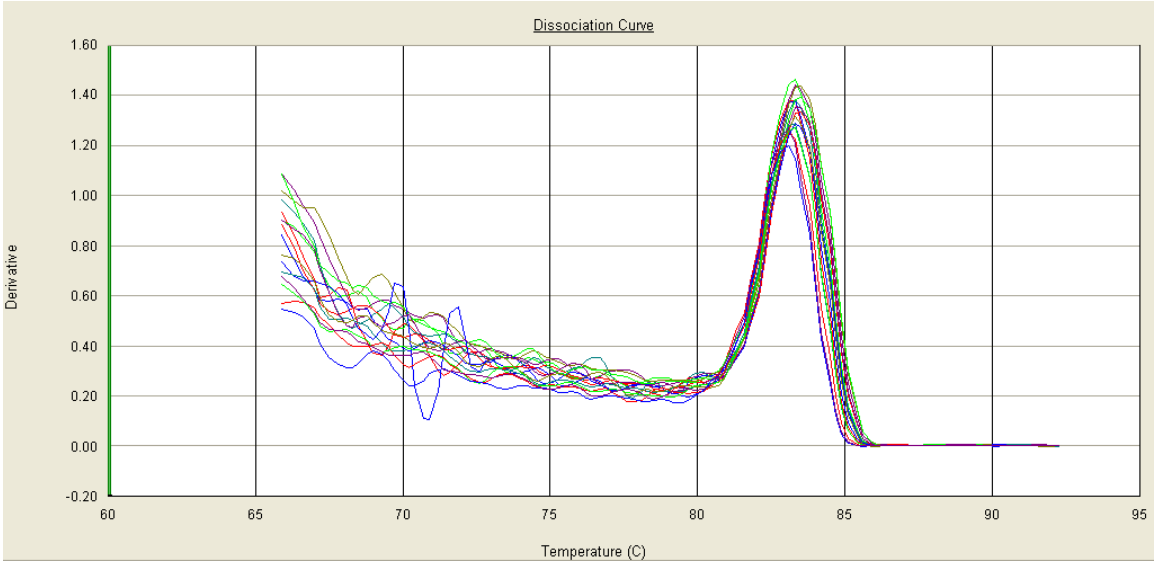


Figure 8.5. Determination of murine GAPDH primer quality and efficiency. 2^ΔCT values were determined for a range of murine cDNA concentrations (A), and then plotted on a scatter graph to determine the linear equation (B), and the overall primer efficiency (A). Dissociation curves for all primers were also analysed to ensure amplification of a single PCR product (C).

References

Abdallah, B.M., Al-Shammary, A., Skagen, P., Abu Dawud, R., Adjaye, J., Aldahmash, A., and Kassem, M. (2015). CD34 defines an osteoprogenitor cell population in mouse bone marrow stromal cells. Stem Cell Research *15*, 449-458.

Adams, P., Bolus, R., Middleton, S., Moores, A.P., and Grierson, J. (2011). Influence of signalment on developing cranial cruciate rupture in dogs in the UK. Journal of Small Animal Practice *52*, 347-352.

Aebersold, R., and Mann, M. (2003). Mass spectrometry-based proteomics. Nature *422*, 198-207.

Alberton, P., Dex, S., Popov, C., Shukunami, C., Schieker, M., and Docheva, D. (2014). Loss of Tenomodulin Results in Reduced Self-Renewal and Augmented Senescence of Tendon Stem/Progenitor Cells. Stem cells and development *24*, 597-609.

Asai, S., Otsuru, S., Candela, M.E., Cantley, L., Uchibe, K., Hofmann, T.J., Zhang, K., Wapner, K.L., Soslowsky, L.J., Horwitz, E.M*., et al.* (2014). Tendon Progenitor Cells in Injured Tendons Have Strong Chondrogenic Potential: The CD105-Negative Subpopulation Induces Chondrogenic Degeneration. Stem cells *32*, 3266-3277.

Babraj, J.A., Cuthbertson, D.J.R., Smith, K., Langberg, H., Miller, B., Krogsgaard, M.R., Kjaer, M., and Rennie, M.J. (2005). Collagen synthesis in human musculoskeletal tissues and skin. American Journal of Physiology - Endocrinology And Metabolism *289*, E864.

Baddoo, M., Hill, K., Wilkinson, R., Gaupp, D., Hughes, C., Kopen, G.C., and Phinney, D.G. (2003). Characterization of mesenchymal stem cells isolated from murine bone marrow by negative selection. Journal of cellular biochemistry *89*, 1235-1249.

Baghaban Eslaminejad, M., and Fallah, N. (2013). Effects of BIO on proliferation and chondrogenic differentiation of mouse marrow-derived mesenchymal stem cells. Veterinary Research Forum *4*, 69-76.

Baird, A.E.G., Carter, S.D., Innes, J.F., Ollier, W., and Short, A. (2014a). Genome-wide association study identifies genomic regions of association for cruciate ligament rupture in Newfoundland dogs. Animal Genetics *45*, 542-549.

Baird, A.E.G., Carter, S.D., Innes, J.F., Ollier, W.E., and Short, A.D. (2014b). Genetic basis of cranial cruciate ligament rupture (CCLR) in dogs. Connective tissue research *55*, 275-281.

Bantscheff, M., Schirle, M., Sweetman, G., Rick, J., and Kuster, B. (2007). Quantitative mass spectrometry in proteomics: a critical review. Analytical and Bioanalytical Chemistry *389*, 1017-1031.

Barboni, B., Curini, V., Russo, V., Mauro, A., Di Giacinto, O., Marchisio, M., Alfonsi, M., and Mattioli, M. (2012). Indirect co-culture with tendons or tenocytes can program amniotic epithelial cells towards stepwise tenogenic differentiation. PLoS One *7*, e30974.

Barsby, T., Bavin, E.P., and Guest, D.J. (2014). Three-Dimensional Culture and Transforming Growth Factor Beta3 Synergistically Promote Tenogenic Differentiation of Equine Embryo-Derived Stem Cells. Tissue engineering Part A *20*, 2604-2613.

Benjamin, M., and Ralphs, J.R. (1998). Fibrocartilage in tendons and ligaments — an adaptation to compressive load. Journal of anatomy *193*, 481-494.

Bentzinger, C.F., Wang, Y.X., von Maltzahn, J., Soleimani, V.D., Yin, H., and Rudnicki, M.A. (2013). Fibronectin regulates Wnt7a signaling and satellite cell expansion. Cell stem cell *12*, 75-87.

Bergh, M.S., Sullivan, C., Ferrell, C.L., Troy, J., and Budsberg, S.C. (2014). Systematic Review of Surgical Treatments for Cranial Cruciate Ligament Disease in Dogs. Journal of the American Animal Hospital Association *50*, 315-321.

Berman, M.B. (1993). Regulation of corneal fibroblast MMP-1 collagenase secretion by plasmin. Cornea *1993*, 5.

Bi, Y., Ehirchiou, D., Kilts, T.M., Inkson, C.A., Embree, M.C., Sonoyama, W., Li, L., Leet, A.I., Seo, B.M., Zhang, L.*, et al.* (2007). Identification of tendon stem/progenitor cells and the role of the extracellular matrix in their niche. Nature medicine *13*, 1219-1227.

Bianco, P., Barker, R., Brüstle, O., Cattaneo, E., Clevers, H., Daley, G.Q., De Luca, M., Goldstein, L., Lindvall, O., Mummery, C.*, et al.* (2013). Regulation of stem cell therapies under attack in Europe: for whom the bell tolls. The EMBO Journal *32*, 1489-1495.

Birch, H.L., Bailey, A.J., and Goodship, A.E. (1998). Macroscopic 'degeneration' of equine superficial digital flexor tendon is accompanied by a change in extracellular matrix composition. Equine veterinary journal *30*, 534-539.

Birch, H.L., Thorpe, C.T., and Rumian, A.P. (2013). Specialisation of extracellular matrix for function in tendons and ligaments. Muscles, ligaments and tendons journal *3*, 12-22.

Bouton, M.-C., Boulaftali, Y., Richard, B., Arocas, V., Michel, J.-B., and Jandrot-Perrus, M. (2012). Emerging role of serpinE2/protease nexin-1 in hemostasis and vascular biology. Blood *119*, 2452-2457.

Brandau, O., Meindl, A., Fässler, R., and Aszódi, A. (2001). A novel gene, tendin, is strongly expressed in tendons and ligaments and shows high homology with chondromodulin-I. Developmental Dynamics *221*, 72-80.

Braun, J., Hack, A., Weis-Klemm, M., Conrad, S., Treml, S., Kohler, K., Walliser, U., Skutella, T., and Aicher, W.K. (2010). Evaluation of the osteogenic and chondrogenic differentiation capacities of equine adipose tissue-derived mesenchymal stem cells. American Journal of Veterinary Research *71*, 1228-1236.

Brown, J.P., Galassi, T.V., Stoppato, M., Schiele, N.R., and Kuo, C.K. (2015). Comparative analysis of mesenchymal stem cell and embryonic tendon progenitor cell response to embryonic tendon biochemical and mechanical factors. Stem cell research & therapy *6*, 89.

Buck, M.R., Karustis, D.G., Day, N.A., Honn, K.V., and Sloane, B.F. (1992). Degradation of extracellular-matrix proteins by human cathepsin B from normal and tumour tissues. Biochemical Journal *282*, 273-278.

Bueno-Betí, C., Novella, S., Lázaro-Franco, M., Pérez-Cremades, D., Heras, M., Sanchís, J., and Hermenegildo, C. (2013). An affordable method to obtain cultured endothelial cells from peripheral blood. Journal of Cellular and Molecular Medicine *17*, 1475-1483.

Burden, N., Chapman, K., Sewell, F., and Robinson, V. (2015). Pioneering Better Science through the 3Rs: An Introduction to the National Centre for the Replacement, Refinement, and Reduction of Animals in Research (NC3Rs). Journal of the American Association for Laboratory Animal Science : JAALAS *54*, 198-208.

Burk, J., Gittel, C., Heller, S., Pfeiffer, B., Paebst, F., Ahrberg, A., and Brehm, W. (2014). Gene expression of tendon markers in mesenchymal stromal cells derived from different sources. BMC Research Notes *7*, 826.

Cadby, J.A., Buehler, E., Godbout, C., van Weeren, P.R., and Snedeker, J.G. (2014). Differences between the Cell Populations from the Peritenon and the Tendon Core with Regard to Their Potential Implication in Tendon Repair. PLoS ONE *9*, e92474.

Calvi, L.M., Adams, G.B., Weibrecht, K.W., Weber, J.M., Olson, D.P., Knight, M.C., Martin, R.P., Schipani, E., Divieti, P., Bringhurst, F.R.*, et al.* (2003). Osteoblastic cells regulate the haematopoietic stem cell niche. Nature *425*, 841-846.

Caplan, A.I. (1991). Mesenchymal stem cells. Journal of Orthopaedic Research *9*, 641-650.

Caplan, A.I. (2017). Mesenchymal Stem Cells: Time to Change the Name! STEM CELLS Translational Medicine *6*, 1445-1451.

Caplan, A.I., and Correa, D. (2011). THE MSC: AN INJURY DRUGSTORE. Cell stem cell *9*, 11-15.

Carvalho, A.d.M., Badial, P., Alvarez, L.E.C., Yamada, A.L.M., Borges, A., Deffune, E., Hussni, C., and Garcia Alves, A. (2013). Equine tendonitis therapy using mesenchymal stem cells and platelet concentrates: a randomized controlled trial. Stem cell research & therapy *4*, 85.

Çelebi, B., Mantovani, D., and Pineault, N. (2011). Effects of extracellular matrix proteins on the growth of haematopoietic progenitor cells. Biomedical materials *6*, 055011.

Chang, X., M., Liu, H.W., Jin, Y., Liu, Y., and He, H.X. (2009). Isolation and identification of dog periodontal ligament stem cells. West China Journal of Stomatology *27*, 79-83.

Chen, J., Zhang, W., Liu, Z., Zhu, T., Shen, W., Ran, J., Tang, Q., Gong, X., Backman, L.J., Chen, X.*, et al.* (2016). Characterization and comparison of post-natal rat Achilles tendon-derived stem cells at different development stages. Scientific Reports *6*, 22946.

Chen, L., Dong, S.-W., Liu, J.-P., Tao, X., Tang, K.-L., and Xu, J.-Z. (2012a). Synergy of tendon stem cells and platelet-rich plasma in tendon healing. Journal of Orthopaedic Research *30*, 991-997.

Chen, L., Dong, S.-W., Tao, X., Liu, J.-P., Tang, K.-L., and Xu, J.-Z. (2012b). Autologous Platelet-Rich Clot Releasate Stimulates Proliferation and Inhibits Differentiation of Adult Rat Tendon Stem Cells towards Nontenocyte Lineages. The Journal of international medical research *40*, 1399-1409.

Chen, L., Liu, J., Tao, X., Wang, G., Wang, Q., and Liu, X. (2015a). The role of Pin1 protein in aging of human tendon stem/progenitor cells. Biochemical and biophysical research communications *464*, 487-492.

Chen, L., Liu, J.P., Tang, K.L., Wang, Q., Wang, G.D., Cai, X.H., and Liu, X.M. (2014). Tendon Derived Stem Cells Promote Platelet-Rich Plasma Healing in Collagenase-Induced Rat Achilles Tendinopathy. Cellular Physiology and Biochemistry *34*, 2153-2168.

Chen, L., Wang, G.-D., Liu, J.-P., Wang, H.-S., Liu, X.-M., Wang, Q., and Cai, X.-H. (2015b). miR-135a modulates tendon stem/progenitor cell senescence via suppressing ROCK1. Bone *71*, 210-216.

Chen, W., Tang, H., Zhou, M., Hu, C., Zhang, J., and Tang, K. (2015c). Dexamethasone inhibits the differentiation of rat tendon stem cells into tenocytes by targeting the scleraxis gene. The Journal of Steroid Biochemistry and Molecular Biology *152*, 16-24.

Chen, X.-D., Dusevich, V., Feng, J.Q., Manolagas, S.C., and Jilka, R.L. (2007). Extracellular Matrix Made by Bone Marrow Cells Facilitates Expansion of Marrow-Derived Mesenchymal Progenitor Cells and Prevents Their Differentiation Into Osteoblasts. J Bone Miner Res *22*, 1943-1956.

Chen, X., Wei, S., Ji, Y., Guo, X., and Yang, F. (2015d). Quantitative proteomics using SILAC: Principles, applications, and developments. Proteomics *15*, 3175-3192.

Cheng, B., Ge, H., Zhou, J., and Zhang, Q. (2014a). TSG-6 mediates the effect of tendon derived stem cells for rotator cuff healing. European Review for Medical and Pharmacological Sciences *18*, 247-251.

Cheng, M.-T., Liu, C.-L., Chen, T.-H., and Lee, O.K. (2010). Comparison of Potentials Between Stem Cells Isolated from Human Anterior Cruciate Ligament and Bone Marrow for Ligament Tissue Engineering. Tissue engineering *16*, 2237-2253.

Cheng, M.-T., Liu, C.-L., Chen, T.-H., and Lee, O.K. (2014b). Optimization of Culture Conditions for Stem Cells Derived From Human Anterior Cruciate Ligament and Bone Marrow. Cell transplantation *23*, 791-803.

Cheng, M.T., Yang, H.W., Chen, T.H., and Lee, O.K.S. (2009). Isolation and characterization of multipotent stem cells from human cruciate ligaments. Cell Proliferation *42*, 448-460.

Chiquet-Ehrismann, R., Orend, G., Chiquet, M., Tucker, R.P., and Midwood, K.S. (2014). Tenascins in stem cell niches. Matrix Biology *37*, 112-123.

Chockalingam, P.S., Glasson, S.S., and Lohmander, L.S. (2013). Tenascin-C levels in synovial fluid are elevated after injury to the human and canine joint and correlate with markers of inflammation and matrix degradation. Osteoarthr Cartilage *21*, 339-345.

Choi, H.R., Cho, K.A., Kang, H.T., Lee, J.B., Kaeberlein, M., Suh, Y., Chung, I.K., and Park, S.C. (2011). Restoration of senescent human diploid fibroblasts by modulation of the extracellular matrix. Aging Cell *10*, 148-157.

Chua, K.-H., Zaman Wan Safwani, W.K., Hamid, A.A., Shuhup, S.K., Mohd Haflah, N.H., and Mohd Yahaya, N.H. (2014). Retropatellar fat pad–derived stem cells from older osteoarthritic patients have lesser differentiation capacity and expression of stemness genes. Cytotherapy *16*, 599-611.

Cianfarani, F., Tommasi, R., Failla, C.M., Viviano, M.T., Annessi, G., Papi, M., Zambruno, G., and Odorisio, T. (2006). Granulocyte/macrophage colony-stimulating factor treatment of human chronic ulcers promotes angiogenesis associated with de novo vascular endothelial growth factor transcription in the ulcer bed. British Journal of Dermatology *154*, 34-41.

Cimino, F., Volk, B.S., and Setter, D. (2010). Anterior Cruciate Ligament Injury: Diagnosis, Management, and Prevention. American Family Physician *82*, 917-922.

Clark, J.M., and Sidles, J.A. (1990). The interrelation of fiber bundles in the anterior cruciate ligament. Journal of Orthopaedic Research *8*, 180-188.

Claydon, A.J., and Beynon, R. (2012). Proteome Dynamics: Revisiting Turnover with a Global Perspective. Molecular & Cellular Proteomics : MCP *11*, 1551-1565.

Clegg, P.D., Strassburg, S., and Smith, R.K. (2007). Cell phenotypic variation in normal and damaged tendons. Int J Exp Pathol *88*, 227-235.

Co, C., Vickaryous, M.K., and Koch, T.G. (2014). Membrane culture and reduced oxygen tension enhances cartilage matrix formation from equine cord blood mesenchymal stromal cells in vitro. Osteoarthr Cartilage *22*, 472-480.

Coleman, B.D., Khan, K.M., Maffulli, N., Cook, J.L., and Wark, J.D. (2000). Studies of surgical outcome after patellar tendinopathy: clinical significance of methodological deficiencies and guidelines for future studies. Scandinavian Journal of Medicine & Science in Sports *10*, 2-11.

Collier, T.S., Sarkar, P., Franck, W.L., Rao, B.M., Dean, R.A., and Muddiman, D.C. (2010). Direct Comparison of Stable Isotope Labeling by Amino Acids in Cell Culture and Spectral Counting for Quantitative Proteomics. Analytical Chemistry *82*, 8696-8702.

Colter, D.C., Sekiya, I., and Prockop, D.J. (2001). Identification of a subpopulation of rapidly self-renewing and multipotential adult stem cells in colonies of human marrow stromal cells. Proceedings of the National Academy of Sciences of the United States of America *98*, 7841-7845.

Comerford, E.J., Smith, K., and Hayashi, K. (2011). Update on the aetiopathogenesis of canine cranial cruciate ligament disease. Veterinary and Comparative Orthopaedics and Traumatology (VCOT) *24*, 91-98.

Comerford, E.J., Tarlton, J.F., Innes, J.F., Johnson, K.A., Amis, A.A., and Bailey, A.J. (2005). Metabolism and composition of the canine anterior cruciate ligament relate to differences in knee joint mechanics and predisposition to ligament rupture. Journal of Orthopaedic Research *23*, 61-66.

Conze, P., van Schie, H.T.M., Weeren, R.v., Staszyk, C., Conrad, S., Skutella, T., Hopster, K., Rohn, K., Stadler, P., and Geburek, F. (2014). Effect of autologous adipose tissue-derived mesenchymal stem cells on neovascularization of artificial equine tendon lesions. Regenerative medicine *9*, 743-757.

Cook, J.L., Kuroki, K., Visco, D., Pelletier, J.P., Schulz, L., and Lafeber, F.P.J.G. (2010). The OARSI histopathology initiative – recommendations for histological assessments of osteoarthritis in the dog. Osteoarthr Cartilage *18, Supplement 3*, S66-S79.

Correa, P.M., Zuckermann, J., Fischer, G.B., and Castro, M.S. (2016). Immunosuppressive serum levels in allogeneic hematopoietic stem cell transplantation: pharmaceutical care contribution. Pharmacy Practice *14*, 683.

Corso, A., Mangiacavalli, S., Cocito, F., Pascutto, C., Ferretti, V.V., Pompa, A., Ciampichini, R., Pochintesta, L., and Mantovani, L.G. (2013). Long Term Evaluation of the Impact of Autologous Peripheral Blood Stem Cell Transplantation in Multiple Myeloma: A Cost-Effectiveness Analysis. PLoS ONE *8*, e75047.

Creemers, L.B., Hoeben, K.A., Jansen, D.C., Buttle, D.J., Beertsen, W., and Everts, V. (1998). Participation of intracellular cysteine proteinases, in particular cathepsin B, in degradation of collagen in periosteal tissue explants. Matrix Biology *16*, 575-584.

Crockett, R., Centrella, M., McCarthy, T., and Grant Thomson, J. (2010). Effects of cyclic strain on rat tail tenocytes. Mol Biol Rep *37*, 2629-2634.

Csaki, C., Matis, U., Mobasheri, A., Ye, H., and Shakibaei, M. (2007). Chondrogenesis, osteogenesis and adipogenesis of canine mesenchymal stem cells: a biochemical, morphological and ultrastructural study. Histochemistry and cell biology *128*, 507-520.

Dai, L., Hu, X., Zhang, X., Zhu, J., Zhang, J., Fu, X., Duan, X., Ao, Y., and Zhou, C. (2015). Different tenogenic differentiation capacities of different mesenchymal stem cells in the presence of BMP-12. Journal of Translational Medicine *13*, 200.

Dapeng, L., Xiaojie, L., Ping, G., Yan, D., and Gang, S. (2014). Erk1/2 signalling is involved in the differentiation of periodontal ligament stem cells to Schwann cells in dog. Archives of Oral Biology *59*, 487-491.

Dawson, A.G., Hutchison, J.D., and Sutherland, A.G. (2016). Is Anterior Cruciate Reconstruction Superior to Conservative Treatment? Journal of Knee Surgery *29*, 74-79.

de Hoog, C.L., and Mann, M. (2004). PROTEOMICS. Annual Review of Genomics and Human Genetics *5*, 267-293.

De Schauwer, C., Meyer, E., Van de Walle, G.R., and Van Soom, A. (2011). Markers of stemness in equine mesenchymal stem cells: a plea for uniformity. Theriogenology *75*, 1431-1443.

de Windt, T.S., Vonk, L.A., Slaper-Cortenbach, I.C.M., Nizak, R., van Rijen, M.H.P., and Saris, D.B.F. (2017). Allogeneic MSCs and Recycled Autologous Chondrons Mixed in a One-Stage Cartilage Cell Transplantion: A First-in-Man Trial in 35 Patients. Stem cells *35*, 1984-1993.

Docheva, D., Hunziker, E.B., Fässler, R., and Brandau, O. (2005). Tenomodulin Is Necessary for Tenocyte Proliferation and Tendon Maturation. Molecular and cellular biology *25*, 699-705.

Doherty, M.K., Hammond, D.E., Clague, M.J., Gaskell, S.J., and Beynon, R.J. (2009). Turnover of the Human Proteome: Determination of Protein Intracellular Stability by Dynamic SILAC. Journal of Proteome Research *8*, 104-112.

Doherty, M.K., Whitehead, C., McCormack, H., Gaskell, S.J., and Beynon, R.J. (2005). Proteome dynamics in complex organisms: Using stable isotopes to monitor individual protein turnover rates. PROTEOMICS *5*, 522-533.

Dominici, M., Le Blanc, K., Mueller, I., Slaper-Cortenbach, I., Marini, F., Krause, D., Deans, R., Keating, A., Prockop, D., and Horwitz, E. (2006). Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy *8*, 315-317.

Dowthwaite, G.P., Bishop, J.C., Redman, S.N., Khan, I.M., Rooney, P., Evans, D.J.R., Haughton, L., Bayram, Z., Boyer, S., Thomson, B*., et al.* (2004). The surface of articular cartilage contains a progenitor cell population. Journal of Cellular Science *117*, 889-897.

Duerr, F.M., Martin, K.W., Rishniw, M., Palmer, R.H., and Selmic, L.E. (2014). Treatment of canine cranial cruciate ligament disease. A survey of ACVS Diplomates and primary care veterinarians. Veterinary and Comparative Orthopaedics and Traumatology (VCOT) *27*, 478-483.

Durgam, S., Schuster, B., Cymerman, A., Stewart, A., and Stewart, M. (2016a). Differential Adhesion Selection for Enrichment of Tendon-Derived Progenitor Cells During In Vitro Culture. Tissue Engineering Part C: Methods *22*, 801-808.

Durgam, S.S., Stewart, A.A., Sivaguru, M., Wagoner Johnson, A.J., and Stewart, M.C. (2016b). Tendon-derived progenitor cells improve healing of collagenase-induced flexor tendinitis. Journal of Orthopaedic Research *34*, 2162-2171.

Eleuterio, E., Trubiani, O., Sulpizio, M., Di Giuseppe, F., Pierdomenico, L., Marchisio, M., Giancola, R., Giammaria, G., Miscia, S., Caputi, S*., et al.* (2013). Proteome of human stem cells from periodontal ligament and dental pulp. PLoS One *8*, e71101.

Ely, E.R., Avella, C.S., Price, J.S., Smith, R.K.W., Wood, J.L.N., and Verheyen, K.L.P. (2009). Descriptive epidemiology of fracture, tendon and suspensory ligament injuries in National Hunt racehorses in training. Equine veterinary journal *41*, 372-378.

Eslaminejad, M.B., Nikmahzar, A., Taghiyar, L., Nadri, S., and Massumi, M. (2006). Murine mesenchymal stem cells isolated by low density primary culture system. Development, Growth & Differentiation *48*, 361-370.

Estany, S., Vicens-Zygmunt, V., Llatjós, R., Montes, A., Penín, R., Escobar, I., Xaubet, A., Santos, S., Manresa, F., Dorca, J*., et al.* (2014). Lung fibrotic tenascin-C upregulation is associated with other extracellular matrix proteins and induced by TGFβ1. BMC Pulmonary Medicine *14*, 120-120.

Farndale, R.W., Buttle, D.J., and Barrett, A.J. (1986). Improved quantitation and discrimination of sulphated glycosaminoglycans by use of dimethylmethylene blue. Biochimica et Biophysica Acta (BBA) - General Subjects *883*, 173-177.

Farrar, C.A., Kupiec-Weglinski, J.W., and Sacks, S.H. (2013). The Innate Immune System and Transplantation. Cold Spring Harbor Perspectives in Medicine *3*, a015479.

Figueroa, D., Espinosa, M., Calvo, R., Scheu, M., Vaisman, A., Gallegos, M., and Conget, P. (2014). Anterior cruciate ligament regeneration using mesenchymal stem cells and collagen type I scaffold in a rabbit model. Knee Surg, Sports Traumatol, Arthrosc *22*, 1196-1202.

Foudi, A., Hochedlinger, K., Van Buren, D., Schindler, J.W., Jaenisch, R., Carey, V., and Hock, H. (2009). Defining Hematopoietic Stem and Progenitor Cell Turnover by Analysis of Histone 2B-GFP Dilution. Nature biotechnology *27*, 84-90.

Franchi, M., Trire, A., Quaranta, M., Orsini, E., and Ottani, V. (2007). Collagen structure of tendon relates to function. TheScientificWorldJournal *7*, 404-420.

Francois, D., Venisse, L., Marchal-Somme, J., Jandrot-Perrus, M., Crestani, B., Arocas, V., and Bouton, M.-C. (2014). Increased expression of protease nexin-1 in fibroblasts during idiopathic pulmonary fibrosis regulates thrombin activity and fibronectin expression. Lab Invest *94*, 1237-1246.

Frank, C.B. (2004). Ligament structure, physiology and function. Journal of musculoskeletal & neuronal interactions *4*, 199-201.

Fu, W., Li, Q., Tang, X., Chen, G., Zhang, C., and Li, J. (2015). Mesenchymal stem cells reside in anterior cruciate ligament remnants in situ. International orthopaedics *40*, 1523-1530.

Fujii, S., Maeda, H., Wada, N., Tomokiyo, A., Saito, M., and Akamine, A. (2008). Investigating a clonal human periodontal ligament progenitor/stem cell line in vitro and in vivo. Journal of Cellular Physiology *215*, 743-749.

Fullár, A., Firneisz, G., Regős, E., Dudás, J., Szarvas, T., Baghy, K., Ramadori, G., and Kovalszky, I. (2016). Response of Hepatic Stellate Cells to TGFB1 Differs from the Response of Myofibroblasts. Decorin Protects against the Action of Growth Factor. Pathology & Oncology Research, 1-8.

Gao, H., Li, B., Zhao, L., and Jin, Y. (2015). Influence of nanotopography on periodontal ligament stem cell functions and cell sheet based periodontal regeneration. International Journal of Nanomedicine *10*, 4009-4027.

Garcion, E., Faissner, A., and ffrench-Constant, C. (2001). Knockout mice reveal a contribution of the extracellular matrix molecule tenascin-C to neural precursor proliferation and migration. Development *128*, 2485-2496.

Garcion, E., Halilagic, A., Faissner, A., and French-Constant, C. (2004). Generation of an environmental niche for neural stem cell development by the extracellular matrix molecule tenascin C. Development *131*, 3423-3432.

Garwood, J., Garcion, E., Dobbertin, A., Heck, N., Calco, V., Ffrench-Constant, C., and Faissner, A. (2004). The extracellular matrix glycoprotein Tenascin-C is expressed by oligodendrocyte precursor cells and required for the regulation of maturation rate, survival and responsiveness to platelet-derived growth factor. European Journal of Neuroscience *20*, 2524-2540.

Gattazzo, F., Urciuolo, A., and Bonaldo, P. (2014). Extracellular matrix: A dynamic microenvironment for stem cell niche. Biochimica et biophysica acta.

Gay, I.C., Chen, S., and MacDougall, M. (2007). Isolation and characterization of multipotent human periodontal ligament stem cells. Orthodontics & Craniofacial Research *10*, 149-160.

Gianotti, S.M., Marshall, S.W., Hume, P.A., and Bunt, L. (2009). Incidence of anterior cruciate ligament injury and other knee ligament injuries: A national population-based study. Journal of Science and Medicine in Sport *12*, 622-627.

Goldin, M., and Malanga, G.A. (2013). Tendinopathy: a review of the pathophysiology and evidence for treatment. Phys Sportsmed *41*, 36-49.

Goseki, T., Shimizu, N., Iwasawa, T., Takiguchi, H., and Abiko, Y. (1996). Effects of in vitro cellular aging on alkaline phosphatase, cathepsin activities and collagen secretion of human periodontal ligament derived cells. Mechanisms of Ageing and Development *91*, 171-183.

Grau, S., Richards, P.J., Kerr, B., Hughes, C., Caterson, B., Williams, A.S., Junker, U., Jones, S.A., Clausen, T., and Ehrmann, M. (2006). The Role of Human HtrA1 in Arthritic Disease. Journal of Biological Chemistry *281*, 6124-6129.

Greenbaum, D., Colangelo, C., Williams, K., and Gerstein, M. (2003). Comparing protein abundance and mRNA expression levels on a genomic scale. Genome Biology *4*, 117-117.

Grogan, S.P., Miyaki, S., Asahara, H., D'Lima, D.D., and Lotz, M.K. (2009). Mesenchymal progenitor cell markers in human articular cartilage: normal distribution and changes in osteoarthritis. Arthritis research & therapy *11*, R85-R85.

Güngörmüş, C., and Kolankaya, D. (2008). Characterization of type I, III and V collagens in high-density cultured tenocytes by triple-immunofluorescence technique. Cytotechnology *58*, 145-152.

Guo, J., Chan, K.-M., Zhang, J.-F., and Li, G. (2016). Tendon-derived stem cells undergo spontaneous tenogenic differentiation. Experimental Cell Research *341*, 1-7.

Guo, M., Mathieu, P.A., Linebaugh, B., Sloane, B.F., and Reiners, J.J. (2002). Phorbol Ester Activation of a Proteolytic Cascade Capable of Activating Latent Transforming Growth Factor-β: A PROCESS INITIATED BY THE EXOCYTOSIS OF CATHEPSIN B. Journal of Biological Chemistry *277*, 14829-14837.

Haddad-Weber, M., Prager, P., Kunz, M., Seefried, L., Jakob, F., Murray, M.M., Evans, C.H., NÖTh, U., and Steinert, A.F. (2010). BMP12 and BMP13 gene transfer induce ligamentogenic differentiation in mesenchymal progenitor and anterior cruciate ligament cells. Cytotherapy *12*, 505-513.

Hakki, S.S., Kayis, S.A., Hakki, E.E., Bozkurt, S.B., Duruksu, G., Unal, Z.S., Turaç, G., and Karaoz, E. (2014). Comparison of MSCs Isolated From Pulp and Periodontal Ligament. Journal of Periodontology *86*, 283-291.

Harumiya, S., Gibson, M.A., and Koshihara, Y. (2002). Antisense Suppression of Collagen VI Synthesis Results in Reduced Expression of Collagen I in Normal Human Osteoblast-like Cells. Bioscience, Biotechnology, and Biochemistry *66*, 2743-2747.

Hasegawa, A., Nakahara, H., Kinoshita, M., Asahara, H., Koziol, J., and Lotz, M.K. (2013). Cellular and extracellular matrix changes in anterior cruciate ligaments during human knee aging and osteoarthritis. Arthritis research & therapy *15*, R29-R29.

Hattori, Y., Furuita, K., Ohki, I., Ikegami, T., Fukada, H., Shirakawa, M., Fujiwara, T., and Kojima, C. (2013). Utilization of lysine 13C-methylation NMR for protein–protein interaction studies. Journal of Biomolecular NMR *55*, 19-31.

Heckmann, M., Aumailley, M., Chu, M.-L., Timpl, R., and Krieg, T. (1992). Effect of transforming growth factors-β on collagen type VI expression in human dermal fibroblasts. FEBS Letters *310*, 79-82.

Heinemeier, K.M., Schjerling, P., Heinemeier, J., Magnusson, S.P., and Kjaer, M. (2013). Lack of tissue renewal in human adult Achilles tendon is revealed by nuclear bomb 14C. The FASEB Journal *27*, 2074-2079.

Hill, C.L., Seo, G.S., Gale, D., Totterman, S., Gale, M.E., and Felson, D.T. (2005). Cruciate ligament integrity in osteoarthritis of the knee. Arthritis & Rheumatism *52*, 794-799.

Hillmann, A., Ahrberg, A.B., Brehm, W., Heller, S., Josten, C., Paebst, F., and Burk, J. (2015). Comparative Characterization of Human and Equine Mesenchymal Stromal Cells: A Basis for Translational Studies in the Equine Model. Cell transplantation *25*, 109-124.

Hoedt, E., Zhang, G., and Neubert, T.A. (2014). Stable Isotope Labeling by Amino Acids in Cell Culture (SILAC) for Quantitative Proteomics. In Advancements of Mass Spectrometry in Biomedical Research, pp. 93-106.

Huang, T.F., Chen, Y.T., Yang, T.H., Chen, L.L., Chiou, S.H., Tsai, T.H., Tsai, C.C., Chen, M.H., Ma, H.L., and Hung, S.C. (2008). Isolation and characterization of mesenchymal stromal cells from human anterior cruciate ligament. Cytotherapy *10*, 806-814.

Ilic, Mirna Z., Carter, P., Tyndall, A., Dudhia, J., and Handley, Christopher J. (2005). Proteoglycans and catabolic products of proteoglycans present in ligament. Biochemical Journal *385*, 381-388.

Innes, J.F., and Clegg, P. (2010). Comparative rheumatology: what can be learnt from naturally occurring musculoskeletal disorders in domestic animals? Rheumatology *49*, 1030-1039.

Ireland, D., Harrall, R., Curry, V., Holloway, G., Hackney, R., Hazleman, B., and Riley, G. (2001). Multiple changes in gene expression in chronic human Achilles tendinopathy. Matrix Biology *20*, 159-169.

Ishida, Y., Kubota, H., Yamamoto, A., Kitamura, A., Bächinger, H.P., and Nagata, K. (2006). Type I Collagen in Hsp47-null Cells Is Aggregated in Endoplasmic Reticulum and Deficient in N-Propeptide Processing and Fibrillogenesis. Molecular Biology of the Cell *17*, 2346-2355.

Islam, M.S., Kusakabe, M., Horiguchi, K., Iino, S., Nakamura, T., Iwanaga, K., Hashimoto, H., Matsumoto, S., Murata, T., Hori, M.*, et al.* (2014). PDGF and TGF-β promote tenascin-C expression in subepithelial myofibroblasts and contribute to intestinal mucosal protection in mice. British Journal of Pharmacology *171*, 375-388.

Ito, S., and Nagata, K. (2016). Biology of Hsp47 (Serpin H1), a collagen-specific molecular chaperone. Seminars in Cell & Developmental Biology.

Jaiswal, N., Haynesworth, S.E., Caplan, A.I., and Bruder, S.P. (1997). Osteogenic differentiation of purified, culture-expanded human mesenchymal stem cells in vitro. Journal of cellular biochemistry *64*, 295-312.

Jelinsky, S.A., Archambault, J., Li, L., and Seeherman, H. (2010). Tendon-selective genes identified from rat and human musculoskeletal tissues. Journal of Orthopaedic Research *28*, 289-297.

Jiang, D., Jiang, Z., Zhang, Y., Wang, S., Yang, S., Xu, B., Yang, M., and Li, Z. (2014). Effect of Young Extrinsic Environment Stimulated by Hypoxia on the Function of Aged Tendon Stem Cell. Cell Biochem Biophys *70*, 967-973.

Jiang, D., Xu, B., Yang, M., Zhao, Z., Zhang, Y., and Li, Z. (2013). Efficacy of tendon stem cells in fibroblast-derived matrix for tendon tissue engineering. Cytotherapy *16*, 662-673.

Jiang, D., Yang, S., Gao, P., Zhang, Y., Guo, T., Lin, H., and Geng, H. (2015). Combined effect of ligament stem cells and umbilical-cord-blood-derived CD34+ cells on ligament healing. Cell and tissue research *362*, 587-595.

Jiang, J., Huang, L., Yu, W., Wu, X., Zhou, P., and Li, X. (2012). Overexpression of HTRA1 Leads to Down-Regulation of Fibronectin and Functional Changes in RF/6A Cells and HUVECs. PLoS ONE *7*, e46115.

Jones, A., Kumar, S., Zhang, N., Tong, Z., Yang, J.-H., Watt, C., Anderson, J., Amrita, Fillerup, H., McCloskey, M.*, et al.* (2011). Increased expression of multifunctional serine protease, HTRA1, in retinal pigment epithelium induces polypoidal choroidal vasculopathy in mice. Proceedings of the National Academy of Sciences of the United States of America *108*, 14578-14583.

Jones, G.C., Corps, A.N., Pennington, C.J., Clark, I.M., Edwards, D.R., Bradley, M.M., Hazleman, B.L., and Riley, G.P. (2006). Expression profiling of metalloproteinases and tissue inhibitors of metalloproteinases in normal and degenerate human achilles tendon. Arthritis & Rheumatism *54*, 832-842.

Kajikawa, Y., Morihara, T., Watanabe, N., Sakamoto, H., Matsuda, K.-i., Kobayashi, M., Oshima, Y., Yoshida, A., Kawata, M., and Kubo, T. (2007). GFP chimeric models exhibited a biphasic pattern of mesenchymal cell invasion in tendon healing. Journal of Cellular Physiology *210*, 684-691.

Kanaya, A., Deie, M., Adachi, N., Nishimori, M., Yanada, S., and Ochi, M. (2007). Intra-articular Injection of Mesenchymal Stromal Cells in Partially Torn Anterior Cruciate Ligaments in a Rat Model. Arthroscopy: The Journal of Arthroscopic & Related Surgery *23*, 610-617.

Kannus, P. (2000). Structure of the tendon connective tissue. Scandinavian Journal of Medicine & Science in Sports *10*, 312-320.

Kasashima, Y., Takahashi, T., Smith, R.K.W., Goodship, A.E., Kuwano, A., Ueno, T., and Hirano, S. (2004). Prevalence of superficial digital flexor tendonitis and suspensory desmitis in Japanese Thoroughbred flat racehorses in 1999. Equine veterinary journal *36*, 346-350.

Katsara, O., Mahaira, L.G., Iliopoulou, E.G., Moustaki, A., Antsaklis, A., Loutradis, D., Stefanidis, K., Baxevanis, C.N., Papamichail, M., and Perez, S.A. (2011). Effects of Donor Age, Gender, and In Vitro Cellular Aging on the Phenotypic, Functional, and Molecular Characteristics of Mouse Bone Marrow-Derived Mesenchymal Stem Cells. Stem cells and development *20*, 1549-1561.

Kawasaki, T., Sumita, Y., Egashira, K., Ohba, S., Kagami, H., Tran, S.D., and Asahina, I. (2015). Transient Exposure to Hypoxic and Anoxic Oxygen Concentrations Promotes Either Osteogenic or Ligamentogenic Characteristics of PDL Cells. BioResearch Open Access *4*, 175-187.

Kerever, A., Schnack, J., Vellinga, D., Ichikawa, N., Moon, C., Arikawa-Hirasawa, E., Efird, J.T., and Mercier, F. (2007). Novel extracellular matrix structures in the neural stem cell niche capture the neurogenic factor fibroblast growth factor 2 from the extracellular milieu. Stem cells *25*, 2146-2157.

Kharaz, Y.A., Tew, S.R., Peffers, M., Canty-Laird, E.G., and Comerford, E. (2016). Proteomic differences between native and tissue-engineered tendon and ligament. PROTEOMICS *16*, 1547-1556.

Kilian, O., Dahse, R., Alt, V., Zardi, L., Hentschel, J., Schnettler, R., and Kosmehl, H. (2008). mRNA Expression and Protein Distribution of Fibronectin Splice Variants and High-Molecular Weight Tenascin-C in Different Phases of Human Fracture Healing. Calcif Tissue Int *83*, 101-111.

Kim, S.S., Kwon, D.W., Im, I., Kim, Y.D., Hwang, D.S., Holliday, L.S., Donatelli, R.E., Son, W.S., and Jun, E.S. (2012). Differentiation and characteristics of undifferentiated mesenchymal stem cells originating from adult premolar periodontal ligaments. Korean journal of orthodontics *42*, 307-317.

Kjaer, M. (2004). Role of Extracellular Matrix in Adaptation of Tendon and Skeletal Muscle to Mechanical Loading. Physiological Reviews *84*, 649-698.

Klein, M.B., Yalamanchi, N., Pham, H., Longaker, M.T., and Chan, J. (2002). Flexor tendon healing in vitro: Effects of TGF-β on tendon cell collagen production. The Journal of hand surgery *27*, 615-620.

Kohler, J., Popov, C., Klotz, B., Alberton, P., Prall, W.C., Haasters, F., Müller-Deubert, S., Ebert, R., Klein-Hitpass, L., Jakob, F.*, et al.* (2013). Uncovering the cellular and molecular changes in tendon stem/progenitor cells attributed to tendon aging and degeneration. Aging Cell *12*, 988-999.

Kowalski, T.J., Leong, N.L., Dar, A., Wu, L., Kabir, N., Khan, A.Z., Eliasberg, C.D., Pedron, A., Karayan, A., Lee, S.*, et al.* (2015). Hypoxic culture conditions induce increased metabolic rate and collagen gene expression in ACL-derived cells. Journal of Orthopaedic Research *34*, 985-994.

Kraus-Hansen, A.E., Fackelman, G.E., Becker, C., Williams, R.M., and Pipers, F.S. (1992). Preliminary studies on the vascular anatomy of the equine superficial digital flexor tendon. Equine veterinary journal *24*, 46-51.

Kraus, A., Woon, C., Raghavan, S., Megerle, K., Pham, H., and Chang, J. (2013). Co-culture of human adipose-derived stem cells with tenocytes increases proliferation and induces differentiation into a tenogenic lineage. Plastic and reconstructive surgery *132*, 754e-766e.

Kristjánsson, B., Limthongkul, W., Yingsakmongkol, W., Thantiworasit, P., Jirathanathornnukul, N., and Honsawek, S. (2016). Isolation and Characterization of Human Mesenchymal Stem Cells From Facet Joints and Interspinous Ligaments. Spine *41*, E1-7.

Lane, S.W., Williams, D.A., and Watt, F.M. (2014). Modulating the stem cell niche for tissue regeneration. Nature Biotechnology *32*, 795-803.

Langberg, H., Skovgaard, D., Petersen, L.J., Bülow, J., and Kjær, M. (1999). Type I collagen synthesis and degradation in peritendinous tissue after exercise determined by microdialysis in humans. The Journal of Physiology *521*, 299-306.

Lee, C.H., Lee, F.Y., Tarafder, S., Kao, K., Jun, Y., Yang, G., and Mao, J.J. (2015a). Harnessing endogenous stem/progenitor cells for tendon regeneration. The Journal of Clinical Investigation *125*, 2690-2701.

Lee, D.-H., Ng, J., Kim, S.-B., Sonn, C.H., Lee, K.-M., and Han, S.-B. (2015b). Effect of Donor Age on the Proportion of Mesenchymal Stem Cells Derived from Anterior Cruciate Ligaments. PLoS ONE *10*, e0117224.

Lee, I.C., Wang, J.-H., Lee, Y.-T., and Young, T.-H. (2007). The differentiation of mesenchymal stem cells by mechanical stress or/and co-culture system. Biochemical and biophysical research communications *352*, 147-152.

Lee, J.S., An, S.Y., Kwon, I.K., and Heo, J.S. (2014a). Transdifferentiation of human periodontal ligament stem cells into pancreatic cell lineage. Cell Biochemistry and Function *32*, 605-611.

Lee, J.S., Kim, H.S., Park, S.Y., Kim, T.W., Jung, J.S., Lee, J.B., and Kim, C.S. (2014b). Synergistic effects of a calcium phosphate/fibronectin coating on the adhesion of periodontal ligament stem cells onto decellularized dental root surfaces. Cell transplantation *24*, 1767-1779.

Lee, J.S., Yi, J.K., An, S.Y., and Heo, J.S. (2014c). Increased Osteogenic Differentiation of Periodontal Ligament Stem Cells on Polydopamine Film Occurs via Activation of Integrin and PI3K Signaling Pathways. Cell Physio Biochem *34*, 1824-1834.

Lee, W.Y., Lui, P.P., and Rui, Y.F. (2012). Hypoxia-mediated efficient expansion of human tendon-derived stem cells in vitro. Tissue engineering Part A *18*, 484-498.

Lei, J., Hui, D., Huang, W., Liao, Y., Yang, L., Liu, L., Zhang, Q., Qi, G., Song, W., Zhang, Y.*, et al.* (2013). Heterogeneity of the biological properties and gene expression profiles of murine bone marrow stromal cells. The International Journal of Biochemistry & Cell Biology *45*, 2431-2443.

Lejard, V., Blais, F., Guerquin, M.-J., Bonnet, A., Bonnin, M.-A., Havis, E., Malbouyres, M., Bidaud, C.B., Maro, G., Gilardi-Hebenstreit, P.*, et al.* (2011). EGR1 and EGR2 Involvement in Vertebrate Tendon Differentiation. Journal of Biological Chemistry *286*, 5855-5867.

Lempainen, L., Johansson, K., Banke, I.J., Ranne, J., Mäkelä, K., Sarimo, J., Niemi, P., and Orava, S. (2015). Expert opinion: diagnosis and treatment of proximal hamstring tendinopathy. Muscles, ligaments and tendons journal *5*, 23-28.

Lengner, C.J., Welstead, G.G., and Jaenisch, R. (2008). The pluripotency regulator Oct4: A role in somatic stem cells? Cell Cycle *7*, 725-728.

Li, R., and Zhang, Q. (2015). HtrA1 may regulate the osteogenic differentiation of human periodontal ligament cells by TGF-β1. Journal of Molecular Histology *46*, 137-144.

Li, X., Gong, P., and Liao, D. (2010). In vitro neural/glial differentiation potential of periodontal ligament stem cells. Archives of Medical Science : AMS *6*, 678-685.

Li, X., Wu, Z., Ni, J., Liu, Y., Meng, J., Yu, W., Nakanishi, H., and Zhou, Y. (2016a). Cathepsin B Regulates Collagen Expression by Fibroblasts via Prolonging TLR2/NF-κB Activation. Oxidative Medicine and Cellular Longevity *2016*, 7894247.

Li, X., Zhao, D., Guo, Z., Li, T., Qili, M., Xu, B., Qian, M., Liang, H., E, X., Chege Gitau, S.*, et al.* (2016b). Overexpression of SerpinE2/protease nexin-1 Contribute to Pathological Cardiac Fibrosis via increasing Collagen Deposition. Scientific Reports *6*, 37635.

Li, Z.Y., Wang, C.Q., Lu, G., Pan, X.Y., and Xu, K.L. (2014). Effects of Bone Marrow Mesenchymal Stem Cells on Hematopoietic Recovery and Acute Graft-Versus-Host Disease in Murine Allogeneic Umbilical Cord Blood Transplantation Model. Cell Biochem Biophys *70*, 115-122.

Lichtwark, G.A., and Wilson, A.M. (2005). In vivo mechanical properties of the human Achilles tendon during one-legged hopping. Journal of Experimental Biology *208*, 4715-4725.

Linon, E., Spreng, D., Rytz, U., and Forterre, S. (2014). Engraftment of autologous bone marrow cells into the injured cranial cruciate ligament in dogs. The Veterinary Journal *202*, 448-454.

Little, D., Thompson, J.W., Dubois, L.G., Ruch, D.S., Moseley, M.A., and Guilak, F. (2014). Proteomic differences between male and female anterior cruciate ligament and patellar tendon. PLoS One *9*, e96526.

Liu, J., Chen, L., Zhou, Y., Liu, X., and Tang, K. (2014a). Insulin-Like Growth Factor-1 and Bone Morphogenetic Protein-2 Jointly Mediate Prostaglandin E2-Induced Adipogenic Differentiation of Rat Tendon Stem Cells. PLoS ONE *9*, e85469.

Liu, J., Tao, X., Chen, L., Han, W., Zhou, Y., and Tang, K. (2015a). CTGF Positively Regulates BMP12 Induced Tenogenic Differentiation of Tendon Stem Cells and Signaling. Cellular Physiology and Biochemistry *35*, 1831-1845.

Liu, J., Wang, L., Liu, W., Li, Q., Jin, Z., and Jin, Y. (2014b). Dental Follicle Cells Rescue the Regenerative Capacity of Periodontal Ligament Stem Cells in an Inflammatory Microenvironment. PLoS ONE *9*, e108752.

Liu, N.Q., Dekker, L.J.M., Stingl, C., Güzel, C., De Marchi, T., Martens, J.W.M., Foekens, J.A., Luider, T.M., and Umar, A. (2013). Quantitative Proteomic Analysis of Microdissected Breast Cancer Tissues: Comparison of Label-Free and SILAC-based Quantification with Shotgun, Directed, and Targeted MS Approaches. Journal of Proteome Research *12*, 4627-4641.

Liu, W., Watson, S.S., Lan, Y., Keene, D.R., Ovitt, C.E., Liu, H., Schweitzer, R., and Jiang, R. (2010). The atypical homeodomain transcription factor Mohawk controls tendon morphogenesis. Molecular and cellular biology *30*, 4797-4807.

Liu, X., Chen, W., Zhou, Y., Tang, K., and Zhang, J. (2015b). Mechanical Tension Promotes the Osteogenic Differentiation of Rat Tendon-derived Stem Cells Through the Wnt5a/Wnt5b/JNK Signaling Pathway. Cellular Physiology and Biochemistry *36*, 517-530.

Liu, X., Wu, H., Byrne, M., Krane, S., and Jaenisch, R. (1997). Type III collagen is crucial for collagen I fibrillogenesis and for normal cardiovascular development. Proceedings of the National Academy of Sciences of the United States of America *94*, 1852-1856.

Liu, Y., Zheng, Y., Ding, G., Fang, D., Zhang, C., Bartold, P.M., Gronthos, S., Shi, S., and Wang, S. (2008). Periodontal Ligament Stem Cell-Mediated Treatment for Periodontitis in Miniature Swine. Stem cells *26*, 1065-1073.

Livak, K.J., and Schmittgen, T.D. (2001). Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2−ΔΔCT Method. Methods *25*, 402-408.

Lo, I.K.Y., Ou, Y., Rattner, J.-P., Hart, D.A., Marchuk, L.L., Frank, C.B., and Rattner, J.B. (2002). The cellular networks of normal ovine medial collateral and anterior cruciate ligaments are not accurately recapitulated in scar tissue. Journal of anatomy *200*, 283-296.

Loeser, R.F. (2009). Aging and Osteoarthritis: The Role of Chondrocyte Senescence and Aging Changes in the Cartilage Matrix. Osteoarthr Cartilage *17*, 971-979.

Lohberger, B., Kaltenegger, H., Stuendl, N., Rinner, B., Leithner, A., and Sadoghi, P. (2015). Impact of cyclic mechanical stimulation on the expression of extracellular matrix proteins in human primary rotator cuff fibroblasts. Knee Surgery, Sports Traumatology, Arthroscopy, 1-8.

Lovati, A., Corradetti, B., Lange Consiglio, A., Recordati, C., Bonacina, E., Bizzaro, D., and Cremonesi, F. (2011). Characterisation and Differentiation of equine tendon-derived progenitor cells. Journal of Biological Regulators and Homeostatic Agents *25*, 75-84.

Lovati, A.B., Corradetti, B., Cremonesi, F., Bizzaro, D., and Consiglio, A.L. (2012). Tenogenic differentiation of equine mesenchymal progenitor cells under indirect co-culture. The International journal of artificial organs *35*, 996-1005.

Lui, P.P. (2013). Identity of tendon stem cells – how much do we know? J Cell Mol Med *17*, 55-64.

Lui, P.P., and Wong, Y. (2013). Higher BMP/Smad sensitivity of tendon-derived stem cells (TDSCs) isolated from the collagenase-induced tendon injury model: possible mechanism for their altered fate in vitro. BMC musculoskeletal disorders *14*, 248-256.

Lui, P.P.Y., Maffulli, N., Rolf, C., and Smith, R.K.W. (2011). What are the validated animal models for tendinopathy? Scandinavian Journal of Medicine & Science in Sports *21*, 3-17.

Lui, P.P.Y., Wong, O.T., and Lee, Y.W. (2014). Application of Tendon-Derived Stem Cell Sheet for the Promotion of Graft Healing in Anterior Cruciate Ligament Reconstruction. The American Journal of Sports Medicine *42*, 681-689.

Lui, P.P.Y., Wong, O.T., and Lee, Y.W. (2016). Transplantation of tendon-derived stem cells pre-treated with connective tissue growth factor and ascorbic acid in vitro promoted better tendon repair in a patellar tendon window injury rat model. Cytotherapy *18*, 99-112.

Luo, Q., Song, G., Song, Y., Xu, B., Qin, J., and Shi, Y. (2009). Indirect co-culture with tenocytes promotes proliferation and mRNA expression of tendon/ligament related genes in rat bone marrow mesenchymal stem cells. Cytotechnology *61*, 1-10.

Luo, Y., Zeng, H.-Q., Shen, Y., Zhang, P., Lou, S.-F., Chen, L., and Deng, J.-C. (2016). Allogeneic hematopoietic stem cell transplantation following donor CIK cell infusion: A phase I study in patients with relapsed/refractory hematologic malignancies. Leukemia Research *48*, 6-10.

Lupatov, A.Y., Vdovin, A.S., Vakhrushev, I.V., Poltavtseva, R.A., and Yarygin, K.N. (2015). Comparative Analysis of the Expression of Surface Markers on Fibroblasts and Fibroblast-Like Cells Isolated from Different Human Tissues. Bull Exp Biol Med *158*, 537-543.

Maffulli, N., Wong, J., and Almekinders, L.C. (2003). Types and epidemiology of tendinopathy. Clinical Sports Medicine *22*, 675-692.

Malara, A., Currao, M., Gruppi, C., Celesti, G., Viarengo, G., Buracchi, C., Laghi, L., Kaplan, D.L., and Balduini, A. (2014). Megakaryocytes contribute to the bone marrow-matrix environment by expressing fibronectin, type IV collagen and laminin. Stem cells *32*, 926-937.

Manescu, A., Giuliani, A., Mohammadi, S., Tromba, G., Mazzoni, S., Diomede, F., Zini, N., Piattelli, A., and Trubiani, O. (2015). Osteogenic potential of dualblocks cultured with human periodontal ligament stem cells: in vitro and synchrotron microtomography study. Journal of Periodontal Research *51*, 112-124.

Matheson, S., Larjava, H., and Häkkinen, L. (2005). Distinctive localization and function for lumican, fibromodulin and decorin to regulate collagen fibril organization in periodontal tissues. Journal of Periodontal Research *40*, 312-324.

Matsumoto, T., Ingham, S.M., Mifune, Y., Osawa, A., Logar, A., Usas, A., Kuroda, R., Kurosaka, M., Fu, F.H., and Huard, J. (2012). Isolation and characterization of human anterior cruciate ligament-derived vascular stem cells. Stem cells and development *21*, 859-872.

Matthiesen, R., and Jensen, O.N. (2008). Analysis of Mass Spectrometry Data in Proteomics. In Bioinformatics: Structure, Function and Applications, pp. 105-122.

Mayor, R.B. (2012). Treatment of athletic tendonopathy. Conn Med *76*, 471-475.

Mazzieri, R., Masiero, L., Zanetta, L., Monea, S., Onisto, M., Garbisa, S., and Mignatti, P. (1997). Control of type IV collagenase activity by components of the urokinase-plasmin

system: a regulatory mechanism with cell-bound reactants. The EMBO Journal *16*, 2319-2332.

McDermott, B.T., Ellis, S., Bou-Gharios, G., Clegg, P.D., and Tew, S.R. (2016). RNA binding proteins regulate anabolic and catabolic gene expression in chondrocytes. Osteoarthr Cartilage *24*, 1263-1273.

Mendias, C.L., Gumucio, J.P., Bakhurin, K.I., Lynch, E.B., and Brooks, S.V. (2012). Physiological loading of tendons induces scleraxis expression in epitenon fibroblasts. Journal of Orthopaedic Research *30*, 606-612.

Mercado-Gómez, O., Landgrave-Gómez, J., Arriaga-Avila, V., Nebreda-Corona, A., and Guevara-Guzmán, R. (2014). Role of TGF-β signaling pathway on Tenascin C protein upregulation in a pilocarpine seizure model. Epilepsy Research *108*, 1694-1704.

Merl, J., Ueffing, M., Hauck, S.M., and von Toerne, C. (2012). Direct comparison of MS-based label-free and SILAC quantitative proteome profiling strategies in primary retinal Müller cells. PROTEOMICS *12*, 1902-1911.

Mienaltowski, M., Adams, S., and Birk, D. (2014). Tendon proper- and peritenon-derived progenitor cells have unique tenogenic properties. Stem Cell Research and Therapy *5*, 86.

Mienaltowski, M.J., Adams, S.M., and Birk, D.E. (2013). Regional differences in stem cell/progenitor cell populations from the mouse achilles tendon. Tissue engineering Part A *19*, 199-210.

Miller, B.F., Olesen, J.L., Hansen, M., Døssing, S., Crameri, R.M., Welling, R.J., Langberg, H., Flyvbjerg, A., Kjaer, M., Babraj, J.A*., et al.* (2005). Coordinated collagen and muscle protein synthesis in human patella tendon and quadriceps muscle after exercise. The Journal of Physiology *567*, 1021-1033.

Moles, A.D., Hill, T.P., and Glyde, M. (2009). Triple tibial osteotomy for treatment of the canine cranial cruciate ligament-deficient stifle joint. Surgical findings and postoperative complications in 97 stifles. Veterinary and Comparative Orthopaedics and Traumatology (VCOT) *22*, 473-478.

Mölsä, S.H., Hyytiäinen, H.K., Hielm-Björkman, A.K., and Laitinen-Vapaavuori, O.M. (2014). Long-term functional outcome after surgical repair of cranial cruciate ligament disease in dogs. BMC Veterinary Research *10*, 266.

Mueller, A.J., Tew, S.R., Vasieva, O., Clegg, P.D., and Canty-Laird, E.G. (2016). A systems biology approach to defining regulatory mechanisms for cartilage and tendon cell phenotypes. Scientific Reports *6*, 33956.

Murawski, C.D., van Eck, C.F., Irrgang, J.J., Tashman, S., and Fu, F.H. (2014). Operative Treatment of Primary Anterior Cruciate Ligament Rupture in Adults. J Bone Joint Surg *96*, 685-694.

Murdoch, A.D., Grady, L.M., Ablett, M.P., Katopodi, T., Meadows, R.S., and Hardingham, T.E. (2007). Chondrogenic Differentiation of Human Bone Marrow Stem Cells in Transwell Cultures: Generation of Scaffold-Free Cartilage. Stem cells *25*, 2786-2796.

Murphy, J.M., Dixon, K., Beck, S., Fabian, D., Feldman, A., and Barry, F. (2002). Reduced chondrogenic and adipogenic activity of mesenchymal stem cells from patients with advanced osteoarthritis. Arthritis & Rheumatism *46*, 704-713.

Murwantoko, Yano, M., Ueta, Y., Murasaki, A., Kanda, H., Oka, C., and Kawaichi, M. (2004). Binding of proteins to the PDZ domain regulates proteolytic activity of HtrA1 serine protease. Biochemical Journal *381*, 895-904.

Naba, A., Clauser, K.R., Ding, H., Whittaker, C.A., Carr, S.A., and Hynes, R.O. (2016). The extracellular matrix: Tools and insights for the “omics” era. Matrix Biology *49*, 10-24.

Nadri, S., and Soleimani, M. (2007). Isolation murine mesenchymal stem cells by positive selection. In Vitro CellDevBiol-Animal *43*, 276-282.

Nadri, S., Soleimani, M., Hosseni, R.H., Massumi, M., Atashi, A., and Izadpanah, R. (2007). An efficient method for isolation of murine bone marrow mesenchymal stem cells. Int J Dev Biol *51*, 723-729.

Nagura, I., Kokubu, T., Mifune, Y., Inui, A., Takase, F., Ueda, Y., Kataoka, T., and Kurosaka, M. (2016). Characterization of progenitor cells derived from torn human rotator cuff tendons by gene expression patterns of chondrogenesis, osteogenesis, and adipogenesis. Journal of Orthopaedic Surgery and Research *11*, 40.

Nagy, K.A., Girard, I.A., and Brown, T.K. (1999). ENERGETICS OF FREE-RANGING MAMMALS, REPTILES, AND BIRDS. Annual Review of Nutrition *19*, 247-277.

Nakahara, H., Hasegawa, A., Otabe, K., Ayabe, F., Matsukawa, T., Onizuka, N., Ito, Y., Ozaki, T., Lotz, M.K., and Asahara, H. (2013). Transcription factor Mohawk and the pathogenesis of human anterior cruciate ligament degradation. Arthritis and rheumatism *65*, 10.1002/art.38020.

Nakamura-Ishizu, A., and Suda, T. (2013). Hematopoietic stem cell niche: An interplay among a repertoire of multiple functional niches. Biochimica et Biophysica Acta (BBA) - General Subjects *1830*, 2404-2409.

Navabazam, A.R., Nodoshan, F.S., Sheikhha, M.H., Miresmaeili, S.M., Soleimani, M., and Fesahat, F. (2013). Characterization of mesenchymal stem cells from human dental pulp, preapical follicle and periodontal ligament. Iran Journal of Reproductive Medicine *11*, 235-242.

Neame, P.J., Kay, C.J., McQuillan, D.J., Beales, M.P., and Hassell, J.R. (2000). Independent modulation of collagen fibrillogenesis by decorin and lumican. Cellular and Molecular Life Sciences CMLS *57*, 859-863.

Ni, M., Lui, P.P.Y., Rui, Y.F., Lee, Y.W., Lee, Y.W., Tan, Q., Wong, Y.M., Kong, S.K., Lau, P.M., Li, G*., et al.* (2012). Tendon-derived stem cells (TDSCs) promote tendon repair in a rat patellar tendon window defect model. Journal of Orthopaedic Research *30*, 613-619.

Ni, M., Rui, Y.F., Tan, Q., Liu, Y., Xu, L.L., Chan, K.M., Wang, Y., and Li, G. (2013). Engineered scaffold-free tendon tissue produced by tendon-derived stem cells. Biomaterials *34*, 2024-2037.

Niimori, D., Kawano, R., Felemban, A., Niimori-Kita, K., Tanaka, H., Ihn, H., and Ohta, K. (2012). Tsukushi controls the hair cycle by regulating TGF-β1 signaling. Developmental Biology *372*, 81-87.

Niimori, D., Kawano, R., Niimori-Kita, K., Ihn, H., and Ohta, K. (2014). Tsukushi is involved in the wound healing by regulating the expression of cytokines and growth factors. Journal of cell communication and signaling *8*, 173-177.

Ning, L.-J., Zhang, Y.-J., Zhang, Y., Qing, Q., Jiang, Y.-L., Yang, J.-L., Luo, J.-C., and Qin, T.-W. (2015). The utilization of decellularized tendon slices to provide an inductive microenvironment for the proliferation and tenogenic differentiation of stem cells. Biomaterials *52*, 539-550.

Nohmi, S., Yamamoto, Y., Mizukami, H., Ishibashi, Y., Tsuda, E., Maniwa, K., Yagihashi, S., Motomura, S., Toh, S., and Furukawa, K.-I. (2012). Post injury changes in the properties of

mesenchymal stem cells derived from human anterior cruciate ligaments. International orthopaedics *36*, 1515-1522.

Nourissat, G., Berenbaum, F., and Duprez, D. (2015). Tendon injury: from biology to tendon repair. Nature Reviews Rheumatology *11*, 223-233.

Oka, C., Tsujimoto, R., Kajikawa, M., Koshiba-Takeuchi, K., Ina, J., Yano, M., Tsuchiya, A., Ueta, Y., Soma, A., Kanda, H*., et al.* (2004). HtrA1 serine protease inhibits signaling mediated by Tgfbeta family proteins. Development *131*, 1041-1053.

Oleggini, R., Gastaldo, N., and Di Donato, A. (2007). Regulation of elastin promoter by lysyl oxidase and growth factors: Cross control of lysyl oxidase on TGF-β1 effects. Matrix Biology *26*, 494-505.

Olnes, M.J., Scheinberg, P., Calvo, K.R., Desmond, R., Tang, Y., Dumitriu, B., Parikh, A.R., Soto, S., Biancotto, A., Feng, X*., et al.* (2012). Eltrombopag and Improved Hematopoiesis in Refractory Aplastic Anemia. The New England journal of medicine *367*, 11-19.

Omachi, T., Sakai, T., Hiraiwa, H., Hamada, T., Ono, Y., Nakashima, M., Ishizuka, S., Matsukawa, T., Oda, T., Takamatsu, A*., et al.* (2014). Expression of tenocyte lineage-related factors in regenerated tissue at sites of tendon defect. Journal of Orthopedic Science *20*, 380-389.

Otabe, K., Nakahara, H., Hasegawa, A., Matsukawa, T., Ayabe, F., Onizuka, N., Inui, M., Takada, S., Ito, Y., Sekiya, I*., et al.* (2015). The transcription factor Mohawk controls tenogenic differentiation of bone marrow mesenchymal stem cells in vitro and in vivo. Journal of Orthopaedic Research *33*, 1-8.

Patterson-Kane, J.C., and Rich, T. (2014). Achilles Tendon Injuries in Elite Athletes: Lessons in Pathophysiology from Their Equine Counterparts. ILAR Journal *55*, 86-99.

Pauly, S., Klatte, F., Strobel, C., Schmidmaier, G., Greiner, S., Scheibel, M., and Wildemann, B. (2010). Charaterization of tendon cell cultures of the human rotator cuff. European Cells and Materials *20*, 84-97.

Peffers, M.J., Cillero-Pastor, B., Eijkel, G.B., Clegg, P.D., and Heeren, R.M.A. (2014a). Matrix assisted laser desorption ionization mass spectrometry imaging identifies markers of ageing and osteoarthritic cartilage. Arthritis research & therapy *16*, R110-R110.

Peffers, M.J., Fang, Y., Cheung, K., Wei, T.K.J., Clegg, P.D., and Birch, H.L. (2015a). Transcriptome analysis of ageing in uninjured human Achilles tendon. Arthritis research & therapy *17*, 33.

Peffers, M.J., McDermott, B., Clegg, P.D., and Riggs, C.M. (2015b). Comprehensive protein profiling of synovial fluid in osteoarthritis following protein equalization. Osteoarthr Cartilage *23*, 1204-1213.

Peffers, M.J., Thorpe, C.T., Collins, J.A., Eong, R., Wei, T.K.J., Screen, H.R.C., and Clegg, P.D. (2014b). Proteomic Analysis Reveals Age-related Changes in Tendon Matrix Composition, with Age- and Injury-specific Matrix Fragmentation. The Journal of Biological Chemistry *289*, 25867-25878.

Peister, A., Mellad, J.A., Larson, B.L., Hall, B.M., Gibson, L.F., and Prockop, D.J. (2004). Adult stem cells from bone marrow (MSCs) isolated from different strains of inbred mice vary in surface epitopes, rates of proliferation, and differentiation potential. Blood *103*, 1662-1668.

Perera, K.A., and Tonge, C.H. (1981). Metabolic turnover of collagen in the mouse molar periodontal ligament during tooth eruption. Journal of anatomy *133*, 359-370.

Phinney, D.G., Kopen, G., Isaacson, R.L., and Prockop, D.J. (1999). Plastic adherent stromal cells from the bone marrow of commonly used strains of inbred mice: Variations in yield, growth, and differentiation. Journal of cellular biochemistry *72*, 570-585.

Pietschmann, M.F., Frankewycz, B., Schmitz, P., Docheva, D., Sievers, B., Jansson, V., Schieker, M., and Müller, P.E. (2013). Comparison of tenocytes and mesenchymal stem cells seeded on biodegradable scaffolds in a full-size tendon defect model. Journal of Materials Science: Materials in Medicine *24*, 211-220.

Polur, I., Lee, P.L., Servais, J.M., Xu, L., and Li, Y. (2010). Role of HTRA1, a serine protease, in the progression of articular cartilage degeneration. Histology and histopathology *25*, 599-608.

Popov, C., Burggraf, M., Kreja, L., Ignatius, A., Schieker, M., and Docheva, D. (2015). Mechanical stimulation of human tendon stem/progenitor cells results in upregulation of matrix proteins, integrins and MMPs, and activation of p38 and ERK1/2 kinases. BMC molecular biology *16*, 6.

Prateeptongkum, E., Klingelhöffer, C., Müller, S., Ettl, T., and Morsczeck, C. (2015). Characterization of progenitor cells and stem cells from the periodontal ligament tissue derived from a single person. Journal of Periodontal Research *51*, 265-272.

Pratt, J.M., Petty, J., Riba-Garcia, I., Robertson, D.H.L., Gaskell, S.J., Oliver, S.G., and Beynon, R.J. (2002). Dynamics of Protein Turnover, a Missing Dimension in Proteomics. Molecular & Cellular Proteomics *1*, 579-591.

Qian, H., Buza-Vidas, N., Hyland, C.D., Jensen, C.T., Antonchuk, J., Månsson, R., Thoren, L.A., Ekblom, M., Alexander, W.S., and Jacobsen, S.E.W. (2007). Critical Role of Thrombopoietin in Maintaining Adult Quiescent Hematopoietic Stem Cells. Cell Stem Cell *1*, 671-684.

Quan, G., Choi, J.-Y., Lee, D.-S., and Lee, S.-C. (2005). TGF-β1 up-regulates transglutaminase two and fibronectin in dermal fibroblasts: a possible mechanism for the stabilization of tissue inflammation. Archives of Dermatological Research *297*, 84-90.

Rada, J.A., Cornuet, P.K., and Hassell, J.R. (1993). Regulation of Corneal Collagen Fibrillogenesis In Vitro by Corneal Proteoglycan (Lumican and Decorin) Core Proteins. Experimental Eye Research *56*, 635-648.

Radcliffe, C.H., Flaminio, M.J., and Fortier, L.A. (2010). Temporal analysis of equine bone marrow aspirate during establishment of putative mesenchymal progenitor cell populations. Stem Cell Development *19*, 269-282.

Ranera, B., Lyahyai, J., Romero, A., Vazquez, F.J., Remacha, A.R., Bernal, M.L., Zaragoza, P., Rodellar, C., and Martin-Burriel, I. (2011). Immunophenotype and gene expression profiles of cell surface markers of mesenchymal stem cells derived from equine bone marrow and adipose tissue. Veterinary immunology and immunopathology *144*, 147-154.

Razzaque, M.S., and Ahmed, A.R. (2002). COLLAGENS, COLLAGEN-BINDING HEAT SHOCK PROTEIN 47 AND TRANSFORMING GROWTH FACTOR-β1 ARE INDUCED IN CICATRICIAL PEMPHIGOID: POSSIBLE ROLE(S) IN DERMAL FIBROSIS. Cytokine *17*, 311-316.

Rees, S.G., Flannery, C.R., Little, C.B., Hughes, C.E., Caterson, B., and Dent, C.M. (2000). Catabolism of aggrecan, decorin and biglycan in tendon. Biochemical Journal *350*, 181-188.

Reynolds, J.A., Haque, S., Williamson, K., Ray, D.W., Alexander, M.Y., and Bruce, I.N. (2016). Vitamin D improves endothelial dysfunction and restores myeloid angiogenic cell function via reduced CXCL-10 expression in systemic lupus erythematosus. Scientific Reports *6*, 22341.

Richter, H., and Hörmann, H. (1983). A large cathepsin D-derived fragment from the central part of the fibronectin subunit chains. FEBS Letters *155*, 317-320.

Riley, G.P., Curry, V., DeGroot, J., van El, B., Verzijl, N., Hazleman, B.L., and Bank, R.A. (2002). Matrix metalloproteinase activities and their relationship with collagen remodelling in tendon pathology. Matrix Biology *21*, 185-195.

Riley, G.P., Harrall, R.L., Constant, C.R., Chard, M.D., Cawston, T.E., and Hazleman, B.L. (1994). Glycosaminoglycans of human rotator cuff tendons: changes with age and in chronic rotator cuff tendinitis. Annals of the Rheumatic Diseases *53*, 367-376.

Rodríguez-Lozano, F.J., García-Bernal, D., Aznar-Cervantes, S., Ros-Roca, M.A., Algueró, M.C., Atucha, N.M., Lozano-García, A.A., Moraleda, J.M., and Cenis, J.L. (2014). Effects of composite films of silk fibroin and graphene oxide on the proliferation, cell viability and mesenchymal phenotype of periodontal ligament stem cells. Journal of materials science Materials in medicine *25*, 2731-2741.

Rozemuller, H., Prins, H.J., Naaijkens, B., Stall, J., Buhring, H.J., and Martens, A.C. (2010). Prospective Isolation of Mesenchymal Stem Cells from Multiple Mammalian Species Using Cross-Reacting Anti-Human Monoclonal Antibodies. Stem Cell Development *19*, 1911-1921.

Rui, Y.F., Lui, P.P., Chan, L.S., Chan, K.M., Fu, S.C., and Gang, L.I. (2011a). Does erroneous differentiation of tendon-derived stem cells contribute to the pathogenesis of calcifying tendinopathy? Chinese Medical Journal *124*, 606-610.

Rui, Y.F., Lui, P.P., Li, G., Fu, S.C., Lee, Y.W., and Chan, K.M. (2010). Isolation and characterization of multipotent rat tendon-derived stem cells. Tissue engineering Part A *16*, 1549-1558.

Rui, Y.F., Lui, P.P., Ni, M., Chan, L.S., Lee, Y.W., and Chan, K.M. (2011b). Mechanical loading increased BMP-2 expression which promoted osteogenic differentiation of tendon-derived stem cells. Journal of Orthopaedic Research *29*, 390-396.

Rui, Y.F., Lui, P.P., Wong, Y.M., Tan, Q., and Chan, K.M. (2013a). Altered Fate of Tendon-Derived Stem Cells Isolated from a Failed Tendon-Healing Animal Model of Tendinopathy. Stem cells and development *22*, 1076-1085.

Rui, Y.F., Lui, P.P., Wong, Y.M., Tan, Q., and Chan, K.M. (2013b). BMP-2 stimulated non-tenogenic differentiation and promoted proteoglycan deposition of tendon-derived stem cells (TDSCs) in vitro. Journal of Orthopaedic Research *31*, 746-753.

Rui, Y.F., Lui, P.P.Y., Ni, M., Chan, L.S., Lee, Y.W., and Chan, K.M. (2011c). Mechanical loading increased BMP-2 expression which promoted osteogenic differentiation of tendon-derived stem cells. Journal of Orthopaedic Research *29*, 390-396.

Rumian, A.P., Wallace, A.L., and Birch, H.L. (2007). Tendons and ligaments are anatomically distinct but overlap in molecular and morphological features—a comparative study in an ovine model. Journal of Orthopaedic Research *25*, 458-464.

Runesson, E., Ackermann, P., Karlsson, J., and Eriksson, B.I. (2015). Nucleostemin- and Oct 3/4-positive stem/progenitor cells exhibit disparate anatomical and temporal expression during rat Achilles tendon healing. BMC musculoskeletal disorders *16*, 212.

Russo, V., Mauro, A., Martelli, A., Di Giacinto, O., Di Marcantonio, L., Nardinocchi, D., Berardinelli, P., and Barboni, B. (2014). Cellular and molecular maturation in fetal and adult ovine calcaneal tendons. Journal of anatomy *226*, 126-142.

Ruzzini, L., Abbruzzese, F., Rainer, A., Longo, U.G., Trombetta, M., Maffulli, N., and Denaro, V. (2013). Characterization of age-related changes of tendon stem cells from adult human tendons. Knee Surg Sport Tr A *22*, 2856-2866.

Sagar, B.M.M., Rentala, S., Gopal, P.N.V., Sharma, S., and Mukhopadhyay, A. (2006). Fibronectin and laminin enhance engraftibility of cultured hematopoietic stem cells. Biochemical and biophysical research communications *350*, 1000-1005.

Salingcarnboriboon, R., Yoshitake, H., Tsuji, K., Obinata, M., Amagasa, T., Nifuji, A., and Noda, M. (2003). Establishment of tendon-derived cell lines exhibiting pluripotent mesenchymal stem cell-like property. Experimental Cell Research *287*, 289-300.

Samiric, T., Ilic, M.Z., and Handley, C.J. (2004a). Characterisation of proteoglycans and their catabolic products in tendon and explant cultures of tendon. Matrix Biology *23*, 127-140.

Samiric, T., Ilic, M.Z., and Handley, C.J. (2004b). Large aggregating and small leucine-rich proteoglycans are degraded by different pathways and at different rates in tendon. European Journal of Biochemistry *271*, 3612-3620.

Santoro, A., Conde, J., Scotece, M., Abella, V., Lois, A., Lopez, V., Pino, J., Gomez, R., Gomez-Reino, J.J., and Gualillo, O. (2015). SERPINE2 Inhibits IL-1α-Induced MMP-13 Expression in Human Chondrocytes: Involvement of ERK/NF-κB/AP-1 Pathways. PLOS ONE *10*, e0135979.

Sato, N., Taniguchi, T., Goda, Y., Kosaka, H., Higashino, K., Sakai, T., Katoh, S., Yasui, N., Sairyo, K., and Taniguchi, H. (2016). Proteomic Analysis of Human Tendon and Ligament: Solubilization and Analysis of Insoluble Extracellular Matrix in Connective Tissues. Journal of Proteome Research *15*, 4709-4721.

Scheltema, R.A., Hauschild, J.-P., Lange, O., Hornburg, D., Denisov, E., Damoc, E., Kuehn, A., Makarov, A., and Mann, M. (2014). The Q Exactive HF, a benchtop mass spectrometer with a pre-filter, high-performance quadrupole and an ultra-high-field Orbitrap analyzer. Mol Cell Proteomics *13*, 3698-3708.

Schneider, P.R.A., Buhrmann, C., Mobasheri, A., Matis, U., and Shakibaei, M. (2011). Three-dimensional high-density co-culture with primary tenocytes induces tenogenic differentiation in mesenchymal stem cells. Journal of orthopaedic research : official publication of the Orthopaedic Research Society *29*, 1351-1360.

Schulze-Tanzil, G., Mobasheri, A., Clegg, P.D., Sendzik, J., John, T., and Shakibaei, M. (2004). Cultivation of human tenocytes in high-density culture. Histochemistry and cell biology *122*, 219-228.

Schwanhausser, B., Busse, D., Li, N., Dittmar, G., Schuchhardt, J., Wolf, J., Chen, W., and Selbach, M. (2011). Global quantification of mammalian gene expression control. Nature *473*, 337-342.

Schwanhäusser, B., Gossen, M., Dittmar, G., and Selbach, M. (2009). Global analysis of cellular protein translation by pulsed SILAC. PROTEOMICS *9*, 205-209.

Schwartz, A., Watson, J.N., and Hutchinson, M.R. (2015). Patellar Tendinopathy. Sports Health *7*, 415-420.

Schweitzer, R., Chyung, J.H., Murtaugh, L.C., Brent, A.E., Rosen, V., Olson, E.N., Lassar, A., and Tabin, C.J. (2001). Analysis of the tendon cell fate using Scleraxis, a specific marker for tendons and ligaments. Development *128*, 3855-3866.

Scott, P.G., and Pearson, H. (1981). Cathepsin D: Specificity of Peptide-Bond Cleavage in Type-I Collagen and Effects on Type-III Collagen and Procollagen. European Journal of Biochemistry *114*, 59-62.

Sedigh, H.S., Saffarpour, A., Jamshidi, S., Ashouri, M., Nassiri, S.M., Shafiri, D., Torkzaban, P., and Kajbafzadeh, A. (2010). Canine Periodontal Stem Cells: Isolation, Differentiation Potential and Electronic Microscopic Characterization. Iran Journal of Veterinary Surgery *5*, 19-28.

Segawa, Y., Muneta, T., Makino, H., Nimura, A., Mochizuki, T., Ju, Y.-J., Ezura, Y., Umezawa, A., and Sekiya, I. (2009). Mesenchymal stem cells derived from synovium, meniscus, anterior cruciate ligament, and articular chondrocytes share similar gene expression profiles. Journal of Orthopaedic Research *27*, 435-441.

Seo, B.-M., Miura, M., Gronthos, S., Mark Bartold, P., Batouli, S., Brahim, J., Young, M., Gehron Robey, P., Wang, C.Y., and Shi, S. (2004). Investigation of multipotent postnatal stem cells from human periodontal ligament. The Lancet *364*, 149-155.

Sharma, P., and Maffulli, N. (2006). Biology of tendon injury: healing, modeling and remodeling. Journal of musculoskeletal & neuronal interactions *6*, 181-190.

Shepherd, J.H., and Screen, H.R.C. (2013). Fatigue loading of tendon. Int J Exp Pathol *94*, 260-270.

Shi, Y., Fu, Y., Tong, W., Geng, Y., Lui, P.P., Tang, T., Zhang, X., and Dai, K. (2012a). Uniaxial mechanical tension promoted osteogenic differentiation of rat tendon-derived stem cells (rTDSCs) via the Wnt5a-RhoA pathway. Journal of cellular biochemistry *113*, 3133-3142.

Shi, Y., Fu, Y., Tong, W., Geng, Y., Lui, P.P.Y., Tang, T., Zhang, X., and Dai, K. (2012b). Uniaxial mechanical tension promoted osteogenic differentiation of rat tendon-derived stem cells (rTDSCs) via the Wnt5a-RhoA pathway. Journal of cellular biochemistry *113*, 3133-3142.

Shikh Alsook, M.K., Gabriel, A., Piret, J., Waroux, O., Tonus, C., Connan, D., Baise, E., and Antoine, N. (2015). Tissues from equine cadaver ligaments up to 72 hours of post-mortem: a promising reservoir of stem cells. Stem cell research & therapy *6*, 253.

Shimada, A., Wada, S., Inoue, K., Ideno, H., Kamiunten, T., Komatsu, K., Kudo, A., Nakamura, Y., Sato, T., Nakashima, K*., et al.* (2014). Efficient expansion of mouse primary tenocytes using a novel collagen gel culture method. Histochemistry and cell biology *142*, 205-215.

Singhatanadgit, W., Donos, N., and Olsen, I. (2009). Isolation and Characterization of Stem Cell Clones from Adult Human Ligament. Tissue Eng Pt A *15*, 2625-2636.

Sinha, K.M., Yasuda, H., Zhou, X., and deCrombrugghe, B. (2014). Osterix and NO66 histone demethylase control the chromatin architecture of Osterix target genes during osteoblast differentiation. J Bone Miner Res *29*, 855-865.

Smith, R.K.W., Werling, N.J., Dakin, S.G., Alam, R., Goodship, A.E., and Dudhia, J. (2013). Beneficial Effects of Autologous Bone Marrow-Derived Mesenchymal Stem Cells in Naturally Occurring Tendinopathy. PLoS ONE *8*, e75697.

Soleimani, M., and Nadri, S. (2009). A protocol for isolation and culture of mesenchymal stem cells from mouse bone marrow. Nature Protocols *4*, 102-106.

Soteriou, D., Iskender, B., Byron, A., Humphries, J.D., Borg-Bartolo, S., Haddock, M.-C., Baxter, M.A., Knight, D., Humphries, M.J., and Kimber, S.J. (2013). Comparative Proteomic Analysis of Supportive and Unsupportive Extracellular Matrix Substrates for Human Embryonic Stem Cell Maintenance. J Biol Chem *288*, 18716-18731.

Sottile, J., and Hocking, D.C. (2002). Fibronectin Polymerization Regulates the Composition and Stability of Extracellular Matrix Fibrils and Cell-Matrix Adhesions. Molecular Biology of the Cell *13*, 3546-3559.

Sottocornola, R., and Lo Celso, C. (2012). Dormancy in the stem cell niche. Stem cell research & therapy *3*, 10-10.

Stanco, D., ViganÒ, M., Perucca Orfei, C., Di Giancamillo, A., Thiebat, G., Peretti, G., and de Girolamo, L. (2014). In vitro characterization of stem/progenitor cells from semitendinosus and gracilis tendons as a possible new tool for cell-based therapy for tendon disorders. Joints *2*, 159-168.

Steinert, A.F., Kunz, M., Prager, P., Barthel, T., Jakob, F., Noth, U., Murray, M.M., Evans, C.H., and Porter, R.M. (2011). Mesenchymal stem cell characteristics of human anterior cruciate ligament outgrowth cells. Tissue engineering Part A *17*, 1375-1388.

Stiehler, M., Rauh, J., Bünger, C., Jacobi, A., Vater, C., Schildberg, T., Liebers, C., Günther, K.-P., and Bretschneider, H. (2016). In vitro characterization of bone marrow stromal cells from osteoarthritic donors. Stem Cell Research *16*, 782-789.

Su, F., Liu, S.-S., Ma, J.-L., Wang, D.-S., E, L.-L., and Liu, H.-C. (2015). Enhancement of periodontal tissue regeneration by transplantation of osteoprotegerin-engineered periodontal ligament stem cells. Stem cell research & therapy *6*, 22.

Sun, S., Guo, Z., Xiao, X., Liu, B., Liu, X., Tang, P.-H., and Mao, N. (2003). Isolation of Mouse Marrow Mesenchymal Progenitors by a Novel and Reliable Method. Stem cells *21*, 527-535.

Takayama, K., Kawakami, Y., Mifune, Y., Matsumoto, T., Tang, Y., Cummins, J.H., Greco, N., Kuroda, R., Kurosaka, M., Wang, B.*, et al.* (2015). The effect of blocking angiogenesis on anterior cruciate ligament healing following stem cell transplantation. Biomaterials *60*, 9-19.

Takemitsu, H., Zhao, D., Yamamoto, I., Harada, Y., Michishita, M., and Arai, T. (2012). Comparison of bone marrow and adipose tissue-derived canine mesenchymal stem cells. BMC Veterinary Research *8*, 150.

Tan, Q., Lui, P.P., and Lee, Y.W. (2013). In Vivo Identity of Tendon Stem Cells and the Roles of Stem Cells in Tendon Healing. Stem cells and development *22*, 128-140.

Tan, Q., Lui, P.P., and Rui, Y.F. (2012a). Effect of in vitro passaging on the stem cell-related properties of tendon-derived stem cells-implications in tissue engineering. Stem cells and development *21*, 790-800.

Tan, Q., Lui, P.P., Rui, Y.F., and Wong, Y.M. (2012b). Comparison of potentials of stem cells isolated from tendon and bone marrow for musculoskeletal tissue engineering. Tissue engineering Part A *18*, 840-851.

Tanaka, S., Maekawa, A., Matsubara, L., Imanishi, A., Yano, M., Roeder, R.G., Hasegawa, N., Asano, S., and Ito, M. (2016). Periostin supports hematopoietic progenitor cells and niche-dependent myeloblastoma cells in vitro. Biochemical and biophysical research communications *478*, 1706-1712.

Tang, Z., Yang, L., Xue, R., Zhang, J., Wang, Y., Chen, P.C., and Sung, K.L.P. (2009). Differential expression of matrix metalloproteinases and tissue inhibitors of metalloproteinases in anterior cruciate ligament and medial collateral ligament fibroblasts after a mechanical injury: Involvement of the p65 subunit of NF-κB. Wound Repair Regen *17*, 709-716.

Tao, X., Liu, J., Chen, L., Zhou, Y., and Tang, K. (2015). EGR1 Induces Tenogenic Differentiation of Tendon Stem Cells and Promotes Rabbit Rotator Cuff Repair. Cellular Physiology and Biochemistry *35*, 699-709.

Tempfer, H., Wagner, A., Gehwolf, R., Lehner, C., Tauber, M., Resch, H., and Bauer, H.C. (2009). Perivascular cells of the supraspinatus tendon express both tendon- and stem cell-related markers. Histochemistry and cell biology *131*, 733-741.

Temponi, E.F., de Carvalho Júnior, L.H., Sonnery-Cottet, B., and Chambat, P. (2015). Partial tearing of the anterior cruciate ligament: diagnosis and treatment. Revista Brasileira de Ortopedia *50*, 9-15.

Theobald, P., Benjamin, M., Nokes, L., and Pugh, N. (2005). Review of the vascularisation of the human Achilles tendon. Injury *36*, 1267-1272.

Thorpe, C.T., Birch, H.L., Clegg, P.D., and Screen, H.R.C. (2013). The role of the non-collagenous matrix in tendon function. Int J Exp Pathol *94*, 248-259.

Thorpe, C.T., Riley, G.P., Birch, H.L., Clegg, P.D., and Screen, H.R.C. (2014). Effect of fatigue loading on structure and functional behaviour of fascicles from energy-storing tendons. Acta Biomaterialia *10*, 3217-3224.

Thorpe, C.T., Streeter, I., Pinchbeck, G.L., Goodship, A.E., Clegg, P.D., and Birch, H.L. (2010). Aspartic Acid Racemization and Collagen Degradation Markers Reveal an Accumulation of Damage in Tendon Collagen That Is Enhanced with Aging. The Journal of Biological Chemistry *285*, 15674-15681.

To, W.S., and Midwood, K.S. (2010). Cryptic domains of tenascin-C differentially control fibronectin fibrillogenesis. Matrix Biology *29*, 573-585.

Tocharus, J., Tsuchiya, A., Kajikawa, M., Ueta, Y., Oka, C., and Kawaichi, M. (2004). Developmentally regulated expression of mouse HtrA3 and its role as an inhibitor of TGF-β signaling. Development, Growth & Differentiation *46*, 257-274.

Tokunaga, T., Shukunami, C., Okamoto, N., Taniwaki, T., Oka, K., Sakamoto, H., Ide, J., Mizuta, H., and Hiraki, Y. (2015). FGF-2 Stimulates the Growth of Tenogenic Progenitor Cells to Facilitate the Generation of Tenomodulin-Positive Tenocytes in a Rat Rotator Cuff Healing Model. The American Journal of Sports Medicine *43*, 2411-2422.

Tong, W.Y., Shen, W., Yeung, C.W.F., Zhao, Y., Cheng, S.H., Chu, P.K., Chan, D., Chan, G.C.F., Cheung, K.M.C., Yeung, K.W.K*., et al.* (2012). Functional replication of the tendon tissue microenvironment by a bioimprinted substrate and the support of tenocytic differentiation of mesenchymal stem cells. Biomaterials *33*, 7686-7698.

Torii, D., Tsutsui, T.W., Watanabe, N., and Konishi, K. (2014). Bone morphogenetic protein 7 induces cementogenic differentiation of human periodontal ligament-derived mesenchymal stem cells. Odontology, 1-9.

Tozer, S., and Duprez, D. (2005). Tendon and ligament: Development, repair and disease. Birth Defects Research Part C: Embryo Today: Reviews *75*, 226-236.

Trompeter, A., Gill, K., Appleton, M.A.C., and Palmer, S.H. (2009). Predicting anterior cruciate ligament integrity in patients with osteoarthritis. Knee Surgery, Sports Traumatology, Arthroscopy *17*, 595-599.

Tropel, P., Noël, D., Platet, N., Legrand, P., Benabid, A.-L., and Berger, F. (2004). Isolation and characterisation of mesenchymal stem cells from adult mouse bone marrow. Experimental Cell Research *295*, 395-406.

Tsai, R.Y.L., and McKay, R.D.G. (2000). Cell Contact Regulates Fate Choice by Cortical Stem Cells. The Journal of Neuroscience *20*, 3725-3735.

Tsuzaki, M., Bynum, D., Almekinders, L., Faber, J., and Banes, A.J. (2005). Mechanical loading stimulates ecto-ATPase activity in human tendon cells. Journal of cellular biochemistry *96*, 117-125.

Tucker, R.P., Ferralli, J., Schittny, J.C., and Chiquet-Ehrismann, R. (2013). Tenascin-C and tenascin-W in whisker follicle stem cell niches: possible roles in regulating stem cell proliferation and migration. Journal of Cell Science *126*, 5111-5115.

Turk, V., Stoka, V., Vasiljeva, O., Renko, M., Sun, T., Turk, B., and Turk, D. (2012). Cysteine cathepsins: From structure, function and regulation to new frontiers. Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics *1824*, 68-88.

Uefuji, A., Matsumoto, T., Matsushita, T., Ueha, T., Zhang, S., Kurosaka, M., and Kuroda, R. (2014). Age-Related Differences in Anterior Cruciate Ligament Remnant Vascular-Derived Cells. Am J Sport Med *42*, 1478-1486.

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., and Speleman, F. (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biology *3*, research0034.0031-research0034.0011.

Verrecchia, F., and Mauviel, A. (2004). TGF-beta and TNF-alpha: antagonistic cytokines controlling type I collagen gene expression. Cell Signal *16*, 873-880.

Violini, S., Ramelli, P., Pisani, L., Gorni, C., and Mariani, P. (2009). Horse bone marrow mesenchymal stem cells express embryo stem cell markers and show the ability for tenogenic differentiation by in vitro exposure to BMP-12. BMC Cell Biology *10*, 29.

Voss, A., McCarthy, M.B., Hoberman, A., Cote, M.P., Imhoff, A.B., Mazzocca, A.D., and Beitzel, K. (2016). Extracellular Matrix of Current Biological Scaffolds Promotes the Differentiation Potential of Mesenchymal Stem Cells. Arthroscopy: The Journal of Arthroscopic & Related Surgery *32*, 2381-2392.

Wada, S., Ideno, H., Shimada, A., Kamiunten, T., Nakamura, Y., Nakashima, K., Kimura, H., Shinkai, Y., Tachibana, M., and Nifuji, A. (2015). H3K9MTase G9a is essential for the differentiation and growth of tenocytes in vitro. Histochemistry and cell biology *144*, 13-20.

Wang, J.H.C., Jia, F., Yang, G., Yang, S., Campbell, B.H., Stone, D., and Woo, S.L.Y. (2003). Cyclic Mechanical Stretching of Human Tendon Fibroblasts Increases the Production of Prostaglandin E 2 and Levels of Cyclooxygenase Expression: A Novel In Vitro Model Study. Connective tissue research *44*, 128-133.

Wang, P., Liu, X., Xu, P., Lu, J., Wang, R., and Mu, W. (2016). Decorin reduces hypertrophic scarring through inhibition of the TGF-β1/Smad signaling pathway in a rat osteomyelitis model. Experimental and therapeutic medicine *12*, 2102-2108.

Wang, W.-J., Zhao, Y.-M., Lin, B.-C., Yang, J., and Ge, L.-H. (2012). Identification of multipotent stem cells from adult dog periodontal ligament. European Journal of Oral Sciences *120*, 303-310.

Watts, A.E., Yeager, A.E., Kopyov, O.V., and Nixon, A.J. (2011). Fetal derived embryonic-like stem cells improve healing in a large animal flexor tendonitis model. Stem cell research & therapy *2*, 4.

Webster, D.F., and Burry, H.C. (1982). The effects of hypoxia on human skin, lung and tendon cells in vitro. British Journal of Experimental Pathology *63*, 50-55.

Whitehair, J.G., Vasseur, P.B., and Willits, N.H. (1993). Epidemiology of cranial cruciate ligament rupture in dogs. Journal of the American Veterinary Medical Association *203*, 1016-1019.

Wilke, V.L., Conzemius, M.G., Kinghorn, B.P., Macrossan, P.E., Cai, W., and Rothschild, M.F. (2006). Inheritance of rupture of the cranial cruciate ligament in Newfoundlands. Journal of the American Veterinary Medical Association *228*, 61-64.

Wilkins, M.R., Sanchez, J.-C., Gooley, A.A., Appel, R.D., Humphery-Smith, I., Hochstrasser, D.F., and Williams, K.L. (1996). Progress with Proteome Projects: Why all Proteins Expressed by a Genome Should be Identified and How To Do It. Biotechnology and Genetic Engineering Reviews *13*, 19-50.

Williamson, J.R., Edwards, A.V.G., Verano-Braga, T., Schwämmle, V., Kjeldsen, F., Jensen, O.N., and Larsen, M.R. (2016). High-performance hybrid Orbitrap mass spectrometers for quantitative proteome analysis: Observations and implications. Proteomics *16*, 907-914.

Williamson, K.A., Lee, K.J., Humphreys, W.J.E., Comerford, E.J.V., Clegg, P.D., and Canty-Laird, E.G. (2015). Restricted differentiation potential of progenitor cell populations obtained from the equine superficial digital flexor tendon (SDFT). Journal of Orthopaedic Research *33*, 849-858.

Wilson, A., Laurenti, E., Oser, G., van der Wath, R.C., Blanco-Bose, W., Jaworski, M., Offner, S., Dunant, C.F., Eshkind, L., Bockamp, E.*, et al.* (2008). Hematopoietic Stem Cells Reversibly Switch from Dormancy to Self-Renewal during Homeostasis and Repair. Cell *135*, 1118-1129.

Wilson, A.M., McGuigan, M.P., Su, A., and van den Bogert, A.J. (2001). Horses damp the spring in their step. Nature *414*, 895-899.

Wilson, R., Diseberg, A.F., Gordon, L., Zivkovic, S., Tatarczuch, L., Mackie, E.J., Gorman, J.J., and J.F., B. (2010). Comprehensive Profiling of Cartilage Extracellular Matrix Formation and Maturation Using Sequential Extraction and Label-free Quantitative Proteomics. Mol Cell Proteomics *9.6*, 1296-1313.

Winter, A.D., Campbell, M.A., Robinson, H.C., and Handley, C.J. (2000). Catabolism of newly synthesized decorin by explant cultures of bovine ligament. Matrix Biology *19*, 129-138.

Witsberger, T.H., Villamil, J.A., Schultz, L.G., Hahn, A.W., and Cook, J.L. (2008). Prevalence of and risk factors for hip dysplasia and cranial cruciate ligament deficiency in dogs. Journal of the American Veterinary Medical Association *232*, 1818-1824.

Wolf, R.E., Scavelli, T.D., Hoelzler, M.G., Fulcher, R.P., and Bastian, R.P. (2012). Surgical and postoperative complications associated with tibial tuberosity advancement for cranial cruciate ligament rupture in dogs: 458 cases (2007–2009). Journal of the American Veterinary Medical Association *240*, 1481-1487.

Wong, J., Bennett, W., Ferguson, M.W.J., and McGrouther, D.A. (2006). Microscopic and histological examination of the mouse hindpaw digit and flexor tendon arrangement with 3D reconstruction. Journal of anatomy *209*, 533-545.

Woo, S.L.Y., Debski, R.E., Zeminski, J., Abramowitch, S.D., Chan Saw, S.S., and Fenwick, J.A. (2000). Injury and Repair of Ligaments and Tendons. Annual Review of Biomedical Engineering *2*, 83-118.

Wu, H., Zhao, G., Zu, H., Wang, J.H.C., and Wang, Q.-M. (2015a). Aging-related viscoelasticity variation of tendon stem cells (TSCs) characterized by quartz thickness shear mode (TSM) resonators. Sensors and actuators *210*, 369-380.

Wu, R.-X., Bi, C.-S., Yu, Y., Zhang, L.-L., and Chen, F.-M. (2015b). Age-related decline in the matrix contents and functional properties of human periodontal ligament stem cell sheets. Acta Biomaterialia *22*, 70-82.

Xu, Y., Dong, S., Zhou, Q., Mo, X., Song, L., Hou, T., Wu, J., Li, S., Li, Y., Li, P.*, et al.* (2014). The effect of mechanical stimulation on the maturation of TDSCs-poly(L-lactide-co-e-caprolactone)/collagen scaffold constructs for tendon tissue engineering. Biomaterials *35*, 2760-2772.

Xu, Y., Wang, Q., Li, Y., Gan, Y., Li, P., Li, S., Zhou, Y., and Zhou, Q. (2015). Cyclic Tensile Strain Induces Tenogenic Differentiation of Tendon-Derived Stem Cells in Bioreactor Culture. BioMed research international *2015*, 790804.

Yang, G., Rothrauff, B.B., Lin, H., Gottardi, R., Alexander, P.G., and Tuan, R.S. (2013). Enhancement of tenogenic differentiation of human adipose stem cells by tendon-derived extracellular matrix. Biomaterials *34*, 9295-9306.

Yang, J., Zhao, Q., Wang, K., Liu, H., Ma, C., Huang, H., and Liu, Y. (2016). Isolation and biological characterization of tendon-derived stem cells from fetal bovine. In Vitro CellDevBiol-Animal *52*, 846-856.

Yang, Z., Jin, F., Zhang, X., Ma, D., Han, C., Huo, N., Wang, Y., Zhang, Y., Lin, Z., and Jin, Y. (2009). Tissue Engineering of Cementum/Periodontal-Ligament Complex Using a Novel Three-Dimensional Pellet Cultivation System for Human Periodontal Ligament Stem Cells. Tissue Engineering Part C: Methods *15*, 571-581.

Ye, G., Li, C., Xiang, X., Chen, C., Zhang, R., Yang, X., Yu, X., Wang, J., Wang, L., Shi, Q.*, et al.* (2014). Bone morphogenetic protein-9 induces PDLSCs osteogenic differentiation through the ERK and p38 signal pathways. International journal of medical sciences *11*, 1065-1072.

Yin, Z., Chen, X., Chen, J.L., Shen, W.L., Hieu Nguyen, T.M., Gao, L., and Ouyang, H.W. (2010). The regulation of tendon stem cell differentiation by the alignment of nanofibers. Biomaterials *31*, 2163-2175.

Yin, Z., Chen, X., Zhu, T., Hu, J.-j., Song, H.-x., Shen, W.-l., Jiang, L.-y., Heng, B.C., Ji, J.-f., and Ouyang, H.-W. (2013). The effect of decellularized matrices on human tendon stem/progenitor cell differentiation and tendon repair. Acta Biomaterialia *9*, 9317-9329.

Yokota, T., Oritani, K., Mitsui, H., Aoyama, K., Ishikawa, J., Sugahara, H., Matsumura, I., Tsai, S., Tomiyama, Y., Kanakura, Y.*, et al.* (1998). Growth-Supporting Activities of Fibronectin on Hematopoietic Stem/Progenitor Cells In Vitro and In Vivo: Structural Requirement for Fibronectin Activities of CS1 and Cell-Binding Domains. Blood *91*, 3263-3272.

Yoon, J.H., and Halper, J. (2005). Tendon proteoglycans: biochemistry and function. Journal of musculoskeletal & neuronal interactions *5*, 22-34.

Yoshimatsu, M., Uehara, M., and Yoshida, N. (2008). Expression of heat shock protein 47 in the periodontal ligament during orthodontic tooth movement. Archives of Oral Biology *53*, 890-895.

Yu, B., and Wang, Z. (2014). Effect of concentrated growth factors on beagle periodontal ligament stem cells in vitro. Molecular Medicine Reports *9*, 235-242.

Yu, Y., Bi, C.-S., Wu, R.-X., Yin, Y., Zhang, X.-Y., Lan, P.-H., and Chen, F.-M. (2016). Effects of short-term inflammatory and/or hypoxic pretreatments on periodontal ligament stem cells: in vitro and in vivo studies. Cell and tissue research *366*, 311-328.

Zhang, C., Lu, Y., Zhang, L., Liu, Y., Zhou, Y., Chen, Y., and Yu, H. (2015). Influence of different intensities of vibration on proliferation and differentiation of human periodontal ligament stem cells. Archives of Medical Science : AMS *11*, 638-646.

Zhang, H.Y., Liu, R., Xing, Y.J., Xu, P., Li, Y., and Li, C.J. (2013). Effects of hypoxia on the proliferation, mineralization and ultrastructure of human periodontal ligament fibroblasts. Experimental and therapeutic medicine *6*, 1553-1559.

Zhang, J., Li, B., and Wang, J.H. (2011a). The role of engineered tendon matrix in the stemness of tendon stem cells in vitro and the promotion of tendon-like tissue formation in vivo. Biomaterials *32*, 6972-6981.

Zhang, J., Pan, T., Im, H.J., Fu, F.H., and Wang, J.H. (2011b). Differential properties of human ACL and MCL stem cells may be responsible for their differential healing capacity. BMC medicine *9*, 68.

Zhang, J., Pan, T., Liu, Y., and Wang, J.H. (2010). Mouse treadmill running enhances tendons by expanding the pool of tendon stem cells (TSCs) and TSC-related cellular production of collagen. Journal of Orthopaedic Research *28*, 1178-1183.

Zhang, J., and Wang, J.H.-C. (2010a). Platelet-Rich Plasma Releasate Promotes Differentiation of Tendon Stem Cells Into Active Tenocytes. Am J Sport Med *38*, 2477-2486.

Zhang, J., and Wang, J.H. (2010b). Characterization of differential properties of rabbit tendon stem cells and tenocytes. BMC musculoskeletal disorders *11*, 10.

Zhang, J., and Wang, J.H. (2010c). Production of PGE(2) increases in tendons subjected to repetitive mechanical loading and induces differentiation of tendon stem cells into non-tenocytes. Journal of Orthopaedic Research *28*, 198-203.

Zhang, J., and Wang, J.H. (2014). Prostaglandin E2 (PGE2) Exerts Biphasic Effects on Human Tendon Stem Cells. PLoS ONE *9*, e87706.

Zhang, J., and Wang, J.H.C. (2010d). Mechanobiological response of tendon stem cells: Implications of tendon homeostasis and pathogenesis of tendinopathy. Journal of Orthopaedic Research *28*, 639-643.

Zhang, J., and Wang, J.H.C. (2013a). The Effects of Mechanical Loading on Tendons - An In Vivo and In Vitro Model Study. PLoS ONE *8*, e71740.

Zhang, J., and Wang, J.H.C. (2013b). Human Tendon Stem Cells Better Maintain Their Stemness in Hypoxic Culture Conditions. PLoS ONE *8*, e61424.

Zhang, J., and Wang, J.H.C. (2015). Moderate Exercise Mitigates the Detrimental Effects of Aging on Tendon Stem Cells. PLoS ONE *10*, e0130454.

Zhang, J., Xin, L., Shan, B., Chen, W., Xie, M., Yuen, D., Zhang, W., Zhang, Z., Lajoie, G.A., and Ma, B. (2012). PEAKS DB: De Novo Sequencing Assisted Database Search for Sensitive and Accurate Peptide Identification. Molecular & Cellular Proteomics *11*, M111.010587.

Zhou, Y., Fan, W., and Xiao, Y. (2014). The Effect of Hypoxia on the Stemness and Differentiation Capacity of PDLC and DPC. BioMed research international *2014*, 890675.

Zhou, Z., Akinbiyi, T., Xu, L., Ramcharan, M., Leong, D.J., Ros, S.J., Colvin, A.C., Schaffler, M.B., Majeska, R.J., Flatow, E.L.*, et al.* (2010). Tendon-derived stem/progenitor cell aging: defective self-renewal and altered fate. Aging Cell *9*, 911-915.