A Comparative Analysis of Equine Mesenchymal Stromal Cells and Dermal Fibroblasts

A thesis

submitted in fulfilment

of the requirements for the degree

of

Master of Philosophy

in Biological Sciences

at

The University of Waikato

by

Alexandra Anneli Kristina Leander

THE UNIVERSITY OF
WAIKATO
Te Whare Wānanga o Waikato

2017

ABSTRACT

The aim of this study was to isolate and characterise equine mesenchymal stromal cells from various sources and to compare their biological properties to that of dermal fibroblasts. By using multiple conventional methods a comparative analysis of the different populations could be made. The results showed that little distinguishes mesenchymal stromal cells from dermal fibroblasts, they have the same morphology and growth characteristics, the same cell surface markers as demonstrated with immunocytochemistry and flow cytometry and the capacity to undergo trilineage differentiation into adipo-, chondro- and osteogenic lineages essentially becoming fat, cartilage and bone. An allogenic synovial fluid model was shown to induce a chondrogenic phenotype in equine mesenchymal stromal cells as well as dermal fibroblasts. A bio-activation assay was performed to evaluate cytokine production of cell populations after activation with Tumour necrosis factor alpha or inflammatory synovial fluid. All of the analysed cell populations demonstrated up-regulation of production of the anti-inflammatory cytokines IL-10 and TGF-β1, after activation with inflammatory synovial fluid, although significant values could not be obtained for every source. As for Prostaglandin $E_{2,}$ results were obtained for bone marrow stromal cells and dermal fibroblasts populations only. Their effects were in opposite; dermal fibroblast reduced the production of Prostaglandin E_2 post activation. A functional bio-activation assay has shown promise as a method to distinguish equine mesenchymal stromal cells from dermal fibroblast populations.

ACKNOWLEDGEMENTS

Thank you to my University of Waikato supervisor Dr Ray Cursons, your investigative mind is amazing! As a veterinarian I am forever grateful for your contributions to equine research in New Zealand. To Dr Linda Peters, thank you for your support and practical laboratory mentoring and for being there to listen to my rushed updates flying in and out of the lab.

To my industry supervisor and mentor Dr Lee Morris, thank you for believing in me and trusting me with this project. You and your team at EquiBreed NZ have provided me with your extensive knowledge and support to become a scientist and a reproductive veterinarian. I'm very proud to work with you and I truly admire your drive and passion for science.

New Zealand Equine Research Foundation and Mr Kevin and Mrs Joanna Hickman from Valachi Downs Stud, without your support this project could not have been carried out. Thank you for your commitment to the equine industry in New Zealand and the way you encourage scientific research as well as sponsor individuals to achieve for the ultimate benefit of the horse.

To Dr Sari Karppinen and (future Dr) Olivia Patty for your friendship and relaxing Friday lunches. My other laboratory colleagues Steven Evans and Greg Jacobson for your technical help and friendship.

Dr Ray Littler, my statistical analysis say that you will never approach the subject of equine mesenchymal stromal cells ever again! Thank you for explaining things over and over again, I appreciate your humour and patience.

Dr Anna Brooks thank you for your very professional and knowledgeable guidance with flow cytometry and your insights into current ideas relating to human dermal fibroblast populations.

DEDICATION

I would like to dedicate my thesis to my family; Sam my husband and children Anja and Henrik. Thank you for your love and understanding of my work commitments. There will be some more time for family fun in the future!! You mean the world to me, I love you.

I would like this opportunity to recognise my Grandmother Ritva, who has been my devotee and educator since I was a little girl. You have taught me so much about nature and in your kind way you nurtured my inner drive to learn and excel. I've got vivid memories of liquid nitrogen clouds when I watched you prepare frozen semen for insemination of cattle. You inspired me to become a veterinarian and theriogenologist. Though it's a very long distance between us you are always in my heart.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

7-AAD	7-aminoactinomycin D
ACS	Acellular conditioned serum
ADSC	Adipose derived stromal cell
ALEXAFluor	Alexa fluorescent dye
ANOVA	Analysis of variance
APC	Allophycocyanin
ASC	Adult stem cell
AT	Adipose tissue
BAPN	Beta-aminopropionitrile fumarate
BM	Bone marrow
BMP-12	Bone morphogenic protein-12
BMP-6	Bone morphogenetic protein six
BMSC	Bone marrow derived stem cell
BODIPY	Boron-dipyrromethene
Bp	Base pair
BPE	B-phycoerythrin
CCL5	Chemokine ligand 5
CD	Cluster of differentiation
CDET	Common digital extensor tendon
CD-marker	Cluster of differentiation marker
cDNA	Complementary deoxyribonucleic acid
CM	Culture media
CO_2	Carbon dioxide
COX2	Cyclooxygenase 2
DAPI	4', 6-diamidino-2-phenylindole
DF	Dermal fibroblast
DJD	Degenerative joint disease
DMEM	Dulbeccos minimal eagles medium
DMEM LG	Dulbecco’s minimal eagle medium low glucose
DMSC	Dermal mesenchymal cells
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid,
EFG-2	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
EMSC	Embryonic derived stem cells
EMT	Epithelial- mesenchymal transition
ESC	Embryonic stem cell
ESNZ	Equestrian Sport New Zealand

FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FGF-2	Fibroblast growth factor 2
FITC	Fluorescine isothiocyanate
GAG	Glycosaminoglycan
GDP	Gross domestic product
Gent	Gentamicin
GFP	Green fluorescent protein
H&E	Hematoxylin eosin
HEPES	Hydroxyethyl piperazineethanesulfonic acid
HGF	Hepatocyte growth factor
HNDFs	Human dermal fibroblasts
ICC	Immunocytochemistry
IDO	Indolamine 2,3 dioygenase
IFN-γ	Interferon gamma
IGF-1	Insulin- like growth factor 1
IL-1α	Interleukin 1 alpha
IL-1β	Interleukin 1 beta
IL-6	Interleukin 6
IL-10	Interleukin 10
iPS	Induced pluripotent cell
IRAP	Interleukin Receptor antagonist Protein
ISCT	The International Society for Cellular Therapy
I-SF	Inflammatory synovial fluid
LFA-1	Lymphocyte function-associated antigen 1
LPS	Lipopolysaccharide
MCP1	Monocyte chemoattractant protein-
MeOH	Methanol
MET	Mesenchymal–epithelial transition
MHC I	Major histocompatibility complex 1
MHC II	Major histocompatibility complex 2
MMP-9	Metallo-matrix protein 9
MNC	Mononuclear cell
MQ	MilliQ water
MRI	Magnetic Resonance Imaging
mRNA	Messenger ribonucleic acid
MSC	Mesenchymal stem cell (human)
MSC	Mesenchymal stromal cell (equine)
MSI	Musculoskeletal injuries
NaAc	Sodium acetate
NaOAc	Sodium Acetate
NO	Nitric oxide
NSAID	Non-steroidal anti-inflammatories
NZTR	New Zealand Thoroughbred Racing

O_2	Oxygen
OA	Osteo-arthritis
OCD	Ostechondrosis dissecans
OCT	Optimum cutting temperature
P-	Passage minus
P0	Passage zero
P1	Passage one
P2	Passage two
P4	Passage four
P16	Passage sixteen
pB	Peripheral blood
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
pBSC	Peripheral blood stromal cell
PBSNaAz	Phosphate buffered sodium azide
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PGE2	Prostaglandin E_2
PHA	Phytohemagglutinin
PIPES	Piperazine-N,N′-bis(2-ethanesulfonic acid
PPARy2	Peroxisome proliferator-activated receptor
PRP	Platelet rich plasma
q-PCR	Quantitative reverse transcription-polymerase chain reaction
RBC	Red blood cell
RNA	Ribonucleic acid
RPE	R-phycoerythrin
RT	Room temperature
RT-PCR	Real time polymerase chain reaction
RUNX2	Runt-related transcription factor
SAA	Serum Amyloid A
SDFT	Superficial Digital Flexor Tendon
SF	Synovial fluid
SJD	Septic joint disease
SL	Suspensory ligament
SOX9	Transcription factor SOX-9
SPP1	Secreted phosphoprotein 1 (Osteopontin)
SRY	Sex determining region Y
TAE	Tris base, acetic acid and EDTA
TGF-β	Transforming growth factor beta
TGF-β1	Transforming growth factor beta-1
TiES	Thoroughbreds in Equestrian Sport
TNFα	Tumour necrosis factor alpha
TP	Total protein

TSG-6	TNFα stimulated gene/protein 6
UCB	Umbilical cord blood
UMBT	Umbillical tissue derived stromal cell
VEGF-A	Vascular endothelial growth factor A

CHAPTER 1: LITERATURE REVIEW

1.1 INTRODUCTION

Mesenchymal stromal cells (MSC) as a cellular-based regenerative treatment option have created great interest in the equine industry for the last decade (Clegg 2012). Numerous studies have investigated their use for conditions including equine osteoarthritis (Broeckx et al. 2014a; Frisbie et al. 2009; McIlwraith et al. 2011) tendinopathy (Godwin et al. 2012; Renzi et al. 2013), ulcerative keratitis (Marfe et al. 2012) and wound therapy (Spaas et al. 2013a). Musculo-skeletal injuries often involve long recovery periods with high re-injury rates, due to the inherent inability for functional repair of some of these tissues. A treatment that can repair defective cartilage in a joint or aid in the engineering of a new tendon is an enticing prospect for researchers and clinicians.

Mesenchymal stromal cells are derived from many sources with adipose tissue and bone marrow being the most studied in the equine field. However, several other sources are also being explored; among them placental and umbilical cord tissue, peripheral blood, tendons, dental pulp and periodontal tissue. The skin dermis is also a tissue of mesenchymal origin. Recent studies in human medicine on wound healing and skin regeneration are progressively investigating the relationship between dermal fibroblasts and stem cells (Brohem et al. 2013; Feisst et al. 2013; Lorenz et al. 2008). An important feature of MSC is their differentiation potential involves at least three specialised cell types from the mesenchymal tissues; fat, bone and cartilage. Stromal cells from bone marrow, umbilical cord (perivascular tissue) and also dermally derived fibroblasts have been induced to differentiate into adipocytes, chondrocytes and osteocytes.

There are many protocols published for isolation of MSC from the various sources and the methods for identification of MSC also varies between studies. The type of injuries exhibits significant variability. For example clinical trials that treat naturally occurring injuries often only report favourable results consisting of outcome variables like " Return to Full work (RTW)" at various levels and/ or re-injury rates during a set follow up period (Herthel 2001; Pacini et al. 2007; Rich

2014). However, the assessment is subjective rather than objective which some of the authors acknowledge. Other variables like; horse factors, owner factors, veterinary factors, husbandry and rehabilitation protocols can be present, which can confound the results. Other studies in a controlled laboratory setting can eliminate some of these factors. However, there may be other factors at play; the type of injury for example; iatrogenic injuries are created for the purpose of the study; surgical or collagenase methods are accepted methods to create a lesion, but are these models comparable to naturally occurring injuries? The controlled studies have the advantage that the animals are euthanised and histology can be performed to further quantify the degree and quality of healing that has occurred. The timing of euthanasia and histological interpretation varies among studies, which will have an impact on the results. All of the above factors make comparison of results difficult for the clinician and there are no set guidelines on what source of MSC is preferable nor the dose and method of deliverance. The majority of equine MSC studies to date have evaluated autologous mesenchymal stromal cells derived from the patient itself. However, equine MSCs have also been used for allogenic cellular therapy where the cells are derived from a donor (Broeckx et al. 2014a; Ricco et al. 2013; Van Loon et al. 2014). This approach reduces the time lag from injury to treatment which typically require up to 14-21 days to multiply enough cells for treatment, and could provide a cost effective *off-the-shelf* treatment option. Despite many international groups focusing their research on this topic, the understanding of the properties and effects of equine MSC are developing more slowly than the clinical implementation. Ongoing research is imperative so that MSC can be offered as a safe and efficacious treatment modality in equine medicine.

The outline of this literature review is to provide a background of the equine industry in New Zealand and wastage associated with musculo-skeletal injury. The anatomy and pathophysiology of tendons and synovial joints will be discussed in detail as these structures are the main targets for MSC therapy in horses. It will also describe and contrast MSC and fibroblasts with respect to i.) physical properties as defined with conventional methods, as well as ii.) functionality, which include new concepts like immunomodulation. The idea that MSC as well as fibroblasts control the inflammatory response is then further explored in the experimental section. If

the functionality of MSC and fibroblasts could be defined, there is the potential for developing a more efficient stromal cell therapeutic program.

The equine industry in New Zealand

Lameness due to musculoskeletal injuries (MSI) cause reduced or lost performance in equine athletes and have significant negative economic impact to the equine industry. The New Zealand racing industry maintains approximately 8900 Thoroughbreds and 5200 Standardbreds in training for approximately 8-9 months a year. In New Zealand a Thoroughbred in training costs $14-20K per annum and a Standardbred costs $10-15k per annum. Racing contributes $1.6 billion (0.9% of GDP) to the New Zealand economy and directly employs 8,877 people. Export revenue of $167 million is generated by the Thoroughbred Racing industry for New Zealand with the annual yearling auction sales alone boosting the economy by more than $57 million. The returns to owners are $53 million in prize money (NZTR Annual report 2014-2015).

Tanner et al. (2012) reported that based on the number of foals born in New Zealand, the Thoroughbred breeding industry is ranked 8th in the world and the Standardbred breeding industry is ranked 9th. In the 2009/2010 season, 6,488 Thoroughbred mares were served by 167 stallions resulting in 4,132 foals and 3,981 Standardbred mares were served by 92 stallions resulting in 2,801 foals (Tanner et al., 2012). The latest data obtained from 2015 estimated the Thoroughbred foal crop to be 3719 foals, which was up from 3551 foals born in 2014 (NZTR Annual report 2014-2015).

Animal welfare is a priority for New Zealand Thoroughbred Racing (NZTR). They report the number of horses leaving thoroughbred racing and breeding in New Zealand each year to be 800 animals. They have introduced a mandatory notification system of horse retirement and expanded the partnerships with Thoroughbreds in Equestrian Sport (TiES) and Equestrian Sport New Zealand (ESNZ) in endeavour to give retired racehorses a career as sport horses. New Zealand Standardbred Breeders' Association actively promote “Life After Racing” to minimise wastage of horses after their racing career

(http://www.harnessracing.co.nz/life-after-racing). They also support The New Zealand Standardbred Riding Association, which was formed in 1992 with the aim to encourage and promote the ridden Standardbred.

1.1.1.1 Wastage definition

Wastage can be used as a broad term in the equine industry, describing loss from all areas of the supply chain from conception through to racing (Tanner et al. 2012). Wastage can be further defined as, "horses purpose-bred for racing which failed to race or participate to their full potential" (McCarthy 2009). The effect of wastage on the racing industry can be objectively measured through analyses of days lost in training, early retirement from racing or death, the cost of diagnosis and treatment of injuries, opportunity cost of investment (Bailey et al. 1997; Dyson et al. 2008).

1.1.1.2 Wastage factors and musculoskeletal injuries quantified

Several studies in the UK have investigated and quantified wastage factors in Thoroughbred racing. In 1982 (Jeffcott et al) identified 246 cases of musculoskeletal lameness in 163 horses. Fifty-three percent (53%) of horses that raced experienced one or more periods of lameness and in 34 cases (13.8%), the condition was sufficiently severe to force retirement. This study was followed up by Rossdale et al. (1985) who reported the greatest number of days lost to training were caused by lameness (67.6%). Similar figures were reported in Australia by Bailey et al. (1997), where "sore shins" (dorsal metacarpal disease) was the most prevalent MS injury. A later UK study by Wilsher et al. (2006) reported that musculoskeletal injuries accounted for 55% of injuries in 2 year olds and 41% of injuries in 3 year olds in training. Furthermore, Dyson et al. (2008) reported that lameness was the leading cause of days lost from training. Paris and Stout (2010) reported 15-25% of horses requiring spelling from training had joint problems, while tendon and ligament injuries accounted for 46% of horses requiring spelling. Clegg (2012) reported tendon and ligament injuries as the major cause of morbidity and premature retirement in racehorses in the UK.

While the horse is unable to race its' earning potential is lost and the costs of rehabilitation are high. Costs include; feeding, stabling, spelling and veterinary expenses. Following treatment and rest from training a case controlled study of 400

horses in the UK revealed that the re-injury rate of tendon and ligament injury was 53% (O'Meara et al. 2010).

In an early New Zealand study, a survey by Hamlin and Hopkins (2003) investigated trainer-reported health and training-related problems in Standardbred horses and found that trainers perceived that horses were more likely to suffer from infections and illness than musculoskeletal problems. However, two years later Perkins et al. (2005) reported that in New Zealand, out of 1571 horses followed during 3,333 training preparations and 392,290 training days, musculoskeletal injuries were associated with the end of a training preparation or spell period in 834 cases; lameness (N=400), shin soreness (N=207), tendon and ligament conditions (N=98), injury or laceration (N=56), fractures (N=55), and back disorders (N=18). Thus, 97% of these cases involved the limbs. A later study by Bolwell et al. (2011b) found similar results; musculoskeletal injury was responsible for 83.3% of involuntary interruptions to training in a cohort of 2-year-old Thoroughbred racehorses in New Zealand. Wastage can also be quantified by the number of horses that fail to enter training. McCarthy (2009) found that approximately 32% of TB and SB failed to enter training in New Zealand. In New Zealand, a study that followed an entire annual crop of Standardbred foals reported that 51.9% (1,575/3,032) failed to start in a race (Tanner et al. 2010).

1.2 INFLAMMATION

Inflammation is the physiological response by the immune system to tissue trauma or infection. The cells of the immune system are involved in tissue healing and initiate repair by fibrosis. Figure 1 is a simplified diagram of the stages of the inflammatory process

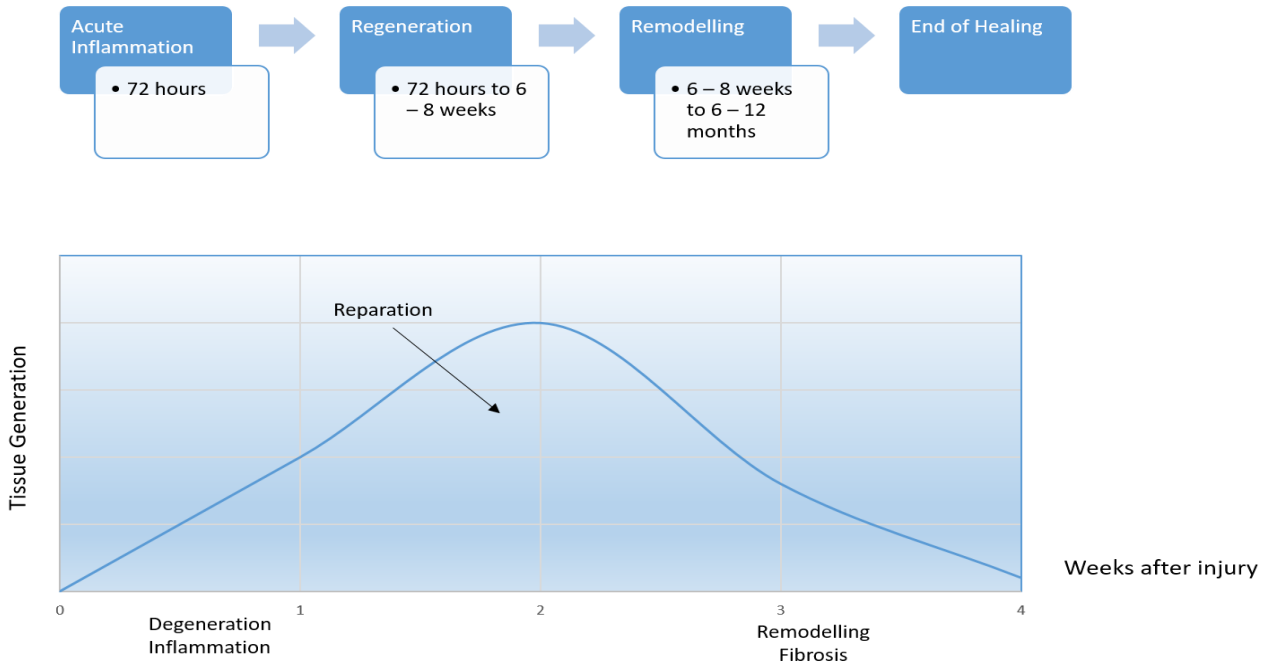


Figure 1 Stages of the inflammatory cascade simplified. Time scale for stages in weeks after initial injury

A cascade of local and systemic events and various cell types and their chemical messengers (cytokines) are involved in inflammation (Prockop 2013) as depicted89 in Figure 2. Prockop (2013) lists four basic components of inflammation i.) exogenous or endogenous stimuli for inflammation such as the products of micro-organisms or injured cells, called “Inducers”, ii.) “Sensors” that consist primarily of macrophages and mast cells that are found locally in the tissue, which express receptors for Inducers. Their response is to produce a variety of pro-inflammatory “Mediators”, iii.) Mediators are chemokines, amines, and eicosanoids released by the Sensors, iv.) “Effectors” are adjacent cells in tissues that respond to Mediators by amplifying their signals to attract the defence system (WBCs). MSC are a type of regulatory cell population which can regulate the generic pathway of inflammation by two negative feedback loops, as depicted in Figure 3.

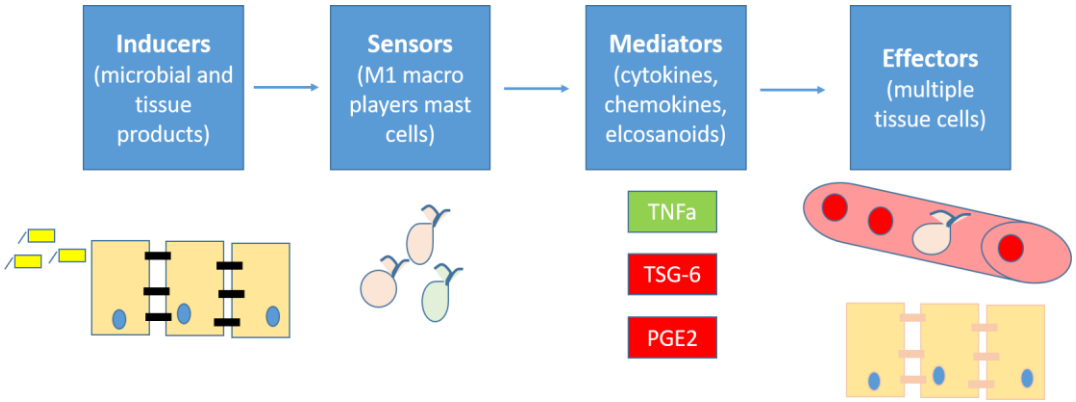


Figure 2 Inflammatory pathway showing the flow-on effects from Inducers – Sensors – Mediators – Target tissues. Adapted from Prockop (2013).

When MSC are activated by certain mediators like TNFα they upregulate COX2 expression and the arachidonic acid pathway, which leads to an increase in secretion of prostaglandin E_2 (PGE_2). Which in turn will affect macrophages (Sensors) and promote a switch from a pro-inflammatory phenotype to a more poorly defined phenotype that will secrete anti-inflammatory mediators like IL-10 and interleukin-1 (IL-1) receptor antagonist. When MSCs being activated by Mediators secreted from Sensors a second negative feedback loop occurs. The activated MSCs increase expression of a number of genes, including the anti-inflammatory protein TNFα stimulated gene/protein 6 (TSG-6). TSG-6 has multiple anti-inflammatory actions on macrophages, and the overall effect is a decrease in the secretion of TNFα and other Mediators that target Effectors. MSC also secrete other Mediators like indolamine 2,3 dioygenase (IDO), Interleukin-10, Transforming growth factor beta (TGF-β), nitric oxide (NO) among others that have direct effects on immune cells (Uccelli and Rosbo 2015) cytokines are discussed further in chapter 1.6.2.

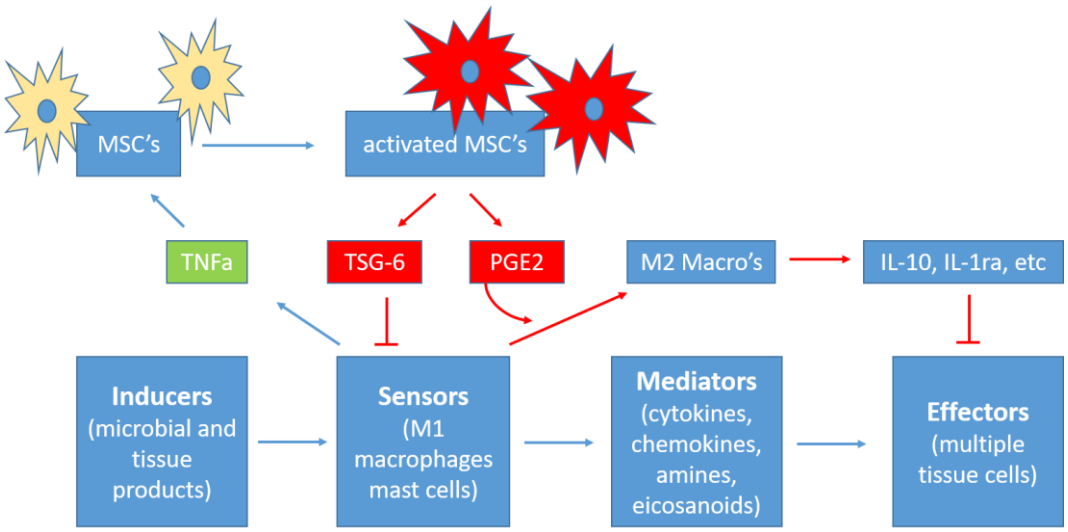


Figure 3 Schematic overview on MSC function during inflammation and the two negative feedback loops via i.) PGE_2 as well as ii.) TSG-6 secretion adapted from Prockop (2013)

Acute inflammation is an important part of the equine immune response and needed for healing to occur. However, chronic, inappropriate and excessive inflammation can lead to further damage (Dakin 2016). For example when a horse presents with acute traumatic tendinopathy of the superficial digital flexor tendon, there is disruption of tendon fibres and visible area of surrounding haemorrhage leading to a clot forming (Dowling et al. 2000). The variables commonly assessed are location, lesion type, tendon and lesion cross sectional area and fibre alignment. When hyper extension trauma has occurred, the injury will change with time. If the inflammatory response is excessive which can happen if the injury is left untreated, a distinct core lesion will become visible on an ultrasonographic exam after 3-5days. An excessive inflammatory response is also present in degenerative joint disease (DJD), osteo-arthritis (OA), where excessive inflammation of the synovium further exacerbate the initial injury leading to the induction of a chronic disease state (McIlwraith et al. 2012) and in autoimmune diseases.

Several cell types in the immune cascade are needed for healing and resolution (Dakin 2016). Tissue-specific progenitor cells or "stem cells" reside in the tendon and are involved in the process of replenishing cells lost due to natural cell turnover but also to assist in the vent of post injury repair (Richardson et al. 2007). The stem cells are activated by inflammatory cytokines (TNFα and IL-1β) and then

themselves become involved in regulating the cascade as well as re-building the tissue (Kamm et al. 2010). In a clinical application the aim is to promote and utilise the anti-inflammatory properties of the MSCs as well as their capacity for self-renewing. In equine tendinopathy, healing by fibrosis (scar tissue formation) is considered inferior due to an inherent weakness of the junction between the scar tissue and the intact tendon.

For example, Figure 4 illustrate the events seen in tendonopathies; a.) In the acute phase swelling, heat and pain is noted on palpation. b.) anatomy of interest c.) there is damage to the tendon fibres and cytokine and chemokine release which initiates an influx of inflammatory cells to the area and activates resident stromal cells. d.) Ultrasonography is not recommend in the first few days post injury as there is limited visibility of the damaged tissue. However, for SDFT core lesions; a distinct area of decreased echogenicity can be seen on the ultrasonography scan three to five days later. This is due to timing of events in the inflammatory process and secreted catabolic enzymes that cause further damage to the tendon tissue. Excessive inflammation can also cause prolonged discomfort to the animal (lameness, swelling, heat and pain). Conservative treatment of tendon injuries includes; rest, cooling therapy (cold water hosing or ice-wraps) and non-steroidal medication and will be further discussed later.

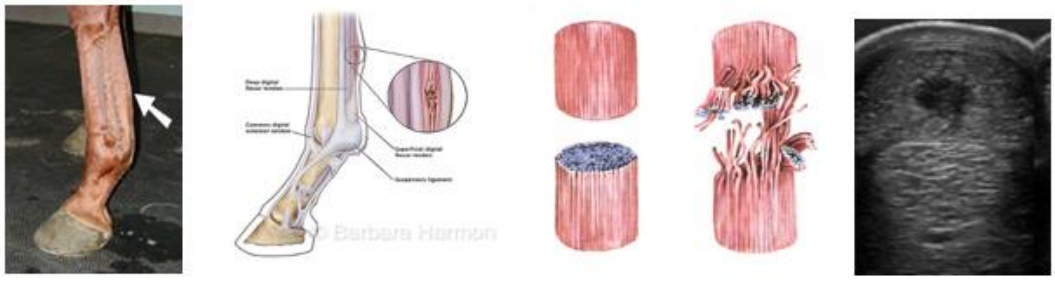


Figure 4 Superficial Digital flexor tendon injury explained a.) Physical appearance "Bowed tendon" b.) Anatomical location of injury c.) Ruptured tendon fibres d.) Ultrasonographic appearance, area of decreased echogenicity as seen on a cross sectional view (left injured, right normal). Pictures retrieved online 05/10/16

a.) http://stable equestrian.net/cms/wpcontent/uploads/2015/06/TendonInjuryText. jpg
b.) http://www.science-art.com/gallery/24357/24357_4302012164337.jpg
c.) http://www.missourifoxtrottersatoz.com/images/fiber%20bundles.jpg
d.)http://www.auntminnieeurope.com/user/images/content_images/sup_ult/2012_12_19_12_02_17_137_2012_12_19_equine_US.jpg

1.3 LAMENESS IN PERFORMANCE HORSES

Lameness is common in the performance horse as discussed above. This section will aim to describe the anatomy and function of tendons and synovial joints as these historically have been the targets of MSC therapy. This will provide an understanding of the complexity of healing these tissues and why clinicians are exploring regenerative treatment options.

1.3.1 TENDON ANATOMY AND FUNCTION

Tendons are elastic, dynamic structures that connect muscle to bone. Their function is to facilitate movement, absorb shock and support the lower limbs (Thorpe et al. 2010). Tendons are composed predominantly of water (approximately 70%), of the remaining 30% dry matter, the cellular component are made up of tenocytes which produce a dense, fibrous extra-cellular matrix of collagen type I (Dowling et al. 2000). Collagen types II, III, IV and V are also present in normal flexor tendons although in smaller quantities and in specific locations. Type II collagen is found within enthesious insertions, the specialised interface between tendon and bone and regions where the tendon changes direction around a bony prominence (Apostolakos et al. 2014). This part of the tendon has a more fibrocartilage-like nature that can withstand compressional as well as tensional forces. Types III, IV and V are found in basement membranes and endotendon. The function of the tendon is reflected in the mechanical properties of the tendon tissue, The collagen fibrils are aligned in the direction of force application (Thorpe et al. 2010), the fibrils have crimp for elasticity and extensive crosslinking, resulting in high tensile strength. The collagen fibril was previously considered to be the primary unit contributing to the strength of the tendon, however more recently, the intra- and inter-fibril collagen cross-links and electrostatic cross-links provided by the non-collagenous proteins like proteoglycans have been considered to contribute significantly to the tendons' biomechanical properties (Dowling et al. 2000). The collagen fibrils group together to form fibrils, fibres and fascicles, to form a functional tendon (Thorpe (2013).

The tenocytes are the cells of the tendon tissue, present only in small numbers in the fascicles between the collagen fibrils. Their function is to produce the collagen

and proteoglycans and remodel the matrix by secretion of enzymes, matrix metalloproteinases, for degradation of injured or defective tendon tissue (Thorpe et al. 2010). The cellularity of the superficial digital flexor tendon (SDFT) and the common digital extensor tendon (CDET) is greatly reduced up to two years of age, then more gradually during ageing (Patterson-Kane et al. 2012)

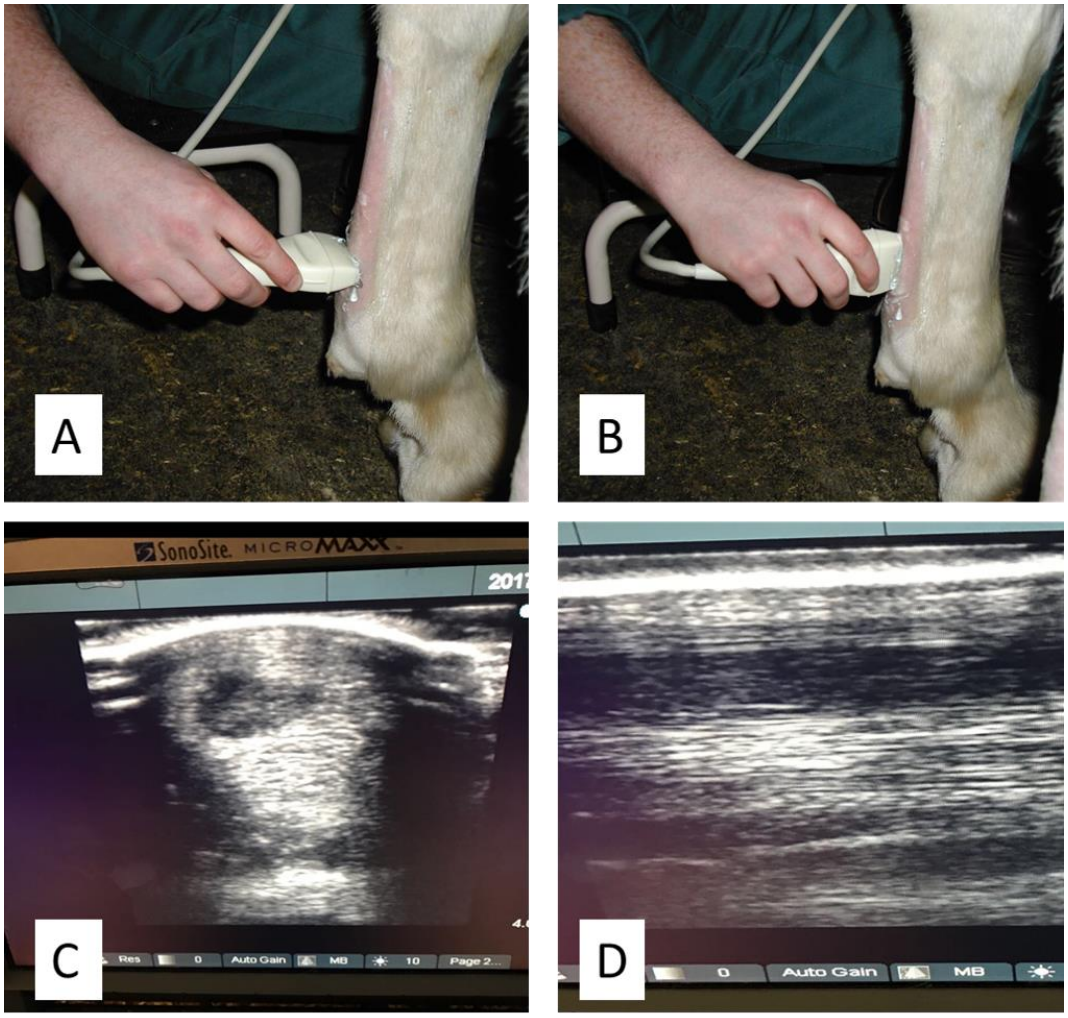


Figure 5 Ultrasound scanning of the equine superficial digital flexor tendon, A.) Transverse scanning B.) Longitudinal scanning C.) Core lesion visible (round hypoechoic area within the SDFT D.) Fibre disruption (elongated hypoechoic area). Images produced by a Sonosite Micro MAXX.

Tendon and ligament injuries are a major cause of lameness in the equine athlete and affect horses at all levels, representing a significant animal welfare concern and economic loss to the equine industry. Figure 5 is an example of a SDFT core lesion

and typical ultrasonograms. General factors implicated causing tendon injuries are fatigue, lack of fitness and poor confirmation (Dowling et al., 2002). In a survey of Thoroughbred racehorses in Hong Kong between 1992-2004 it was reported that the majority of the tendon injuries (97%) occurred in the forelimb tendons (Lam et al. 2007). This can be related to high strains placed on the equine forelimbs, which carry more than 60% of the total weight of the horse when galloping (Thorpe et al. 2010). Excessive and repeated loading like jumping and galloping, cause repetitive micro damage. This localised damage of fibrils occurs when the tendon reaches 80% of failure strain. The micro damage is accompanied by increased gene expression and production of matrix metalloproteinases in the tendon. (Hosaka et al. 2006) showed increased production of MMP-9 and TNF-α *in vitro* when tenocytes where exposed to temperatures over 40°C. The microdamage is accumulative and if not repaired by tenocytes it may result in clinical injury (Thorpe et al. 2010). Other studies have also proposed that exercise-induced hyperthermia is a possible mechanism for tendinopathy (Birch et al., 1997; Patterson-Kane et al., 2012). Increased temperature occurs within viscoelastic materials as energy is released during repeated loading, and hyperthermia is a likely contributor to degenerative changes observed in tendinopathies (Youngstrom 2015). Core temperatures of 43-45°C have been measured in the Superficial Digital Flexor Tendon (SDFT at high speed locomotion (Wilson and Goodship 1994). Tenocytes have shown prolonged heat resistance *in vitro*, however hyperthermic conditions may compromise cell metabolism and cause degeneration of matrix components and predispose to traumatic injury (Birch et al., 1997). Hosaka et al. (2006) showed that exposure of tenocytes to temperatures over 40°C significantly increased the production of MMP-9 *in vitro,* the production of the pro-inflammatory cytokine TNF-α was also increased with prolonged exposure 30-60min above 40°C. Birch et al., (1997) also described a hypoxic environment in the tendon core, which may further compromise cellular activity. Excessive loading may contribute to tendinopathy by activation of prostaglandin E_2-mediated pathways, reducing tendon stem cell population size and encouraging differentiation into non-tenocyte lineages (Youngstrom 2015).

1.3.1.1 **Healing and repair**

When a damaged tendon heals naturally, it heals by the formation of a scar and not neo-tendon, hence it loses a substantial part of its original strength and elasticity (Dakin 2016). Therefore, tendons can be said to recover structurally (*reparation*) but not functionally (*regeneration*). The importance of inflammation in tendon healing is discussed by Dakin (2016), who suggest that excessive and persistent inflammation as the main drive for fibrosis. Fibrotic scar tissue consists of collagen type III, which is less elastic than normal tendon tissue (collage type I). Scar tissue cannot withstand the same loads and stress as the original structure. Tendon injuries carry a high risk of re-injury, some studies reporting 75% (range 23-75%) (Clegg 2012; O'Meara et al. 2010; Renzi et al. 2013) of horses treated conservatively will re-injure within 2 years of the original injury. The re-injury occurs because of reduced failure strains and forces applied to the junction between scar tissue and normal tendon, will cause breakdown sooner compared with a normal healthy tendon, due to a decreased failure strain threshold (Thorpe et al. 2010).

1.3.1.2 **Treatment options for tendinopathies**

Treatment options for tendon and ligament injuries are many and often a combination of several different treatments at the various stages identified in tendon healing. i.) An initial period of stall confinement and rest is required to reduce the discomfort for the horse and attempt to stabilise the injury by not objecting it to further strain. ii.) Physical treatments are performed that aim to reduce swelling of the tendon and minimise inflammation and associated pain. These treatments include initial cold hosing, ice-boots and bandaging iii.) Anti-inflammatory treatments including: systemic non-steroidal anti-inflammatories (NSAIDs), topical dimethylsulphoxide (DMSO) and diclofenac. Other substances have been used for intra-lesional injections; historically Beta-aminopropionitrile fumarate (BAPN) was used (Alves et al. 2001; Dowling et al. 2000), but this is no longer practised (Prof C. Riley, personal communication). However, a recent paper by Jann et al. (2016) reported a favourable outcome on tendon healing using a modified hyaluronic acid gel in an *in vivo* surgical model iv.) A rigorous rehabilitation program is one of the major factors that affect the outcome of tendon healing (Rich 2014). After an initial period of box rest (1-4weeks) the horse is allowed hand walking twice daily with increasing times over a 3-4 month period

(Renzi et al. 2013). Monitoring with repeat ultra sound scans would determine the progression and trotting in a straight line may be introduced after 3-4 months. In general the rehabilitation program would be performed for 9-12months before the horse can resume to full work. Exposure to even and increasing loads by force is required to correctly align and strengthen the newly formed collagen fibres. Many practitioners lose control over their race horse patients when they are spelling or away from the track. It is imperative that communication and regular follow ups are performed to ensure the best outcome for the patient. vi.) Biological treatment options are readily available to the equine practitioner and include; platelet rich plasma (PRP) (Bosch et al. 2011; Textor 2011) or Interleukin Receptor antagonist Protein (IRAP) (Textor 2011), both which are discussed in the following chapter. vii.) Miscellaneous Therapies include; low frequency infrared laser and extracorporeal shockwave therapy (Dowling et al. 2000). Laser treatments performed by lay people are becoming more frequent in New Zealand. A recent study by Duesterdieck-Zellmer et al. (2016) showed that only 1% to 20% and 0.1% to 4% of energy were absorbed by SDFTs and DDFTs, respectively, depending on skin color, skin thickness, and applied wavelength 800 or 970nm. Their results indicated that most laser energy directed through equine skin was absorbed or scattered by the skin. Further study was performed by Husby (2016) with the conclusion that no significant effects was noted on equine fibroblasts *in vitro* using low frequency laser.

Surgical methods which have been described for tendon repair are; superior check ligament desmotomy (Dowling et al. 2000). Hu and Bramlage (2014) reported that 228 of 332 (69%) horses with superficial digital flexor tendonitis of the forelimb that were treated with superior check ligament desmotomy successfully returned to racing. Pin-firing of tendons has been described by Renzi et al. (2013), where thermocautery was applied on the metacarpal flexor area for a few seconds, at an interval of 2cm. The procedure was performed under local anaesthesia. Ten months after pin-firing treatment, 3 horses (25%) were in training and able to return to their previous activity; while the remaining 9 horses (75%) were retired. The method of pin-firing is no longer practiced in New Zealand as it is considered unethical.

There are no traditional treatment options that reliably and efficiently treat tendon injuries hence why a biological therapy that provides anti-inflammatory effects and assist in tissue regeneration is a desirable prospect to clinicians. There is also the need to understand that no single treatment will alone heal a tendon lesion. Patients will need a combination of several treatments like rest, anti-inflammatory treatments and rehabilitation as well as biological treatments to achieve the best outcome.

1.3.2 JOINT DISEASE AND TREATMENT OPTIONS

Joint disease is commonly observed in performance horses (Riggs 2006). Together, joint injury and joint disease represent a large majority of the equine clinician's caseload (Goodrich and Nixon 2006). To understand the development of disease a brief description of joint anatomy and normal function of its components are given.

A synovial joint is composed of several important structures; the synovial membrane, articular (hyaline) cartilage and subchondral bone supported by an interconnected lattice with predominantly vertical arcades of cancellous bone (Goodrich & Nixon 2006). Microscopically, hyaline articular cartilage is composed of approximately 75% water, 15% type II collagen, 10% proteoglycans and a small number of chondrocytes (2%). Proteoglycans, consists of a large number of highly negatively charged polysulphated glycosaminoglycans, which are responsible for drawing water molecules into the extracellular matrix and providing compressive stiffness. The cartilage act as a shock absorber and the synovial fluid lubricates the cartilage surfaces. The synovial membrane and joint capsule are important structures that are involved in joint homeostasis (Kidd et al. 2001).

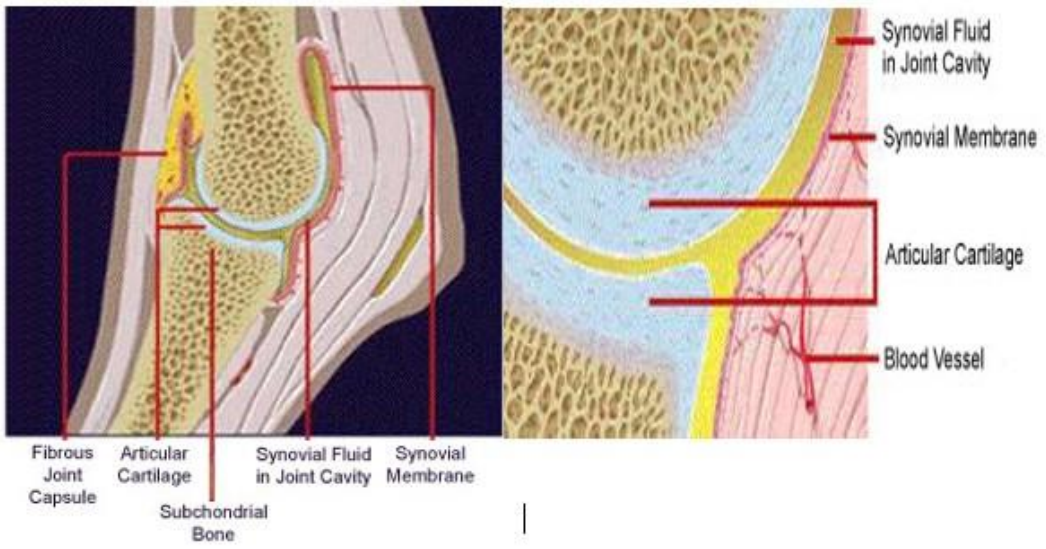


Figure 6 A schematic overview of the anatomy of a synovial joint. Retrieved online 15/10/16 from https://www.horsejournals.com/arthritis-horses-understanding-treating-joint-disease

1.3.2.1 **Arthropathy**

Joint disease is common in horses due to the demands for athletic performance (Riggs 2006). Direct trauma to articular cartilage as well as synovitis and other soft tissue changes are significant components of the pathogenesis of arthropathy (Wayne 1982). Recognition of early changes and appropriate treatment is important. In the young, growing horse we see developmental orthopaedic disease like osteochondrosis (Pool 1993). In the performance and older horse we see osteoarthritis as a major cause of lameness (Kidd et al. 2001). OA is characterised by progressive and permanent disease of the cartilage and associated subchondral bone and synovial tissues. The cartilage is defective due to trauma or degeneration and loses its shock-absorbing ability. Gross findings include fibrillation, erosion and wear lines in the articular cartilage (McIlwraith, 1996). The subchondral bone is subsequently exposed, inflammatory mediators are constantly produced which cause a synovitis with associated pain and discomfort to the horse, expressed as lameness. OA is characterised by the presence of a chronic inflammatory disease process. Interleukin 1 is proposed to be the major inducer of the development of osteoarthritis (McIlwraith et al. 2012).

1.3.2.2 **Conventional treatment options for arthropathies**

Medical treatment methods include physical therapy, use of anti-inflammatory drugs, joint lavage, sodium hyaluronate, and synovectomy (McIlwraith et al. 2012). When there are cartilage and bony changes, surgical approach is preferable and the use of articular cartilage curettage, osteophyte removal, radiation therapy, and surgical arthrodesis is appropriate in some cases.

One option to reduce inflammation is by administration of systemic and/or locally administered (intra-articular) medication. Practitioners commonly use non-steroidal medication such as; phenylbutazone, meloxicam or ketoprofen systemically. Phenylbutazone has been shown to be clinically effective in treating acute synovitis, De Grauw et al. (2014) found that it does not limit inflammation-induced cartilage catabolism and may transiently reduce collagen anabolism. In contrast the same group also studied the effects on meloxicam and found a significant suppression of inflammatory synovial fluid biomarkers, decreased MMP activity and a decrease in the cartilage turnover (Grauw et al. 2009). This was also suggested much earlier by Clegg et al. (1998) who evaluated MMP activity of both NSAIDs and several steroids. Intra-articular administered steroidal anti-inflammatories such as; triamcinolone, betamethasone or methylprednisolone acetate are very commonly used to treat osteoarthritis in the horse (Clegg et al. 1998; Goodrich and Nixon 2006; Souza 2016). In several equine studies of joint inflammation (either by osteochondral chip model or chemically induced arthritis), corticosteroids improved outward signs of joint inflammation; but articular cartilage degeneration and local bone necrosis could be observed histologically (Trotter and McIlwraith 1996). However, McIlwraith (2003) and later (Goodrich and Nixon 2006) showed no detrimental effects in treated joints, their results could possibly be explained due to decreased dosage and exposure intervals compared to initial trials by (Trotter and McIlwraith 1996). Interleukin-1 receptor agonist protein (IRAP) is also used commonly as an intra-articular therapy in OA to counteract the actions of the pro-inflammatory cytokine interleukin-1β (IL-1β), see chapter 4.3.2. A recently developed polyacrylamide gel is currently undergoing clinical trials in New Zealand and Australia. It is proposed that the 2.5% polyacrylamide gel attach to the defective cartilage and the exposed subchondral bone and has visco-elastic

properties, thus forming an artificial pseudo cartilage. Tnibar et al. (2015) showed a decrease in joint effusion and lameness grade assessed over a 24month period.

Supplementation of drugs or nutraceuticals to enhance the joint environment is of commercial significance. Visco-supplementation with non-sulfated glycosamino-glycans; hyaluronic acid, given intra-articular, intra-muscular, intra-venously or orally depending on the preparation. Oral chondroprotectants such as glucosamine and/or chondroitin sulphate are thought to promote viscosity of the synovial fluid composition and aid in lubrication of the joint (Neil et al. 2005). A recent study by Koenig et al. (2014) assessed the effects of intra-venous sodium pentosane polysulfate, N-acetyl glucosamine and sodium hyaluronan in horses with induced OA. They found no significant effect from the treatment and instead synovial fluid total protein concentration and white blood cell count were higher in OA joints of treated horses compared with saline treated horses.

1.3.3 BIOLOGICAL TREATMENT OPTIONS

Biological therapies offer an alternative treatment through the administration of cultured and purified cells, or cellular products, which have naturally occurring anti-inflammatory and regenerative properties (Richardson et al. 2007).

1.3.3.1 **Platelet Rich Plasma (PRP)**

Platelet Rich Plasma is a biological treatment option currently used in practice to assist healing of core lesions in tendons or ligamentous tears (Bosch et al. 2010). This autologous treatment is prepared freshly from the patients' own blood and consists of a highly concentrated solution of platelets (thrombocytes) and growth factors. Platelets are cytoplasmic fragments derived from bone marrow megakaryocytes and contain several types of growth factors and cytokines that might stimulate wound healing (Borena et al. 2015). Platelets activate the inflammatory process by releasing cytokines (such as interleukin (IL)-1α, -1β and -6 and tumour necrosis factor-alpha (TNF-α), enhance collagen production (FGF-2, IGF-1, TGF-β), stimulate fibroblast to myofibroblast transformation (TGF-β), initiate angiogenesis (EGF-2. VEGF-A, TGF-β) and promote re-

epithelialisation (EGF, FGF-2, IGF-1, TGF-α). The aim with the treatment is to reduce inflammation and assist healing (Dunkel et al. 2012). PRP can be prepared overnight so offers a rapid treatment option at time of injury. This treatment option is available in New Zealand; (Arthrex ACP, Arthrex Vet Systems, New Zealand). The treatment has to be used fresh as it contains living platelets and cryopreservation is not appropriate. The main difficulty in assessing the therapeutic effects of PRP is to define which growth factor(s) are responsible for the observed effects since the same mixture of growth factors are present in the injured tissue and associated with natural scar healing (Borena et al. 2015).

1.3.3.2 **Interleukin Receptor-Antagonist Protein (IRAP)**

In joints with osteoarthritis activated fibroblasts produce IL-1, which then induce an increase in production of collagenase and prostaglandin E2 (Textor 2011). The principle behind IRAP is that an antagonist protein (Interleukin 1receptor antagonist protein, IL-1Ra) will bind to IL-1 receptor and block the destructive effects of IL-1. In osteoarthritic joints, there is not sufficient IL-1Ra produced to block the destructive effects of the increased IL-1. The result is inflammation, pain, and eventually cartilage destruction. Autologous blood can be collected in specialist syringes containing coated glass beads and cultured for 24hours to stimulate IRAP production (Hraha et al. 2011). This produces an acellular conditioned serum (ACS) which has many components; cytokines, IRAP and growth factors. In the commercial kit Arthrex IRAP II, monocytes (a type of white blood cell) bind to the glass beads. The cells are then stimulated to produce regenerative and anti-inflammatory proteins without the addition of drugs. This process takes place over an incubation period of 24 hours. Several syringes can be prepared from one blood collection and they can be frozen at -20°C and used for repeated treatments.

1.3.3.3 **Mesenchymal stromal cells (MSC)**

Mesenchymal stem cell therapy is now a readily available option for treatment of joint and tendon injuries in the horse. In New Zealand there is a commercial kit available for extraction of autologous stromal vascular fraction and autologous as well as allogenic bone marrow derived stromal cell treatments are offered at two

equine veterinary clinics; (Veterinary Associates Equine and Farm, Karaka and EquiBreed NZ Ltd, Te Awamutu). Indeed, there are many peer reviewed reports of clinical cases treated with MSCs derived from several sources; adipose tissue (Ricco et al. 2013; Rich 2014) , bone marrow (Ferris et al. 2014; Godwin et al. 2012; Guest et al. 2010b; Herthel 2001; Pacini et al. 2007; Smith et al. 2003) and peripheral blood (Broeckx et al. 2014b).

Improvements have been reported on the rate and quality of tissue regeneration compared to conventional treatment (Frisbie and Smith 2010; Renzi et al. 2013; Rich 2014). Nixon et al. (2012) discuss in their review the primary role of MSC and suggest it to be their indirect effects on tissue homeostasis when used in tendon repair. They reported a consistent finding in the equine model to be that the ongoing degradation process associated with tendon rupture is diminished after treatment with adipose stromal vascular fraction or bone marrow derived MSC. A recent study by Rich (2014) included 83 horses treated with autologous stromal fraction from fat. All horses also followed a stringent rehabilitation program over 12months. This study found that 84.6% horses with fore-limb tendon injuries and 82.1% of horses with hind-limb tendon injuries, returned to full work at the same or higher level than before injury. The horses did not re-injure within the 12months follow up period. An Italian group led by Pacini (2007), showed that 9/11 horses with SDFT injuries treated with autologous equine bone marrow derived stem cells (BMSC), returned to racing with no re-injuries during the two year follow up period. The control group was treated with pin-firing and all horses (15/15) re-injured within 12 months. Godwin et al., (2011) showed 98% of horses (111/113) returned to work after treatment with BMSC, with a lower than average re-injury rate of 27.4% reported. The latest studies by Belgian researchers (Broeckx and Spaas 2011-2013) investigated peripheral blood derived stem cells (pBSC) as a treatment option for tendon or ligament lesions (SDFT or SL) and they found that 96% (24/25) of the treated horses performed at the same or higher level than before the injury. Broeckxs et al., (2013) showed no adverse effects on clinical outcomes using allogenic, tenogenic induced peripheral blood derived MSC injected into equine tendons. Stem cells have also been isolated from equine tendon tissue (Burk et al., 2013, Violini 2009). Violini et al., (2009) and Youngstrom (2015) suggest

that stem cells isolated from tendon, may have an inherited propensity for tendon repair and thus prove to be a superior alternative for tendon repair than MSC from other sources.

1.4 MESENCHYMAL STEM AND STROMAL CELLS

Mesenchymal stem cells were first discovered in bone marrow by (Friedenstein et al. 1987) and he described ‘‘osteogenic stem cells’’ as “clonal and fibroblastic stromal cells that formed adherent colonies in culture and had robust osteogenic potential”. These cells were first termed ‘‘mesenchymal stem cells’’ by Caplan (1991) and their multi-lineage differentiation potential into bone, cartilage and fat was further characterised by Pittenger et al. (1999). Mesenchymal stem cells and stromal cells have been isolated from a wide range of species and tissues using numerous techniques. The mesenchymal tissue origins are illustrated in Figure 7.

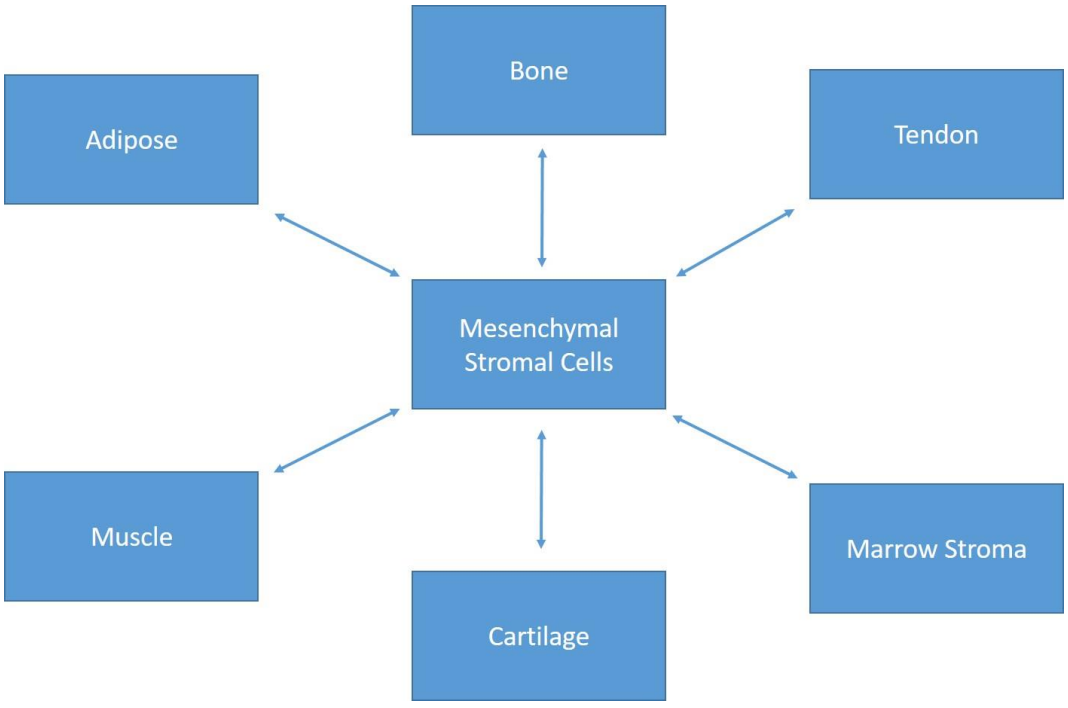


Figure 7 Mesenchymal stem cell tissue origins.

1.4.1 MESENCHYMAL "STEM" AND STROMAL CELLS

1.4.1.1 Definition

In the human field, the term "MSC" is mainly associated with *Mesenchymal Stem Cells* and these cell lines are characterised by long-term self-renewal (*in vivo)* and trilineage differentiation abilities *(in vitro)* (Caplan 1991). The International Society for Cellular Therapy (ISCT) states that the term "stem cell" should be used only for those cells that (i) show long-term survival *in vivo*, (ii) have self-renewal capacities and (iii) possess the ability for tissue re-population with multi lineage differentiation (Horwitz and Dominici 2008). According to human definition criteria these cells should also i.) Express a specific set of cell surface markers and ii.) Have capacity for trilineage differentiation; after specific stimulation become adipocytes, chondrocytes and osteoblasts *in vitro* (De Schauwer et al. 2011; Dominici et al. 2006). These criteria are stated in the publication of the *Minimal Criteria to Define Human MSCs in 2006* by the ISCT (Dominici et al. 2006) and set the standards by which human MSCs can be functionally and phenotypically defined.

In horses, stem cell differentiation capacity is still poorly understood. There are no reports published that provide evidence of long-term self-renewal and full differentiation capacity *in vivo.* In fact a study by Guest et al. (2008) showed less than 5% survival of bone marrow derived stem cells injected into damaged superficial digital flexor tendons at 10 days post injection. Thus, before long term survival *in vivo* has been established the more general term *Mesenchymal Stromal Cells* (MSC) is deemed to be more appropriate in equine medicine (Penny et al. 2012; Ulrich et al. 2012). If a standardised method is used for isolation, characterisation and definition of equine MSC the clinical application would be more predictable and efficacy between studies could be accurately compared. As MSCs become better characterised for their *in vitro* and *in vivo* capacities, it is likely that the definition and nomenclature will continue to evolve (Tessier et al. 2015).

1.4.1.2 **Mesenchymal stromal cells - cell cycle events**

The cell cycle of MSC is the same as for somatic cells; they undergo mitosis to divide into two identical cells. There are two main phases of mitotic division, the interphase and the mitotic phase (Colville and Bassert 2002) Figure 8 below is a schematic representation of the cell cycle

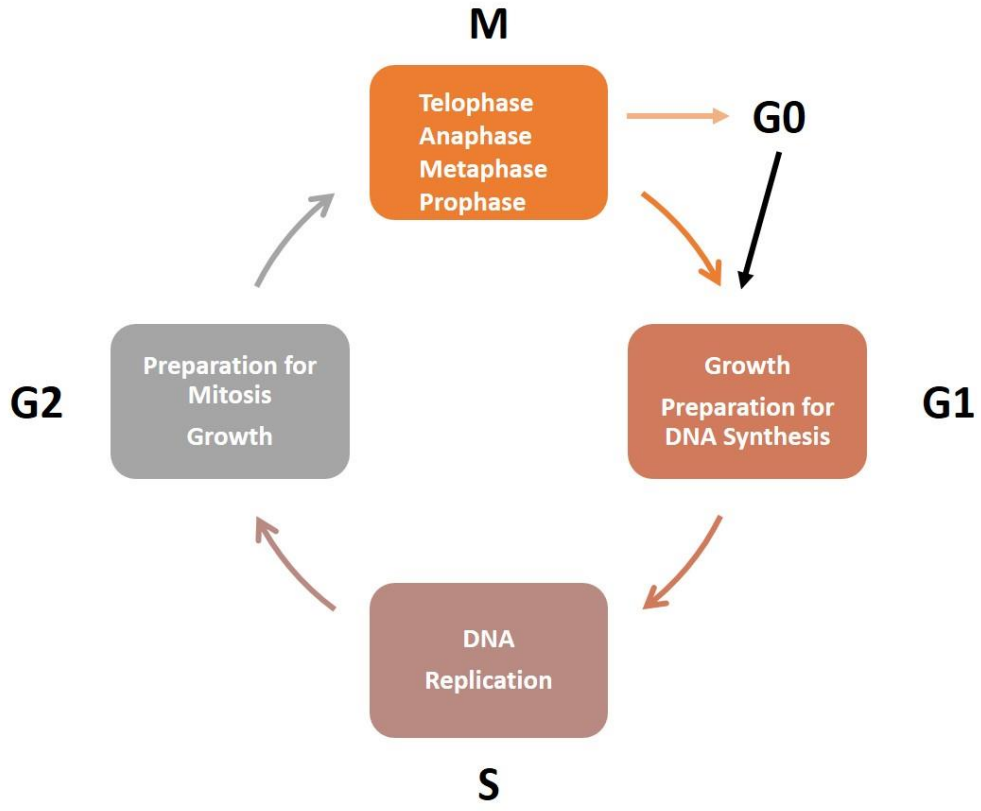


Figure 8 Simplified view of the cell cycle and mitotic division. G_0 = Resting phase G1= Preparation increase in cell size S=Synthesis G2= Growth phase M=Mitosis and cell division

The interphase is the time where the cell carries out normal metabolic functions and no division occurs. The interphase has been subdivided into three consecutive stages. First, the growth one (G_1) phase is a period of intense growth and metabolic activity. Cells that proceed past the restriction point in the G1 phase enter the S phase, whereas those that do not pass the restriction point remain undivided. These undivided cells can withdraw from the cell cycle and enter the G0 phase: a state in which cells are termed quiescent or dormant (Nakamura-Ishizu et al. 2014). The second phase, the synthetic (S) phase involves deoxyribonucleic acid (DNA)

synthesis and replication. The third phase is the growth two (G_2) phase, which involves the synthesis of enzymes and proteins for cell division and growth (Freshney, 2010). On completion of interphase the cell enters the mitotic phase. The cell will actively divide to produce two daughter cells with identical copies of the original cell's DNA. Mitotic division comprises of five phases; prophase, prometaphase, metaphase, anaphase and telophase.

Quiescence is a property that characterises tissue-resident stem cells and they comprise a G0 dormant reserve that can replenish tissues during homeostasis (Nakamura-Ishizu et al. 2014). The regulation of stem cell self-renewal must balance the regenerative needs of tissues that persist throughout life (Orford and Scadden 2008). The non-cycling cells in the G0 phase can either reversibly re-enter the cell cycle and remain dormant or divide, or lose the potential to cycle and become senescent. The number of times a cell can self-replicate is a genetically controlled event known as senescence, which is regulated by several different genes (Freshney, 2010). Cells derived from adult mesenchymal tissue can only be expanded in culture for a limited number of times before they lose their ability to divide. With increasing passages, telomeres become shorter in cultured MSC (Mathon and Lloyd 2001). Telomeres are repetitive DNA sequences located at the linear ends of the chromosomes, and with each cell division they are not fully replicated but become progressively shorter. This corresponds with a decline in the differentiation potential of that cell population.

Cells that commit and become terminally differentiated lose the ability for self-renewal. A mesenchymal–epithelial transition (MET) is the biological process that involves the transition from motile, multipolar or spindle-shaped mesenchymal cells to planar arrays of polarised epithelial cells (Li et al. 2011) Figure 9. Developmental METs have been studied most extensively in embryogenesis. However, MET events are also related to tissue construction and wound healing (Jordan et al. 2011). Stem/stromal cells are involved in all of these processes. The mechanism in which MET occurs is by upregulation of epithelium-associated genes and downregulation of mesenchyme-associated genes, each process has a unique signalling pathway to induce MET. Reverse MET so called epithelial-mesenchymal transition (EMT) has been categorised into three types;

developmental (Type I), fibrosis and wound healing (Type II), and metastatic neoplasia (Jordan et al. 2011; Kalluri and Weinberg 2009).

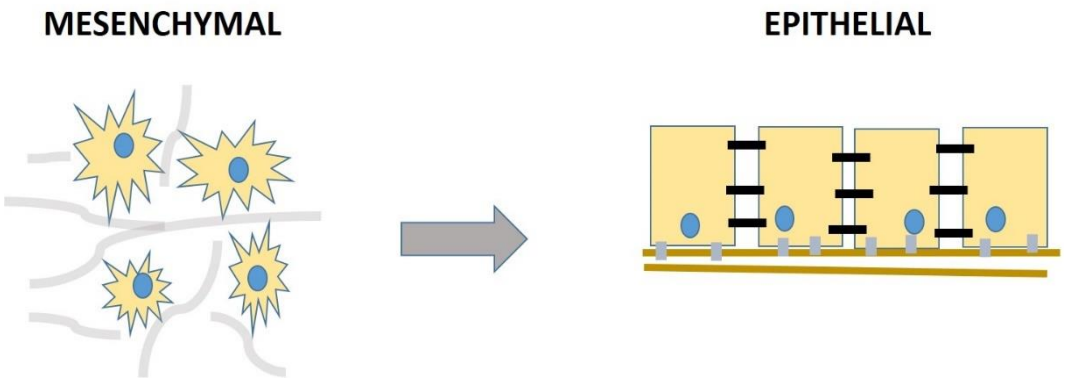


Figure 9 Mesenchymal to epithelial transition simplified.

1.4.2 MESENCHYMAL STROMAL CELL CULTURE METHODS.

There are a myriad of different culture protocols for MSC from various tissue sources published in the literature (Freshney 2010; Masters and Stacey 2007). There is a need for standardisation of protocols to efficiently compare studies and their results. In brief; The tissue is harvested from the animal using a variety of surgical methods suitable for the specific tissue source. After surgical harvest there is further processing involved to mechanically and/or enzymatically digest the tissue. Then a density gradient step involving a carbohydrate like Ficoll or Percoll or Lymphoprep is performed by centrifugation to isolate a heterogeneous suspension of the mononuclear cells (MNCs) from that tissue (Pullen et al. 2016). The primary cultures of MNCs are cultured *in vitro* under standard cell culture conditions; in polystyrene plastic flasks with tissue culture medium and maintained at 37°C in 5% CO_2 (Freshney 2010).

Equine MSC and dermal fibroblasts are multipotent cells that are plastic-adherent when maintained under *in vitro* culture (Radtke et al. 2013; Ranera et al. 2012). The cells are commonly grown in polystyrene cell culture flasks (25 to 75cm^2) at 37°C in 5% CO_2 in a humidified incubator. The MSC form colonies initially, which develop into a monolayer with time. Daily monitoring where the cells are observed for confluence (percentage of cell coverage) is performed. The adherent stromal cells have a spindle-like morphology and grow in a monolayer.

The culture medium used for *in vitro* cell culture generally consists of Dulbeccos Minimal Eagles Medium (DMEM) at a low (1g/1L) or high (4.5g/1L) concentration of glucose, foetal bovine serum between 10-30%, antibiotics (penicillin, streptomycin and/or gentamicin), and an antifungal agent (amphotericin B) (Freshney 2010).

In cell culture, nutrients become depleted and metabolic products, which may be toxic to cells increase in concentration (Masters and Stacey 2007). Frequency of medium change depends on the cell line and the type of medium. It is important to relate the changing of the medium to the health of the cells and the timing of the experiment. The cells rapidly utilize the nutrients and release metabolites into the medium, resulting in a lowering of pH. Buffers are required to standardise pH levels (Masters and Stacey 2007). Most mammalian cells grow at pH levels of 7.2–7.4. The culture media contain bicarbonate, which requires a 5% carbon dioxide atmosphere to maintain the pH at 7.4. HEPES, an organic buffer, is often added to medium to reduce the dependence on bicarbonate. Phenol red is added to medium for ease of pH detection by eye and ranges in colour, optimal pH 7.4 is red in colour.

The medium is changed every 3-4days in adherent culture systems (Freshney 2010). To avoid stress to the cells, the medium is pre-equilibrated in the incubator at 37°C at 5% CO_2. All of the spent medium is removed and replaced with new warm medium at each change (Masters and Stacey 2007). For comparative purposes, it is important to use the same formulation of medium throughout an experiment to eliminate variable. Also, when several cell lines are used, it is essential to grow all the cells in the same medium to exclude this as a variable.

When confluence reaches 60-80% the cells are detached by mechanical or enzymatic release agents (commonly 0.05% Trypsin EDTA) and counted and sub-cultured in new culture flasks and/or cryogenically preserved. Purified trypsin is used in most laboratories at a concentration of 0.25% (wt/vol) in 0.5 mM EDTA (disodium ethylenediaminetetraacetic acid) dissolved in calcium- and magnesium-free phosphate-buffered saline (Masters and Stacey 2007). Optimum activity of trypsin is achieved at 37°C, so pre-warmed trypsin may speed up cell detachment. Long incubation times and high trypsin concentrations tend to damage the cells, causing them to stick together. For passaging the cells are re-seeded in new flasks

for sub-culture, a common cell density is $5.0x10^3$ cells per cm^2 when passaging MSC. The sub-culture process is an attempt to obtain a homogenous population of cells. This population of cells may be morphologically identical but there is great likelihood the cells differ in growth kinetics and differentiation potentials. Thus, any given cell population are assumed to display some level of heterogenicity (Freshney 2010).

During cryopreservation, aliquots of cells are re-suspended in cryogenic media consisting of DMEM, foetal bovine serum or autologous equine serum (5-90%) and dimethyl sulphoxide (DMSO) (5-10%) as a cryoprotective agent (Redmond Hubbard 2014). The MSC are frozen by a controlled freezing protocol at the rate of -1°C per minute in a Mr Frosty container (5100-0001, ThermoFisher Scientific, NZ). It is good laboratory practice to test all cell lines for *Mycoplasma* species, using testing PCR method, that will yield highly sensitive and specific results (Masters and Stacey 2007). Mycoplasma contamination can alter many biochemical processes.

Heterogenicity of cell populations combined with the different techniques used to isolate, culture and define MSC means that there is large experimental variability, inefficient differentiation and often contradictory or incomparable results between studies. The question is if there is a functional difference of MSC derived from different donors and tissues? An attempt can be done to standardise parameters like sex, age and health status, to allow comparison between studies. However, a study by Carter-Arnold et al. (2014) described the in vitro adherence, proliferation and potential for differentiation of equine BMSC harvested from middle-aged mares (10-13 years old). Thus, they were matched for sex and age. The result of this study confirmed than donor-to-donor variation in equine BMSC existed; one out of six donors demonstrated a higher rate of proliferation, enhanced ability for passaging and superior *in vitro* differentiation. Comparatively, equine BMSC from two donors demonstrated a lower rate of proliferation and lack of osteogenic and chondrogenic differentiation. Schnabel et al (2013) showed a large variability in MHC-II expression between ten equine donors of bone marrow derived MSC. However, it is impossible for different studies to use the same donors and cell populations. And

even if matching for sex and age there appear to be variability as per Carter-Arnold et al. (2014). Thus, it should be noted that donor-to-donor variability is unavoidable. Other external factors like; growth conditions, culture media, environmental contamination and operator experience and technique can also affect the outcome (Freshney 2010; Ulrich et al. 2012). Many of these factors can be standardised by using a standard operating procedure (SOP) to minimise variability. Thus, the main cause of variability between studies will ultimately be the individual donors and cell lines derived from those.

1.4.3 IMMUNO-PHENOTYPING OF MESENCHYMAL STROMAL CELLS

1.4.3.1 Cluster of Differentiation (CD) cell surface markers

Mesenchymal stromal cells display cell surface antigens termed cluster of differentiation markers (CD markers). According to definition criteria, human MSC should express the cell surface markers $CD73^+$, $CD90^+$ and CD105+ and lack expression of $CD14^-$ or $CD11b^-$, $CD34^-$, $CD45^-$, $CD79^-$ or $CD19^-$ and $MHC\text{-}II^-$ (Dominici et al. 2006). Besides the markers defined by the ISCT, additional cell surface markers including $CD29^+$, $CD44^+$, $CD106^+$ and $CD166^+$, have been reported to be expressed by human MSC (Dominici et al. 2006). The cluster of differentiation markers on MSCs share many common features with the CD markers on endothelial, epithelial and muscle cells. Thus, a panel of antigens is necessary to identify a cell as a MSC. The MSCs can be analysed and sorted on the basis of their CD markers. The methods used is either immunocytochemistry, where fluorescent labelled antibodies are applied to fixed cells and evaluated with fluorescent microscopy, or flow cytometry.

1.4.3.2 Flow cytometry

Flow cytometry has historically been the gold standard for determining the physical presence of CD markers (De Schauwer et al. 2011). Flow cytometry technology was developed 50 years ago by Mack Fulwyler (Robinson and Roederer 2015). Four years later geneticist Leonard Herzenberg used the discovery of mono-clonal antibodies by César Milstein and Georges Köhler at Cambridge University, together

with the idea of labelling these antibodies with a fluorescent dye to enable cells to be viably sorted into different populations. This was the initial work for the "fluorescence-activated cell sorting" (FACS) technique. Live cells labelled with monoclonal antibodies conjugated to fluorescent dyes, that are maintained in suspension are passed in front of a laser detector (Preffer and Dombkowski 2009). This allows for rapid and simultaneous analysis of multiple parameters such as size, biochemical and antigenicity. Flow cytometry is suitable for cell counting, biomarker detection and also cell or particle sorting to purify populations of interest (Robinson and Roederer 2015). Its application has been of great use in biological research as well as clinical diagnostics, and have led to innumerable advances in immunology and cell biology.

1.4.3.3 **Polymerase chain reaction**

More recently real-time polymerase chain reaction (RT-PCR) has been used to determine the gene expression of the same cell surface markers that traditionally have been evaluated by flow cytometry. The timing is important for PCR analysis as differentiating MSC show a temporal variation in expression of these specific markers (unpublished data Redmond-Hubbard 2014). For example; Wang et al. (2015) did a temporal analysis on days 7, 14, 21 and 28 of expression of osteogenic gene markers including; osteocalcin, osteopontin, Runt-related transcription factor 2 (Runx2), alkaline phosphatase and collagen type I. Quantitative reverse transcription-polymerase chain reaction, q-PCR was used for the analysis, and they reported that human bone marrow derived MSC needed 14days exposure to induction media to express osteopontin and Runx-2 genes and at least 21 days exposure to induction media to get a significant increase in osteocalcin expression. Expression of alkaline phosphatase and collagen type 1 genes reached peak levels on day 21.

1.4.3.4 **Monoclonal antibodies**

In equine veterinary medicine immuno-phenotyping can be difficult due to the lack of commercially available monoclonal "anti-horse" antibodies. Currently there are commercially available antibodies for four equine epitopes; CD13, CD44, CD90 and MHC-II (Burk et al. 2013a; De Schauwer et al. 2011). Studies listed in Table 1 have reported the immuno-phenotype of equine MSC and use this data as the

confirmation of stemness of their stromal cell population. De Schauwer and colleagues (2011) used a qualitative approach and proposed a standardised set of markers of stemness. They performed a large study to validate the cross-reactivity of a large number of human and murine antibodies with cell surface markers present on equine MSC. They found that many of the anti-human and anti-mouse antibodies did not cross react with the equine antigens. For example, none of the anti-human CD34 antibodies recognised equine CD34, still several studies have reported a negative CD34 expression (Iacono et al. 2012; Maia et al. 2013; Radtke et al. 2013). Based on this work De Schauwer et al. (2011) derived a distinct panel of CD markers that are suitable for the characterisation of equine MSC. Their suggestion is that equine MSC should positively express CD29, CD44 and CD90 and lack expression of CD14, CD79α and MHC-II. They used a human mononuclear cells as positive control for CD45 and found that 65.3% of the human MNC expressed CD45. They also compared the immuno-phenotype with gene expression at mRNA level and found that it can be used to support the findings of marker expression, especially when the CD marker was absent or when no suitable antibody was available.

Schnabel et al. (2014) immuno-phenotyped equine bone marrow derived stromal cells (10 donor horses = 10 BMSC cell lines plus three repeat bone marrow aspirates; N=13) as well as one dermal fibroblast (DF) cell line (N=1). They evaluated the expression of following CD-markers: MHC-I, MCH-II, CD29, CD44, CD90, LFA-1 and negative markers CD45RB and major histocompatibility complex I and II (MHC-I/II) with flow cytometry. The major histocompatibility complex (MHC) is a set of cell surface proteins essential for the acquired immune system to recognise foreign molecules. MHC molecules on the cell surface bind to protein fragments derived from invading pathogens and display these to notify T-cells. Gustafson et al. (2004) showed that the equine MHC is a single gene-dense region, similar in structure to the human MHC in contrast to ruminants and pigs, which show a disrupted gene sequence. Lymphocyte function-associated antigen 1, also known as LFA-1 is involved in the binding of T-cells and also on B-cells, macrophages and neutrophils to the antigen presenting cell.

The immuno-phenotype identified by Schnabel et al (2013) was; positive for MHC-I, MHC-II, CD44, CD29 and CD90 and negative for LFA-1 and CD45. This was true for all CD markers but CD90, where one horse (1/10) only had a smaller number (30%) of cells expressing this marker. The expression of LFA-1 showed greater variability, where 6/10 BMSC lines were positive (>10% cells displayed this marker) and the remainder (4/10) were negative. At passage two (P2) 11 out of the 13 samples were positive for MHC-II, with only one (1/10) BMSC line was completely negative for MHC-II and another cell line (1/10) had very small percentage of cells (10.05%) that expressed MHC-II. These results were in contrast to De Schauwer et al. (2011) who suggested per definition equine MSC should be negative for MHC-II. Schnabel et al. (2014) then hypothesised that cell lines would initially be MHC-II positive, but with increasing number of passages (P2-P8) *in vitro*, would lose the expression of this antigen presenting marker. They continued to do a temporal study of MHC-II expression of their BMSC cell lines after passaging P2-P8. Their results showed heterogenicity in the MHC-II expression with 11/13 cell lines being initially positive for MHC-II (>10%) and at passage 8 this number was reduced to 6/13 cell lines remaining positive for MHC-II. They used a modified mixed leukocyte reaction to evaluate the possibility of MSC to provoke an immune response. Their results showed that *MHC-II-mismatched* MSC are capable of inciting an immune reaction *in vitro* and they suggest therefore that immuno-phenotyping of MSC is very important when selecting donor animals for allogenic stem cell treatments to avoid an undesirable immune reaction in the recipient. Pezzanite et al. (2015) injected between $30x10^6$ and $50x10^6$ allogenic bone marrow derived stromal cells intra-dermally in the neck of the recipient horses and then evaluated the recipients serum for any immune response by using the standard two-stage lymphocyte micro-cytotoxicity dye-exclusion test. They found that in some cases there was a strong response and cross reactive antibodies were formed, thus a repeated treatment could result in an excessive inflammatory response. Broeckx et al. (2013) injected allogenic frozen/thawed peripheral blood derived stromal cells (Veno-Cell®, Global Stem Cell Technology, Belgium) intravenously in six horses and performed a temporal haematological study. The concentration of cells was not reported in this study and the carrier fluid was DMEM and 10% DMSO. Results showed the cortisol levels (P=0.0490) and

number of neutrophils (P=0.0042) were significantly higher, while glucose levels were significantly lower (P=0.0330) in all the treated horses. At Day 1, the blood thrombocyte levels were significantly higher in the treated group as compared to the control group (P<0.0001), however this was a transient change and not noted with increasing time. The same authors later performed a study of 291 horses receiving allogenic peripheral blood derived stromal cells (Veno-Cell®, Global Stem Cell Technology, Belgium) at a concentration of $0.2x10^6$ cells as a single or repeated injections as well as one group receiving $1x10^6$ cells (Broeckx et al. 2014a). No negative side effects were reported in this study, however the horses only received a daily clinical exam for ten days and no blood parameters were evaluated.

MSC Source	Positive marker	Negative marker	Reference
AT, BM, Muscle, Periosteal tissue	CD44, CD90	CD45, CD34	Radtke, C.L. *et al.* (2013) Am. J. Vet. Res. **74**:790.
BM	CD44, CD90, Vimentin, PCNA	CD13, CD34	Maia, L. *et al.* (2013) Microsc. Res. Tech. **76**:618.
Amniotic	CD44, CD90, CD105	CD19, CD20, CD28, CD38, CD62L, CD200, CD31, CD62P, CD34, CD41a	Seo, M.-S. *et al.* (2013) J. Vet. Sci. **14**:151.
UCB	CD29, CD44, CD90	CD45, CD73, CD79 alpha, CD105, MHC Class II	De Schauwer, C. *et al.* (2013) Equine Vet. J. **45**:518.
AT, BM	CD29, CD90	-	Ranera, B. *et al.* (2012) BMC Vet. Res. **8**:142.
PB	CD29, CD44, CD90, CD105	CD45, CD79 alpha, MHC Class II	Spaas, J.H. *et al.* (2013) Vet. J. **195**:107.
PB	CD51, CD90, CD105	-	Dhar, M. *et al.* (2012) Equine Vet. J. **44**:600.
Amniotic, UCB, WJ	CD90, CD44, CD105	CD34, CD14, CD45	Iacono, E. *et al.* (2012) Reproduction **143**:455.
UCB	CD29, CD44, CD90	CD79 alpha, MHC-II	De Schauwer, C. *et al.* (2011) Tissue Eng. Part C **17**:1061.
UCB	CD29, CD44, CD90	MHC-I, MHC-II, CD4, CD8, CD11a/18 and CD73	Tessier, L. et al. (2015) PLOS ONE | April 22, 2015
AT,BM, UCB, UCM	CD29, CD44, CD90, MHC-I	CD86, F6B, MHC-II	Carrade, D. et al (2012)

Table 1 Results from individual publications examining surface marker expression (CD Markers) of equine MSCs derived from various tissue sources

1.4.3.5 **Immunocytochemistry (ICC)**

This technique can be used to visualise proteins and peptides inside cells or on the cell surface (Burry 2011). A primary antibody binds to the protein of interest, then a secondary antibody conjugated to a fluorophore is used to bind the primary and the cells are viewed with a fluorescence microscope set at appropriate wavelength. Common fluorophores used are Fluorescein (FITC), AlexaFluor (Invitrogen), BODIPY (Invitrogen), APC, RPE, BPE (Phyco-Biotech, Greensea, Prozyme, Flogen). The newer proprietary formulations of fluorophores are advertised as brighter and both more pH and photo stable. The same cell surface markers as discussed previously can also be assessed with ICC. The need for adequate controls is very important when performing ICC (Burry 2011). For example; to prove the absence of a marker, a positive control from a different cell type that expresses the marker, needs to be included to ensure that the marker is functional and that absence of fluorescence is not due to poor technique or mismatched epitopes.

Barberini et al. (2014) utilised ICC to distinguish adipose, bone marrow and umbilical derived MSC. They found no presence of MHC-II with ICC nor flow cytometry. Reed and Johnson (2008) characterised stromal cells from equine umbilical cord blood with immunocytochemistry. They evaluated markers of stemness, SSEA-1, Oct4, Tra1-60 and Tra1-81. Results demonstrated the presence of Oct4 in the nuclei of greater than 90% of the cells. However, this does not provide proof of a true stem cell population.

1.4.3.6 **Gene expression studies of MSC**

As well as the method of ICC and flowcytometry can assess the protein present inside the cell and on the cell surface, MSC can also be evaluated for the actual gene expression of the same protein. However, immunophenotyping is hampered by the lack of a single specific marker and the limited availability of monoclonal anti-horse antibodies (De Schauwer et al. 2011) and there is also no explicit gene that can define a MSCs. However, genes often referred to as pluripotency genes or "stem cell specific" are; SOX-2, Nanog and Oct-4 (Hackett et al. 2012). These genes as well as tissue specific genes; adipose, chondro and osteo-genic genes, are often assessed after induction of trilineage differentiation as a confirmation of the cell line as a stem cell line. Violini et al. (2009) studied equine BMSC and defined

these as stem cells by their expression of markers, Sox-2 and Nanog. The BMSC also had the capability to differentiate into tenocytes, which expressed tendon-related markers; tenomodulin and decorin.

The method for determination of gene expression is by extraction of m-RNA from the selected cell line and assessment by western blots or reverse transcription polymerase chain reaction (RT-qPCR) (Freshney 2010). Reverse transcription is used to generate a single stranded complementary DNA (cDNA) sample that is put through a series of thermal cycles to amplify the target sequence. Then the PCR product is electrophoresed through an agarose gel and viewed under UV light to determine the presence of the DNA of interest. Detection of gene expression on mRNA level can be a valuable alternative, although the need still exists to test several antibody clones in search for cross-reactivity (De Schauwer et al. 2011). Often studies use a combination of methods for stromal cell identification, however it can be argued that because no specific CD marker or gene have been identified these methods are of limited value. Gene expression studies are more useful as a tool to identify a change in cells after they have undergone differentiation as compared to naïve, undifferentiated cells.

1.4.4 TRILINEAGE DIFFERENTIATION

Differentiation is a diagnostic method applied to MSCs to assess plasticity and ensure the selected cell line fulfil the criteria specified for Stemness of Mesenchymal stromal cells (Dominici et al. 2006). Plasticity is defined as the capacity of a cell to give rise to one or more lineages. Unipotent cells give rise to only one lineage, multipotent cells give rise to more than two lineages, pluripotent cells may give rise to several lineages and totipotent cells are able to give rise to all known cell types (Freshney, 2010). Mesenchymal stem cells are considered multipotent due to their ability to differentiate into more than two lineages. Adipogenic (fat), chondrogenic (cartilage) and osteogenic (bone) lineages are the most commonly differentiated cell types for confirmation of MSC plasticity, although tenogenic (tendon) and myogenic (muscle) (Martinello et al. 2009) and neuronal (Hoynowski et al. 2007) differentiation have also been confirmed for equine MSCs. However the differentiation potential of MSCs is not guaranteed

within a specific population of cells, heterogenicity may still arise even in clonal colonies (Baer and Geiger 2012). Clonal colonies of MSCs are grown from a single precursor cell in the attempt to preserve a specific cell phenotype and its specialised properties (Freshney 2010). Burk et al. (2013b) suggest that cells from certain origins have a higher capacity for differentiation into specific tissues. (Youngstrom 2015) reported that MSC derived from tendon showed a higher capacity for tenogenic differentiation than adipose and bone marrow derived MSC.

The process of trilineage differentiation has been evaluated and confirmed for MSC derived from many equine tissues including fat (de Mattos Carvalho et al. 2009), bone marrow (Parker et al. 2012; Schnabel et al. 2014), peripheral blood (Broeckx et al. 2012; Koerner et al. 2006; J. Spaas et al. 2011) , tendon (Burk et al. 2013b; Youngstrom 2015), aminiotic fluid (A. B. Lovati et al. 2011; Violini et al. 2012) and , umbilical cord blood (T. G. Koch et al. 2007; Mohanty et al. 2014)and Whartons' jelly (Barberini et al. 2014; Cardoso et al. 2012; Hoynowski et al. 2007). Only one paper to our knowledge has evaluated trilineage differentiation in a single equine skin derived fibroblast cell line (DF) (Schnabel et al. 2014).

1.4.4.1 **Adipogenic differentiation**

The MSC are grown 24 or 48well plates and maintained in adipogenic differentiation media for 7-14days. The StemPro adipogenic media (Gibco, Lifetechnologies) have been used by several studies (Barberini et al. 2014; Schnabel et al. 2014; Violini et al. 2012). The detection of adipogenic differentiation is achieved through i.) Determination of morphological characteristics, ii.) The use of specific stains like Oil-Red-O (Sigma), BODIPY (ThermoFisher) and LipidTOX Green (Lifetechnologies) and iii.) Evaluation of gene expression in differentiated cells. Peroxisome proliferator-activated receptor gamma (PPARγ2) and lipoprotein lipase (LPL) are two adipose specific gene markers analysed during differentiation in equine MSCs (Ranera et al. 2012). For confirmation of adipogenic differentiation, oil red O is typically used to stain lipid vesicles deposited within adipocyte cells (Burk et al. 2013b). The use of BODIPY has been reported in one equine study, (Redmond Hubbard 2014). This fluorescent stain has been used with human BMSC to confirm intercellular accumulation of lipid (Fink and Zachar 2011). The commercial kit; StemPro Adipogenesis

Differentiation kit (Gibco, Lifetechnologies) promotes the use of LipidTOX, a fluorescent stain.

1.4.4.2 **Chondrogenic differentiation**

There are several methods described for chondrogenic differentiation. The basic principle is to create a high density cell pellet either by i.) micromass culture (Greco et al. 2011) or ii.) centrifugation of cells in round bottom 96 well plates (Solchaga et al. 2011) or in conical tubes. The chondrogenic media has commonly the additives TGF-β1 or TGF-β3. StemPro Chondrogenic Differentiation media (Gibco, Lifetechnologies) has been used successfully in several equine studies (Barberini et al. 2014; Cardoso et al. 2012; Carvalho et al. 2013; Schnabel et al. 2014). The detection of chondrogenic differentiation is achieved through determination of i.) Morphological characteristics, such as the shape and density of a tertiary structure, ii.) The use of specific stains and iii.) By evaluation of gene expression in differentiated cell pellets. Chondrogenic differentiation is detected by a variety of stains including Alcian Blue (Ahern et al., 2011), toluidine blue (Bunnell et al., 2008) and Safranin O for glycosaminoglycan (GAG) deposits (Burk et al., 2013), hematoxylin and eosin (H&E) for cell structure (Vidal et al., 2008) and Massons trichrome (Burk et al., 2013) for collagen fibre formation. Chondrogenic-specific lineage gene markers include collagen type 1, collagen type 2, aggrecan, bone morphogenic proteins, matrix metalloproteinase-1 and -13, sox9 and cartilage-derived retinoic acid-sensitive protein (Berg et al. 2008; Greco et al. 2011).

1.4.4.3 **Osteogenic differentiation**

Osteogenic differentiation is performed as part of trilineage differentiation and evaluates osteoblastic activity. The evaluation of the differentiation potential is to i.) Determine the presence of mineralised calcium deposits or ii.)alkaline phosphatase activity or iii.) The presence of osteogenic gene markers; osteomodulin, osteocalcin, osterix. Alizarin red is a commonly used stain for the detection of calcium deposits between days 14-21 of culture (Hoynowski et al. 2007; Toupadakis et al. 2010) or Von Kossa stain (Burk et al. 2013b; Colleoni et al. 2009). The intensity of staining can be used in a semi-quantitative measure to

determine osteogenic potential. Staining for alkaline phosphatase activity is also performed successfully between days 4-10 in culture (Toupadakis et al. 2010).

1.4.4.4 **Tenogenic differentiation**

Tenogenic differentiation of MSC is not part of the set criteria for determining stemness, however several groups have evaluated the tenogenic potential of equine mesenchymal stromal cells derived from adipose (Youngstrom 2015), bone marrow (Lovati et al. 2010; Violini et al. 2009; Youngstrom 2015), peripheral blood (Broeckx et al. 2012) and umbilical cord blood (Mohanty et al. 2014). There is no universal differentiation protocol and several agents have been used to induce tenogenesis in equine MSC; bone morphogenic protein-12 (BMP-12), basic fibroblast growth factor, L-ascorbic acid and Tendo-Cell®. The procedure is inherently difficult to achieve *in vitro* as tendon fibrils require a scaffold and tensile force applied to form and align correctly. Factors that regulate tenogenic differentiation of MSC include; growth factors, mechanical stimulation, support from biomaterials or co-culture with tendon fibroblasts (Lui et al. 2011). Tendon engineering is an expanding area and relevant to human medicine, so many groups are researching this concept. Lovati et al., (2012) and Violini et al., (2009) performed tenogenic differentiation in vitro, they evaluated the success with morphological exam and gene expression of tendon related markers tenomodulin and decorin, as well as tenascin-C and collagen type I. Lovati et al., (2009) used low dose (10ng/ml) of basic fibroblast growth factor as well as co-culturing with fresh tendon explants in a transwell co-culture system. Violini et al., (2009) and Mohanty et al., (2014) used bone morphogenic protein-12 (BMP-12) at (50ng/ml) to induce tenogenesis. Youngstrom (2015) created a cyclic strain bioreactor designed to simulate gentle exercise and used decellularised tendon scaffolds for adipose, bone marrow and tendon derived MSC to attach to *in vitro*. The cells migrated into the scaffold and exhibited an elongated, tenocyte-like morphology as evaluated with histology at 10days post seeding. The tensile testing was performed after day 10 in culture and all MSC loaded tendon scaffolds showed higher elastic modulus and greater failure stress compared with matched controls, which consisted of the same scaffolds without added MSC. The bone marrow and tendon derived MSC showed greater failure stress (5MPa vs 1MPa) compared to controls. A study by Liu et al. (2006) used unwoven polyglycolic acid fibres to form a

scaffold, upon which tendon derived tenocytes or dermal fibroblast were cultured for 7days *in vitro.* Then the cellular enriched scaffolds were implanted into lesions created in porcine SDFT *in vivo.* Results showed that fibroblast and tenocyte engineered tendons were similar in gross view, histology and tensile strength, achieving approximately 75% of natural tendon strength after 26 weeks. They proposed that dermal fibroblasts have a promising potential in tendon engineering.

1.4.5 SYNOVIAL FLUID INDUCED DIFFERENTIATION *IN VITRO*

Several equine studies have investigated autologous and allogenic treatment MSCs injected into joints (Broeckx et al. 2014b; Carrade et al. 2011) and tendons (Godwin et al. 2012; Ricco et al. 2013; Van Loon et al. 2014)). To our knowledge no equine studies have yet evaluated long term survival of MSC injected intra-articular *in vivo*. However, two studies have evaluated equine synovial fluid in MSC culture systems *in vitro*, (Boone 2013; Hegewald et al. 2004). Boone (2013) reported an increase in cell proliferation rates correlated with an increased concentration of allogenic synovial fluid in the culture media. Hegewald et al (2004) found that normal autologous synovial fluid induced chondrogenesis of equine BMSC. The synovial fluid induced chondrogenic phenotype showed high levels of expression of type-II collagen but less proteoglycan expression compared to cultures stimulated with TGF-β1. They found that the size of the formed cartilage pellet increased with increased concentration of synovial fluid (up to 50%). Boone (2013) found a significant increase in viability and GAG production of BMSC grown in 50% and 100% allogenic synovial fluid as well as an enhanced chondrogenic potential at 50% concentration.

Human studies by Yang et al. (2006) and Leijs et al. (2012) evaluated the effects of inflammatory synovial fluid on chondrogenesis *in vitro*. Chondrocytes were isolated from the femoral condyle of a healthy donor (post mortem), the cells were passaged and found to de-differentiate into a more fibroblastic phenotype after two passages (Yang et al. 2006). Chondrogenesis was induced with TGF-β1 and the treatment group was also supplemented with 10% allogenic inflammatory synovial fluid, there were no difference noted between treatment and control. However, the

synovial fluid treated cells produced significantly less proteoglycans compared with the control. Leijs et al. (2012) found that MSC from bone marrow cultured in 20% inflammatory synovial fluid upregulated mRNA expression of Interleukin-6 (IL-6) and Indolamine (IDO) and suppressed lymphocyte proliferation. Thus, synovial fluid-activated human BMSC displayed immuno-modulatory effects *in vitro.* These papers show that *in vitro* exposure to allogenic inflammatory synovial fluid can induce chondrogenesis in both human and equine BMSC cell lines and potentially enhance their immuno-modulatory properties.

1.4.6 METHODS OF TRACKING MESENCHYMAL STROMAL CELLS *IN VIVO*

There are very few equine studies to our knowledge that have followed the fate of injected MSC over time. The ways to track stromal cells in the live horse after injection are limited. Labelling of cells can be performed with either,

i.) Fluorescent probes such as green fluorescent protein (GFP). A post-mortem histology exam using fluorescent microscopy will enable viewing of remaining cells in tissue (Guest et al. 2008). This method is not possible to perform *in vivo.*

ii.) Carboxyfluorescein diacetate succinimidyl ester (CFDA-SE; Vybrant CFDA SE Cell Tracer Kit [V-12883]; Molecular Probes, Eugene, was used in a porcine model to label BMSC (Lee et al. 2007).

iii.) Radioactive Technetium-99m and scintigraphy have been used successfully in the live horse to track MSC (Sole et al. 2013; Spriet et al. 2015).

iv.) Magnetic nanoparticles attached to MSC have been used in the live horse combined with Magnetic Resonance Imaging (MRI) (Bourzac et al. 2014; Lange-Consiglio et al. 2011). Currently there is no available MRI machine for equine clinical applications or research in New Zealand.

Guest et al. (2008) labelled bone marrow stem cells (BMSC) and embryonic derived stem cells (EMSC) with green fluorescent protein (GFP) and injected these cells into equine SDFTs and subsequently reported on survival rates and distribution patterns over time. A porcine study reported successful incorporation of labelled MSC into newly formed hyaline cartilage in a surgical osteo-arthritis model as assessed post mortem (Lee et al. 2007). The pigs were sacrificed and six and twelve

weeks and tissue harvested for both morphological and histological analysis. The cell- treated groups showed improved cartilage healing at both time points as compared to controls. However, this mechanism of action of incorporation of injected MSC into newly formed cartilage have not been studies in the horse. The mechanism of MSC becoming incorporated into the architecture of the healing tissue is currently challenged due to recent discoveries; three equine studies have reported that most MSC do not display long term survival when injected into tendons (Becerra et al. 2013; Guest et al. 2008; Sole et al. 2013). Beccera et al. (2013) reported only 24% of injected cells persisted in the tendon after 24hours. This is in accordance with Sole et al. (2013) that reported a median percentage of persistence in the distal limb at 24.1% after regional intra-arterial injection and a slightly lower value of 8.5% for regional intra-venous injection. Guest et al (2008) reported less than 5% survival of injected MSC at 10days, however EMSC showed great survival but also migration in the tissue. The criteria set by Caplan (1991) to define a stem cell, states that the cells have to demonstrate long term survival and incorporation into tissue. The time frame of survival is not specified by the author. However, Caplan (1991) also discuss paracrine effects exerted by MSC on the local tissue environment and how these criteria might be challenged in the future as these effects are further investigated and immuno-modulation is better defined.

1.5 MESENCHYMAL STROMAL CELLS & FIBROBLASTS

Fibroblasts are considered mature mesenchymal cells that are particularly abundant in the connective of each organ and tissue (Blasi et al. 2010). They are adherent proliferating cells which are difficult to remove because they can survive and grow even under extremely selective culture conditions. Therefore, these cells are the most frequent contaminating cell phenotype present in many cell culture systems. Not only is it difficult to apply techniques which successfully eliminate fibroblasts from a culture, it is also particularly complex to distinguish MSCs from fibroblasts in the same culture. Fibroblasts and MSCs have an extremely similar morphological appearance they both proliferate well and have many identical cell surface markers

There is controversy over the relationship between human MSC and fibroblasts (Feisst et al. 2013; Hematti 2012) and human studies have failed to identify any

differences in the morphology, immunophenotype or trilineage differentiation ability between the two cell types (Blasi et al. 2011; Brohem et al. 2013; Covas et al. 2008; Lorenz et al. 2008; Strutz et al. 1995; Ulrich et al. 2012). This could have large implications for clinical use as the common census is that healing by regeneration of tissue is superior to healing by fibrosis. Fibrous tissue has less functionality compared with the original tissue and the re-injury rates are increased and athletic performance is compromised (Burk et al. 2013a).

Feisst et al. (2013) reported that a number of cell populations with stem cell properties have been identified in cell lines grown from human dermis, including; ‘‘skin-derived precursors’’ (SKPs), ‘‘fibroblastic mesenchymal stem-cell-like cells,’’ ‘‘Dermal mesenchymal stem cells’’ (DMSCs), and ‘‘Muse cells’’. However, there is variation in isolation protocols and cell culture methods between studies and the phenotypic and functional properties of these cells differ substantially. Thus, there is currently no consensus regarding MSC populations in human dermis. Cells present within primary dermal fibroblastic cell lines have been shown to be capable of differentiation into mesenchymal lineages.

Some studies have successfully achieved differentiation of fibroblasts into mesenchymal tissues. For example Lorenz et al. (2008) showed that human skin derived fibroblasts could differentiate into adipogenic and osteogenic cells and Hanson et al. (2010) showed that vocal fold cells (a fibroblastic cell type) had the same differentiation potential and the same cell surface markers present as both bone marrow and adipose derived MSC. Tri-lineage differentiation is achieved *in vitro* by the exposure of MSC to supra-physiological concentrations of induction agents specific for the adipogenic, osteogenic or chondrogenic pathways. The common practice of using the tri-lineage differentiation assays as the gold standard for stem cell identification may need to be reviewed as fibroblasts also have been shown to have capacity for differentiation. A study by Blasi et al. 2012 found that human adipose derived-MSCs (AD-MSCs) and human dermal fibroblasts (HNDFs) cultured *in vitro* have very similar morphological appearance, growth rate, and immuno-phenotypic profile. Both cell types expressed typical mesenchymal markers $CD29^{+}$, $CD44^{+}$, $CD90^{+}$ and $CD105^{+}$ and to a minor extent, the adhesion

molecules $CD54^+$, $CD56^+$, $CD106^+$ and $CD166^+$. The same populations were negative for the haematopoetic stem cell markers $CD34^-$, $CD146^-$, $CD133^-$, $CD117^-$as determined by flow cytometry. It can thus be argued that flow cytometry is of limited use as a method for identifying MSC as the same CD markers are present on dermal fibroblasts. Both cell types in this study could be differentiated into osteoblast, adipocyte, and also cardiomyocyte-like cells.

To my knowledge only one equine study has compared the immuno-phenotype and trilineage differentiation potential between bone marrow derived and dermal derived fibroblast. Schnabel et al. (2014) reported an almost identical immuno-phenotype at passage 2 (P2) of a single dermal fibroblast cell line to that of ten BMSC cell lines. The distinguishing feature was that dermal fibroblast cells did not express MHC-II, which was in contrast to the BMSC where 11/13 were positive. Schnabel et al. (2014) did not achieve positive trilineage differentiation in this cell line; however, it appeared to show some chondrogenic differentiation potential, but was negative for adipogenic and osteogenic differentiation as determined with oil-red and Alizarin red staining methods.

Blasi et al. (2012) proposed a method for a functional bio-assay to distinguishing between MSC and fibroblasts in humans. The study investigated the cytokine profile and the exerted paracrine effects on cells of the immune system. They showed that human adipose tissue derived MSCs displayed strong angiogenic and anti-inflammatory activity in contrast to human dermally derived fibroblasts. The anti-inflammatory effects by adipose MSCs on cells from a human monocyte cell line (U937) were seen as a decreased expression of adhesion molecules CD11a and CD11b, both involved in inflammatory cell migration. As well as the reduction of inflammatory chemokines; Chemokine ligand 5 (CCL5) also known as RANTES and Monocyte chemoattractant protein-1 (MCP1), released from the U937 cells. The effects were significantly enhanced after activation of the adipose-MSCs with TNFα (25ng/ml) for 12hours. In comparison the dermal fibroblasts acquired pro-inflammatory activity; increased release of CCL5 and MCP1, with the same treatment. Activation of equine MSC by exposure to the inflammatory cytokine IFN-γ (100ng/ml) for 72hours was reported by Paterson et al. (2014).

Recently a paper by Nicolay et al (2016) reported that cisplatin treatment has a minimal effect on MSCs stem cell characteristics as compared to dermal fibroblasts. Cellular attachment was assessed 24hours after exposure to cisplatin (either 200ng/ml or 1000ng/ml) for 4hours. MSC morphology and attachment was unaffected by treatment and the potential for differentiation was retained. In the dermal fibroblast cell line, the cell attachment was significantly delayed at time 24hours ($p<0.05$) in a dose related manner compared to control. Viability studies also confirmed MSC to have greater resistance to the cytotoxic effects of cisplatin. The authors detected cytoskeletal rearrangements and high expression of individual heat shock proteins in the two MSC cell lines, which may be responsible for the resistance to the cytotoxic effects of cisplatin.

Recent efforts in stem cell research investigate the induction of pluridpotency in somatic cell lines, so called induced pluripotent cell (iPS). Nagy et al. (2011) reported the generation of iPS cells from equine fibroblasts using a *piggyBac* transposon-based method to deliver transgenes containing the reprogramming factors Oct4, Sox-2, Klf4, and c-Myc. The established iPS cell lines express pluripotency markers (Oct4, nanog, Sox-2) and can form teratomas containing all three of the embryonic germ layer derived tissues, when grafted *in vivo* into immunocompromised mice. Thus, the relationship between stem cells and fibroblasts is elusive and more research is needed to categorise these cell lines.

1.6 IMMUNO-MODULATORY EFFECTS OF MESENCHYMAL STROMAL CELLS

1.6.1 DEFINITION

The term *immuno-modulatory* describes a stimulating or suppressant effect on the cells of the immune system. The ability of a certain cell type (here MSC) to exert change on the normal inflammatory cascade by i.) Direct cell to cell contact or ii.) By secretion of cytokines and chemokines or iii.) A combination of both mechanisms (Kyurkchiev et al. 2014)

1.6.2 CYTOKINES

The aforementioned pro- and anti-inflammatory cytokines have been evaluated in several equine studies and reported to be present in diseased joints (Bertone et al. 2001; Kamm et al. 2010; McIlwraith 2005) and in damaged tendon tissue (Hosaka et al. 2002). The progression of osteoarthritis is orchestrated by pro-inflammatory cytokines, IL-1β and TNFα and the prostaglandin PGE_2 (Boone 2013). Figure 10 provides a simplified explanation of the actions of TNFα on the immune system.

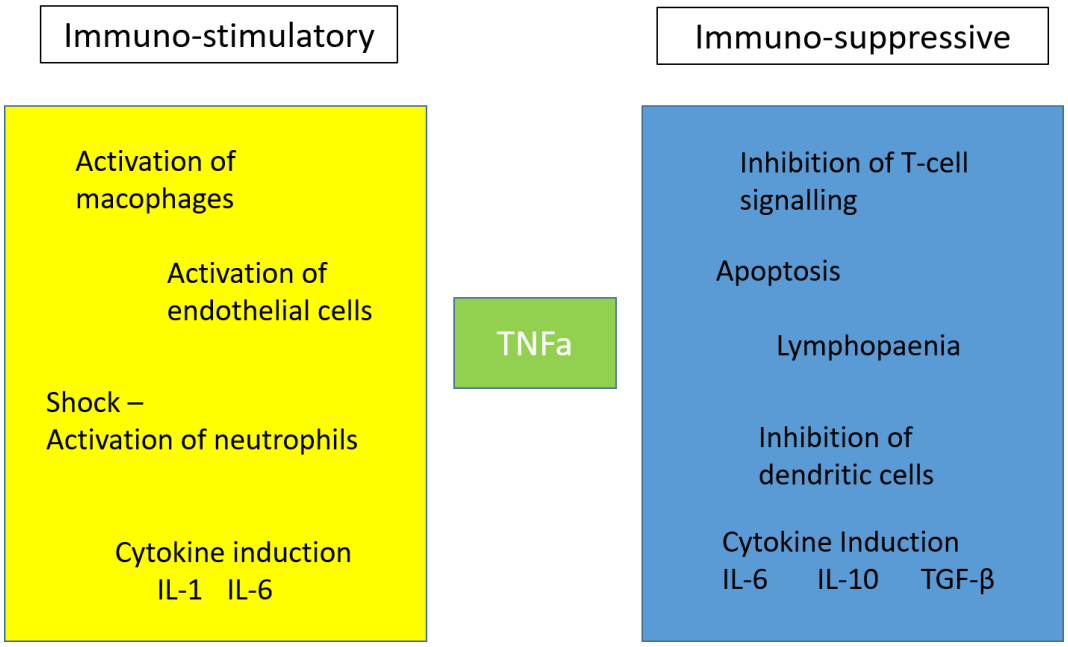


Figure 10 TNFα effect on immune system simplified, adapted from O'Shea et al. (2002)

The secretion of these inflammatory mediators by T cells induce further release of pro-inflammatory cytokines (IL-6, IL-17, IL-18), chemokines and other inflammatory mediators (NO, oncostatin-M, leukaemia inhibitory factor). Both IL-1β and TNFα upregulate expression of the catabolic enzymes involved in extracellular matrix destruction. Kamm et al., (2010) compared the expression of cytokines and other metabolic markers in synovium, synovial fluid and cartilage in normal and osteoarthritic joints. They found TNFα present in synovial fluid, synovium and cartilage, while IL-1β was only present in the cartilage. However, Kamm et al. (2010) argued the concentration was below the range of detection of the ELISA kit (R&D Monoclonocal antibody, author manufactured ELISA kit

developed and used for this trial). Hosaka et al., (2002) demonstrated the presence of IL-1α, IL-1β, TNF-α and IFN-γ antibodies in inflamed equine SDFTs with immunohistochemical staining.

The TGF-β family consists of TGF-β1-3, bone morphogenic proteins (BMP) and activins (Borena et al. 2015). In mammals mainly TGF-β1, -β2 and -β3 isoforms are found, but TGF-β1 predominates in cutaneous wound healing. They are produced by several cell populations; macrophages, fibroblasts, keratinocytes, platelets and MSC. TGF-β is a multifunctional growth factor that attracts new fibroblasts and macrophages to the wound site, stimulates fibroblast proliferation and collagen synthesis, reduces extracellular matrix degradation and modulates the immune system. Indeed, MSCs can directly attenuate inflammatory responses by decreasing the secretion of the pro-inflammatory cytokines such as TNF-α and interferon-γ (IFN-γ) while enhancing the production of anti-inflammatory cytokines including IL-10.

A review article by McIlwraith (2005) analysed biomarkers of equine joint disease and classified them as; i.) *Direct biomarkers*, which originates from cartilaginous or bony structures or are enzymes that are only active in these tissues. ii.) *Indirect biomarkers*, which have the potential to influence the metabolism or integrity of the matrix of the various joint tissues. Proteolytic enzymes and their inhibitors, growth factors and pro-inflammatory cytokines are indirect biomarkers. Bertone et al. (2001) concluded that the presence of Interleukin 6 (IL-6) in synovial fluid was both sensitive and specific as a marker of equine joint disease. They found that Tumour Necrosis Factor alpha (TNFα) and Interleukin-1beta (IL-1β) are no more useful than leucocyte counts in screening for joint disease. TNFα will increase with exercise and repeated arthrocentesis (McIlwraith 2005), hence it can't be considered as a specific marker of joint disease

1.6.3 EFFECT OF MESENCHYMAL STEM CELLS ON CELLS OF THE IMMUNE SYSTEM

MSCs have the unique combination of self-renewal and multi-lineage differentiation capacity as well as immuno-modulatory properties (Kyurkchiev et

al. 2014). MSCs can exert profound immuno-modulatory effects on cells of the innate and adaptive immune systems via a variety of mechanisms, especially cytokine and chemokine secretion (Hoogduijn et al. 2010). Mesenchymal stromal cells have multipotent capacity, which allows them to differentiate from a fibroblastic morphology into osteoblasts, chondrocytes, adipocytes, tenocytes and other cell types *in vitro* (Eggenhofer et al. 2014). MSC were initially thought to migrate to the injured tissue and then undergo differentiation into the type of tissue they were administered to. For example; tendon or cartilage to then form part of the new architecture of that tissue. However, more recent studies (Guest et al. 2010a; Paterson et al. 2014) showed that MSCs do not display long term survival in tendon tissue after implantation. Less than five percent (<5%) of MSC injected into equine superficial digital flexor tendons survived at 10 days (Guest et al. 2010a). The results seen on tissues after MSC therapy are now thought to be achieved through paracrine effects rather than through directed differentiation. The MSC have been shown to have immuno-modulatory effects on T-cells (Bouffi et al. 2010) as well as trophic effects on endogenous stem cells (Guest et al. 2010a; Hoogduijn et al. 2010). The cells communicate by secreted proteins, so called cytokines and these provide the signals to coordinate the inflammatory response. Some cytokines act to broadly provoke the inflammatory response; such as IL-1, IL-6 and TNFα. Zafranskaya et al. (2013) showed that PGE_2 production depends on cell-to-cell contact of MSC and lymphocytes. TGF-β is a negative regulator of proliferation in many cells and has anti-inflammatory actions in some settings. T helper cells are required to stimulate B cell responses as well, with the cytokines IL-10, IL-4 and other cytokines regulating the clonal selection and differentiation of antigen-specific B cells to form antibody-secreting plasma B cells and memory cells. Eggenhofer and colleagues (2014) discuss that *in vitro* culture alters the immunological phenotype of MSCs. They propose that the MCSs can induce a more substantial recruitment of tissue specific repair cells after *in vitro* culture and purification.

Equine mesenchymal stromal cells can produce a range of bioactive trophic factors, inflammatory mediators and adhesion molecules that function to decrease inflammation, inhibit scar formation, inhibit apoptosis and increase angiogenesis

(Kol et al. 2013). Mesenchymal stromal cells need to be activated to perform these functions (Paterson et al., 2013 (Blasi et al. 2011; Carrade et al. 2012)a). MSC growing in normal culture conditions, derived from either adipose, bone marrow, umbilical cord blood and matrix, neither stimulated nor inhibited lymphocyte proliferation (Carrade et al. 2012). The MSC did show continuous expression of TGF-β1. However, when grown in the presence of activated T-cells all the MSC cell lines secreted IL-6 and PGE_2. PGE_2, IL-6, NO, and IDO have been implicated as factors that decrease T-cell proliferation. Activation of MSC has been achieved by;

i.) Presence of TNFα in culture media, 25ng/ml for 12hours (Blasi et al. 2011; Kol et al. 2013; Paterson et al. 2014);
ii.) Presence of IFN-γ in culture media, 100ng/ml for 72hours (Paterson et al. 2014);
iii.) Co-culture of the MSC with activated T-cells (column separation 0.5g nylon wool) (Carrade et al. 2012; Carrade Holt et al. 2014);
iv.) Presence of allogenic mononuclear cells (MNC) (Carrade et al. 2012; Paterson et al. 2014; Tessier et al. 2015); or
v.) Presence of autologous MNC as the stimulator cell. (Tessier et al. 2015).

When activated *in vitro*, equine MSCs exhibit several immunosuppressive effects (Boone 2013). MSC have been shown to decrease lymphocyte proliferation, decrease T-cell production of certain pro-inflammatory cytokines (TNFα and IFN-γ), and increase their production of certain anti-inflammatory cytokines TGF-β1, PGE_2, IL-6 and IL-10, (Boone 2013 (Carrade et al. 2012), Nitric oxide (NO), hepatocyte growth factor and Indoleamine 2,3-dioxygenase (IDO) (De Schauwer et al. 2014; Tessier et al. 2015). Carrade et al. (2012) isolated five MSC lines from each tissue source, adipose tissue, bone marrow, umbilical cord blood and cord matrix. Lymphocyte proliferation was assessed in a mixed leukocyte reaction, and mediator secretion was determined by ELISA. Unstimulated MSCs did not alter lymphocyte proliferation or secrete mediators, except for TGF-β1, which was continuously secreted by MSC of all origins. When activated, MSCs of all tissue types showed anti-inflammatory effects; decreased lymphocyte proliferation, decreased production of TNFα and IFN-γ and increased IL-6 and prostaglandin

PGE_2 secretion. Bone marrow MSCs and umbilical cord blood MSCs also produced nitric oxide (NO), while adipose MSCs and umbilical cord matrix MSCs did not. In contrast to De Schauwer et al. (2014) and Tessier et al. (2015) equine MSCs did not produce IDO in this study. In humans, NO is an upstream regulator of IL-10 and TGF-β1, and a direct regulator of IDO (Carrade et al. 2012). Variable results regarding the production of equine IDO indicates that NO may also participate in upstream regulation of IL-10 or TGF-β1 as well as in its other functions, including vasodilation and the immune response to pathogens. MSCs from multiple tissue sources and species have been shown to produce PGE_2 as part of their immunomodulatory response. Equine MSCs modulate T cell proliferation through a PGE_2 dependent mechanism. T cell proliferation is one indicator that lymphocytes have become activated and the secretion of pro-inflammatory molecules is a critical part of this response, especially TNFα and IFN-γ production. Carrade et al. (2012) suggest that both animal species and tissue source affect the mechanism through which MSCs modulate immune cell function. MSCs from solid-tissue-derived sources inhibited T-cell proliferation through apoptosis in contrast to blood-derived MSCs that inhibited T cell proliferation by cycle arrest.

Paterson et al. (2012) examined the gene expression of embryonic stem cells (EMS) and bone marrow derived stromal cells and also the presence of cytokines; INF-γ, TNFα, IL-6 and IL-10, in the MSC culture media (conditioned media) by ELISA. The stem cells were co-cultured with peripheral blood mononuclear cells (PBMC) to determine their effect on proliferation. They found that embryonic stem cells did not affect baseline proliferation of PBMCs. While bone marrow stromal cells significantly suppressed PBMC baseline proliferation, thus having an anti-inflammatory effect. A greater suppressive effect was noted when the cells types were in direct contact in the dish (P=0.00001) than when PBMC were exposed to the conditioned media. However, the conditioned media still had a significant effect on PBMC and mRNA expression of IL-6 and INF-γ were reduced (P=0.01). Thus, soluble factors also play a role in MSC-mediated immune suppression. Paterson et al. (2012) also found that MSCs secreted IL-6 despite no exposure to PBMCs. Immunomodulation and functionality of activated MSC and DFs is an interesting key concept. I. and Sorrell (2015) suggest that in the event of trauma or infection

tissue specific MSCs are activated and will exert immuno-modulation, which ultimately over time (weeks to months) is seen as specific tissue regeneration. MSCs appear to be local managers of the tissues' innate regenerative potential. If MSC can be activated during controlled conditions in vitro and their function recorded and evaluated, it may provide some insight on how their effects could be managed in vivo to reach desired outcomes.

1.7 AIMS AND OBJECTIVES OF STUDY

The ultimate aim of stem cell research in horses in New Zealand is to provide an effective, proven and readily accessible treatment option for equine athletes suffering from musculoskeletal injury. This study will aim to perform a comparative analysis of stromal cells isolated from various tissues; bone marrow, adipose tissue, peripheral blood and umbilical perivascular tissue (Wharton's jelly) as well as skin derived fibroblast cells, using conventional methods. It also aims to test the immunomodulatory potential of stromal cells of mesenchymal origin *in vitro.*

More specifically these objectives will be carried out:

- To document and assess cell culture characteristics and morphology of MSC derived from adipose, bone marrow, peripheral blood, umbilical cord matrix and dermal tissue. To evaluate cellular morphology and population doubling time for each individual cell line.

- To assess cellular morphology and growth characteristics after incorporation of equine synovial fluid into the culture system.

- To assess the trilineage differentiation potentials for selected cell lines by performing adipogenic, chondrogenic and osteogenic differentiation using commercial differentiation media.

- To compare the immunophenotype of said cell populations by immunocytochemistry and flow cytometry as well as a biological assay.

- To activate stromal cells from bone marrow, umbilical tissue and dermal fibroblasts with either TNFα or allogenic inflammatory synovial fluid and perform an analysis of the cell culture supernatants with equine specific ELISA to determine the production of certain cytokines by activated cells compared to controls.

CHAPTER 2: MATERIALS AND METHODS

2.1 EXPERIMENTAL DESIGN SUMMARY

Ethics approval was gained from the University of Waikato School of Science and Engineering Animal ethics committee (Protocol 952) prior to the commencement of this study. Live animal procedures were minimised by the inclusion of cryopreserved mesenchymal stromal cell samples obtained commercially or via collaborators (Redmond Hubbard 2014). Post mortem dermal and adipose tissue and synovial fluid samples were obtained from horses that were culled for reasons unrelated to this study. Donors are described further in section 2.2.1

2.1.1 EXPERIMENT 1: MORPHOLOGICAL STUDY

The aim was to isolate and culture MSC from various equine sources and DFs at standardised *in vitro* cell culture conditions and to determine the morphology and culture characteristics for these cell populations. The two null hypotheses were tested (see below). The observations of morphology and confluency were performed at set time points and photos were taken to illustrate the similarities between equine MSC and DFs *in vitro.*

H0: The hypothesis is that stromal cells from adipose, bone marrow, peripheral blood and umbilical cord tissue have equivalent morphological characteristics as dermally derived fibroblasts

Population doubling times at passage 4 (P4) were calculated for each source of MSC and DFs.

H0: The hypothesis is that stromal cells from adipose, bone marrow, peripheral blood and umbilical cord tissue have equivalent population dynamics as measured by population doubling times, as dermally derived fibroblasts

Detection of mycoplasma species was performed with PCR and agarose gel electrophoresis on all cell lines to ensure no contamination.

2.1.2 EXPERIMENT 2: IMMUNOPHENOTYPING

The methods of immunocytochemistry and flow cytometry were used to determine the presence of a selected panel of cell surface markers identified in the literature, that currently form part of the laboratory assessment to positively identify a stem cell. To optimise the use of primary and secondary monoclonal antibodies an initial dilution series for markers CD29 and MHC-II was performed. The following hypothesis was investigated;

H0: The hypothesis is that stromal cells from adipose, bone marrow, peripheral blood and umbilical cord tissue have the same qualitative expression of cell surface markers, CD29, CD44, CD90 positive and CD45, MHC-II negative as dermally derived fibroblasts

Flow cytometry was performed under the guidance of Dr Anna Brooks at The Dunbar Laboratory, University of Auckland, NZ. Polychromatic flow cytometry was used to characterise and compare the mesenchymal cell populations from equine adipose tissue, bone marrow, peripheral blood and umbilical cord matrix as well as equine dermis. A panel of five (5) CD markers including; CD29, CD44, CD45, CD90, MHC-II as well as DAPI, was used to label the cells for sorting. Samples for flow cytometry were analysed at the fourth passage (P4), except the commercial DF cell line (P16) and the peripheral blood mononuclear cells (pMNC) (P-1). The pMNC (P-1) were used as a positive control for the negative MSC markers CD45 and MHC-II.

2.1.3 EXPERIMENT 3: TRILINEAGE DIFFERENTIATION

The trilineage differentiation potential where cells are induced to form bone, cartilage and fat is a standard method and accepted determination criteria to identify human stem cells. Trilineage differentiation with commercial induction media (StemPro) was performed for three elected cell populations (BMSC, UMBT and DF). A semi-quantative scoring method was adapted from Redmond Hubbard (2014) which involved a morphological evaluation as well as qualitative staining assays. A new concept that included cluster scoring as a measure of differentiation

of cells grown in culture system incorporating synovial fluid was used to assess change quantitively. The following hypothesis was investigated;

H0: The hypothesis is that stromal cells from bone marrow and umbilical cord tissue have an equal potential for trilineage differentiation as dermally derived fibroblasts

2.1.4 EXPERIMENT 4: SYNOVIAL FLUID MODEL

-Incorporation of allogenic synovial fluid in MSC culture system

The aim was to simulate intra-synovial conditions *in vitro* to understand the effects of this environment on MSC and DFs. It was hypothesised that equine allogenic synovial fluid incorporated into the culture media would have an effect on morphology, confluence and differentiation potential for MSC and DFs *in vitro*. Normal synovial fluid (SF) was obtained from supposedly healthy joints and inflammatory synovial fluid (I-SF) was obtained from diseased joints as defined in section 2.2.1.

The culture characteristics of DF, BMSC and UMBT cell populations grown in 50% normal SF and in 50% I-SF were investigated with the null hypothesis stating;

H0= The hypothesis is that stromal cells from bone marrow, umbilical cord tissue and dermally derived fibroblasts have the equivalent morphological characteristics when grown in standard culture conditions or culture containing 50% normal synovial fluid or culture containing 50% inflammatory synovial fluid

The following outcome variables were measured;

1. Percentage confluence, of cells populations at three different time points; T=24h, T=48h and T=168h
2. Phenotypic change, defined as;
    i.) Number of clusters at T=24, 48 and 168h
    ii.) Number of pellets at T=24, 48 and 168h

2.1.5 EXPERIMENT 5: BIO ACTIVATION ASSAY

-Measuring cytokine production of BMSC, UMBT and DF populations after activation

A comparison of the *in vitro* production of cytokines from mesenchymal stem cells and dermal fibroblasts after activation with tumour necrosis factor alpha (TNFα) or inflammatory synovial fluid (I-SF).

H0: The hypothesis is that activated stromal cells from bone marrow and umbilical cord tissue have equivalent cytokine production as activated dermally derived fibroblasts.

H0: The hypothesis is that activation with TNF alpha of stromal cells from bone marrow or umbilical tissue or dermal tissue derived fibroblasts equals the effect of activation of the same cell populations activated with inflammatory synovial fluid.

An initial temporal assay (ELISA1) of samples collected at four different time points (T= 24, 48, 72 or 168hours) was performed from one selected cell line, from each of bone marrow and dermal fibroblast origin; (BMSC N=1 and DF N=1). This was done to determine an optimal time point for future sample analysis as a full temporal analysis was not possible due to limited funds. The time point T=24hours was selected based on concentrations measured in this experiment, there appeared to be no greater concentration effect measured after this time point, see Appendix 2 for graphs from temporal assay. The following experiment evaluated the cytokine production of each cell population as measured by cytokine concentration in the cell supernatant using equine specific sandwich ELISAs. Cytokine production was measured:

1. After TNF-α activation vs control (CM)
2. After I-SF activation vs control (CM)
3. Per 10^4 cells using population doubling data at P4

2.2 MATERIALS AND METHODS

2.2.1 ANIMALS AND TISSUES COLLECTED

The selection of donors depended on horses available at the facility and horses that were being culled for reasons unrelated to this study, during a certain time frame (April 2015- October 2015), when tissues were collected for the purpose of this study. The horses were examined prior to euthanasia to assess their breed, sex, approximate age, overall health status and lameness score at walk as well as palpation of the carpal, tarsal and stifle joints to detect any heat or effusion. Adipose tissue, dermal tissue and synovial fluid from normal joints were collected within 15minutes *ex vivo* at a local abattoir. The horses were euthanised for reasons unrelated to this study.

Four dermal fibroblast cell lines were acquired, including one commercial cell line; "USA" (*Equus caballus* E.Derm (NBL6, CCL-57™ATCC, USA) and one kindly donated, "Otago" from The University of Otago, New Zealand (Courtesy Dr L. Wise). The remaining adipose and bone marrow derived cell lines were obtained from cryopreserved stock from The University of Waikato cell line inventory (Redmond-Hubbard 2014) or from clinical cases seen at EquiBreed NZ Ltd. For the cryopreserved ADSC and BMSC samples there were no record of age of the donors. However, the sex of these donors was known, all samples were obtained from mares, which were resident at Equibreed NZ.

Peripheral blood samples were collected *in vivo* by jugular veni-puncture by a veterinarian at an equine breeding facility in the Waikato region. Umbilical cords were collected on one Standardbred stud farm and one Thoroughbred stud farm in the Waikato region. The cords were allowed to break off naturally from the foal before the *ex vivo* tissue samples were collected as outlined in Section 2.2.4.1
Table 2 specifies the animal identification and origin of all samples (N=31) used in this study.

Animal ID	Sex	Breed/Age	Tissue	In vivo	Ex vivo	Cryopreserve samples	Commercial cell line
W1	C foal	TB/NA	UMBT		x		
W2	C foal	TB/NA	UMBT		x		
W3	F foal	TB/NA	UMBT		x		
W4	C foal	TB/NA	UMBT		x		
H	F foal	SB/NA	UMBT		x		
Y	F foal	SB/NA	UMBT		x		
T	C foal	SB/NA	UMBT		x		
Q	F foal	SB/NA	UMBT		x		
LH2	M	TB/NR	BMSC			X	
LH3	M	SB/NR	BMSC			X	
LH4	M	SB/NR	BMSC			X	
LH5	M	SB/NR	BMSC			X	
LH6	M	SB/NR	BMSC			X	
Tanga	G	TB/11y	BMSC			X	
USA	F	QH/4y	DF				X
Otago	NR	NR/NR	DF			X	
Copper	M	TB/7y	DF		x		
Becky	M	SB/10y	DF			X	
Eye4F	M	TB/8y	pBSC	X			
PA	NR	SB/NR	pBSC	X			
PM8	M	TB/NR	pBSC			X	
PM13	M	NR/NR	pBSC			X	
LA	G	SB/15y	pBSC		x		
LH4	M	SB/NR	ADSC			X	
LH6	M	SB/NR	ADSC			X	
Copper	M	TB/10y	ADSC		x		
PM13	M	NR/NR	ADSC			X	
Bird	M	SB/10y	pBMNC	X			
Emmy	M	SB/15y	pBMNC	X			
Famietta	M	WB/10y	pBMNC	X			
Guy	M	SB/6y	pBMNC	X			

Table 2 Animal ID and origin of samples. C= Colt F= Filly G= Gelding M= Mare NR= No record. SB= Standardbred TB= Thoroughbred QH= Quarter Horse WB= Warmblood. Adipose Derived stromal cells (ADSC), Bone marrow stromal cells

(BMSC) Dermal fibroblasts (DF), Peripheral blood stromal cells (pBSC),peripheral blood mononuclear cells (pBMNC), Umbilical tissue stromal cells (UMBT).

Apparently normal synovial fluid samples were collected by arthrocentesis *ex vivo.* The joints selected for inclusion were carpal, tarsal and stifle joints, due to the large volume and ease of collection of synovial fluid from these joints. The joints were clipped and prepared aseptically by scrubbing with chlorhexidine for five minutes and wiping with methanol prior to arthrocentesis. 10ml syringes and 19G needles were used and the fluid from each joint was injected into separate 10ml plain tubes and placed in a chilly bag on ice. The inflammatory synovial fluid samples were collected *in vivo* from horses undergoing arthroscopic surgery at an equine hospital, for reasons unrelated to this study (Courtesy Dr G Quinn, Hamilton Veterinary Services, New Zealand). Table 3 defines the origin of the normal and inflammatory synovial fluid used in the experiments, and Table 4 shows the attempt to characterise the fluid with respect to nucleated cell count, total protein and serum amyloid-A content.

Horse ID	**Date**	**Age (approx**	**Diagnosis**	**Carpal vol ml**	**Tarsal vol ml**	**Stifle vol ml**	**Total vol ml**
ACP1	15.07.24	10	Normal	6	6	6	18
ACP2	15.08.04	15	Normal	5	4	7	16
ACP3	15.09.24	8	Normal	4	4	6	14
Pooled Normal							**48**
SX1	15.09.02	1	R OCD	0	5	0	5
SX2	15.09.02	1	Bilat OCD	0	10	0	10
SX3	15.09.03	1	Bilat OCD	0	8	0	8
SX4	15.09.07	3	Carpal chip	3	0	0	3
SX5	15.09.08	1	Bilat OCD	0	10	0	10
SX6	15.09.14	1	L OCD LOCD	0	6	8	14
Pooled I-SF							**50**

Table 3 Origin and definition of synovial fluid samples. Two pooled samples were created one for normal synovial fluid (Pooled Normal) and one for inflammatory synovial fluid (Pooled I-SF).

Horse ID	Culture 24h	Culture 48h	Total Protein	Serum Amyloid A	Smear
ACP1	neg	neg			
ACP2	neg	neg			
Pooled Normal	**neg**	**neg**	**10g/L**	**2mg/L**	**no cells seen**
SX1	neg	neg			
SX2	neg	neg			
SX3	neg	neg			
SX4	neg	neg			
SX5	neg	Neg			
SX6	neg	Neg			
Pooled I-SF	**neg**	**Neg**	**12g/L**	**3mg/L**	**no cells seen**

Table 4 Further description of synovial fluid used in experiments.

2.2.2 PLASTIC CONSUMABLES

The culture flasks used were made from clear polystyrene with good optical properties and treated with Nunclon™ delta surface (ThermoFisher). Nunclon delta is a proprietary cell culture surface treatment used to improve the adhesion ability of a range of cell types. Two flask sizes were used (T25 and T75) and the flasks had angled necks and filter caps to allow airflow and minimise chance of contamination. Falcon™ 15mland 50ml conical centrifuge tubes, as well as 3ml sterile plastic Pasteur pipettes were used in all culture preparation work.

2.2.3 CULTURE MEDIA

The basal culture media consisted of Dulbeccos' minimal essential medium (DMEM) low glucose (ThermoFisher) 10% FBS (ThermoFisher), 1% Antibiotic-antimycotic (Sigma), 0.1% fungizone (Sigma), and 0.05mg/ml gentamicin (ProPharma). All culture media and phosphate buffered saline (PBS) (ThermoFisher) were prepared in the sterile fume hood and filter sterilised with a

0.22µm syringe filter (Minisart, Sartorious stedim) then kept refrigerated at 4°C. When media was used the bottle was wiped down with ethanol before placed in sterile fume hood. The amount of media required for culture work was poured off from the stock bottle, either into sterile culture flasks or 50ml tubes before placed in the incubator to warm to 37°C before adding cells.

2.2.4 PROTOCOLS FOR TISSUE HARVEST AND PREPARATION OF MESENCHYMAL STROMAL CELL LINES FOR CELL CULTURE

An extensive analysis of equine specific peripheral blood protocols as well as umbilical cord tissue protocols were performed before commencement of the experimental phase of this thesis. Appendix 3. details a step by step analysis of the available protocols found in the literature on the harvest and tissue processing methods. For adipose and bone marrow the samples were obtained cryopreserved from a previous study (Redmond Hubbard 2014).

Briefly, all tissue samples were processed to yield a mononucleated (MNC) cell fraction, which is a heterogeneous mixture that contain mesenchymal stromal cells (MSCs), additional mononuclear cells (MNCs) and red blood cells (RBCs). The mononuclear cells were sub-cultured using a sterile cell culture protocol as described below in Section 2.2.5

2.2.4.1 **Umbilical cords (UMBT)**

At the time of foaling, within minutes of the umbilical cord breaking off naturally between the foal and the placenta, a 10-15cm segment of the cord was cut at the distal end previously closest to the foal. The segment was rinsed in 10% iodine then placed in a one litre container containing 300ml of 70% ethanol for 10 minutes to remove environmental contamination. The cord segment was then placed in a sterile 250ml jar with screw top with 100ml of Vigro® embryo flush media (Minitub, Australia) supplemented with 2% Antibiotic-antimycotic (Sigma, USA) and 0.05% gentamicin (ProPharma). The jar was placed into a chilly bin and stored on the farm in the fridge at 4°C before transported to the laboratory. Samples were processed within 12 hours.

In a sterile fume hood the umbilical cord was dissected to obtain the perivascular (Whartons' jelly) tissue, see Figure 11 for gross anatomy of the equine umbilical cord before and after processing.

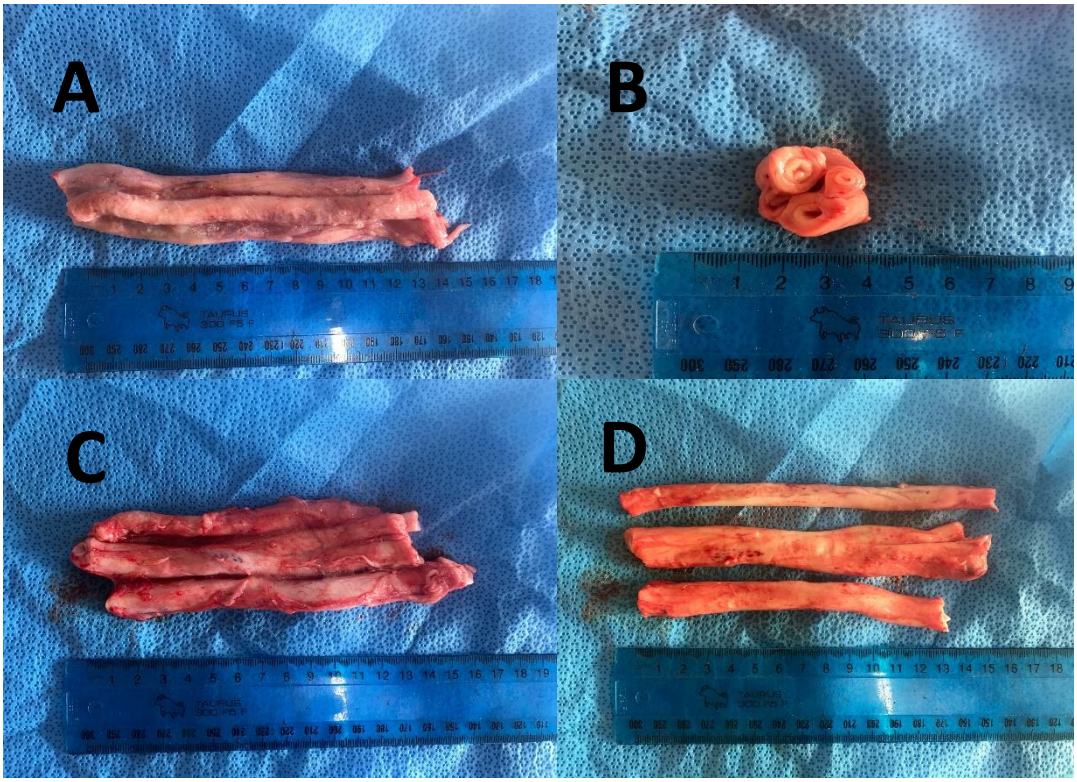


Figure 11 Gross anatomical views of a segment of an equine umbilical cord A.) Unprocessed cord segment B.) Cross section of cord C.) Cord opened up to expose perivascular tissue and Wharton's jelly D.) After removal of perivascular tissue and Wharton's jelly, showing the two umbilical arteries (top and bottom) and umbilical vein (middle)

The protocols of A. B. Lovati et al. (2011) and Cardoso et al. (2012) were modified as described. The perivascular tissue and Wharton's jelly were mechanically minced with fine scissors and the material was weighed and placed into a sterile 50ml tube. The tissue sample was enzymatically digested with 0.75mg/ml collagenase 1, in the shaking incubator for 8hours at 37°C. The enzyme was removed with duplicate washes of 5minutes each in PBS buffer supplemented with 10% FBS. The samples were then centrifuged at 250g for 10 minutes at room temperature (RT) and the supernatant was removed. After the second wash the cell

pellet was re-suspended in 2ml of warm (37°C) basal culture media and cells were counted manually with a haemocytometer and assessed for viability with trypan blue stain. Cells were plated at $1x10^6$ cells/cm^2 in T25 flasks with 10ml of basal culture media. The flasks were placed in the Heal Force, (HF 151, Acorn Scientific) incubator at 37°C with 5% CO_2 and 90% humidity. These are the standard cell culture growth conditions used for the purposes of this thesis. The flasks were observed daily and after an initial 48h period of allowing for cell attachment, the all of the spent media was replaced and then medium was changed every 3-4 days until 60-80% confluence.

When confluence was estimated to be 60-80%, the cells were trypsinised using 5ml of warm (37°C) trypsin (0.05% trypsin EDTA, Sigma). The cells were gently swirled in the flask for 30 seconds then excess liquid was poured off and the flask allowed to stand for 2-4minutes in the 37°C incubator. When cells were observed to detach from the plastic, the flask was removed from the incubator and 5ml warm culture media containing 10% FBS was added to neutralise the enzyme. The cellular suspension was removed with a 3mm sterile transfer pipette and added to a 15ml conical falcon tube. The tube was centrifuged at 250g for 5minutes at RT to pellet the cells. The supernatant was removed and cells re-suspended in 1ml of warm culture media. After counting (refer to section 2.2.5.1), the cells were either passaged or cryopreserved (section 2.2.6). Cells were passaged and re-seeded at $5.0x10^3$ cells per cm^2 or frozen in aliquots of $1.0x10^6$ cells in one millilitre vials in 1ml of cryogenic media (75% DMEM (low glucose) with 25% FBS and 5% DMSO).

2.2.4.2 **Peripheral Blood (pBSC)**

Peripheral blood (20ml) was collected in EDTA tubes. The tubes were kept at 4°C and blood samples processed within 4hours of collection. Briefly, the tubes were centrifuged at 1000g for 20minutes at RT. The buffy coat was gently removed by a sterile 3ml pipette and transferred to a 15ml sterile tube. Sterile PBS was added 1:1 (v/v) and mixed. The sample mixture was layered gently onto an equal volume of Lymphoprep® (MediRay, NZ). Then the tubes were centrifuged at 600g for 15minutes at RT. The interphase was collected and washed with 1X PBS buffer at 200g for 10minutes at RT. The resulting fractional layers are seen in Figure 12.

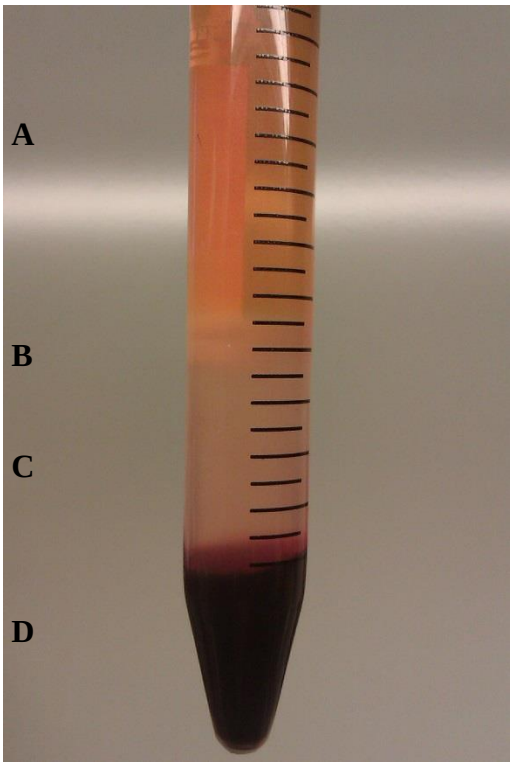


Figure 12 Peripheral blood separation using Lymphoprep. A.) PBS fraction, B.) MSC Interface, C.) Lymphoprep fraction, D.) Red blood cell fraction. From Redmond Hubbard (2014) with permission.

The wash steps were repeated three times with the supernatant discarded after each wash. After the final wash the cells were re-suspended in 2ml of warm culture media (DMEM (low glucose), 20% FBS, 10^{-11}M dexamethasone, 50ug/ml gentamicin, 250ng/ml fungizone, 10µl/ml antibiotic-antimycotic). A 10µl sample of the cellular suspension was added to 20µl of trypan blue stain and vortexed. Then 10µl of this mixture was added to each of two chambers on the haemocytometer and both chambers counted (five squares each) then averaged. The mononuclear cell counts were recorded and cells plated at $5x10^5$ cells/cm^2 in 10ml media in T25 flasks (Nunc Delta, 25cm^2, Thermo Fisher Scientific). The flasks were incubated at 37°C at 5% CO_2 and 90% humidity. The flasks were observed daily and after allowing 96hours for initial cell attachment the media was replaced, then every 3-4 days. At estimated 60-80% confluence the cells were trypsinsed as described in section 2.2.6. The cells were either passaged; re-seeded in new flasks at $5.0x10^3$ cells/cm^2 or cryopreserved.

2.2.4.3 **Skin derived Dermal Fibroblasts**

A skin biopsy (2x2cm^2) was harvested using sterile technique. The hair was clipped and then further shaved with a disposable shaver, then scrubbed with chlorhexidine surgical scrub for 5minutes before wiped with methylated spirit. A number 20 scalpel blade was used to cut the skin layers and forceps were used to hold and remove the sample. The skin biopsy was placed in Vigro® embryo flush media and placed in a chilly bin on ice for transportation to the laboratory. At the laboratory the skin biopsy was further dissected in a sterile fume hood and the hair and epidermis were removed with a number 10 scalpel blade and discarded. The dermal layer was cut into fine pieces and minced with scissors. The tissue was weighed and placed in a 50ml sterile tube and covered with DMEM (low glucose), 0.05mg/ml gentamicin and 1mg/ml collagenase type 1. The tube was incubated in a shaking incubator for 1hour at 37°C. The whole content; the dermal digest, was poured into a T25 flask with 20ml of basal culture media (DMEM (low glucose) 10% FBS, 1% Antibiotic-antimycotic, 0.1% fungizone, 0.05mg/ml gentamicin). The flasks were placed in the incubator under standard cell culture conditions; 37°C at 5% CO_2 and 90% humidity. The flasks were observed daily and after 48hours of initial cell attachment the media was replaced, then again every 3-4 days. At an estimated percentage of confluence at 60-80%, the cells were trypsinised and passaged and/or cryopreserved (section 2.2.6).

2.2.5 STANDARD CELL CULTURE PROTOCOLS

Cell culture work was performed under sterile conditions at the University of Waikato (PC2 laboratory, Room C2:03). An aseptic cell culture technique was strictly followed and performed in a laminar flow hood to avoid environmental contamination of cultures. All cell lines used in this study were screened by PCR to eliminate mycoplasma as outlined in section 2.2.7.

2.2.5.1 **Cell counting protocol**

A 0.100mm (0.0025mm2) Neubauer improved bright-line haemocytometer (Fortuna, Germany) was used to manually perform cell counts Figure 13.

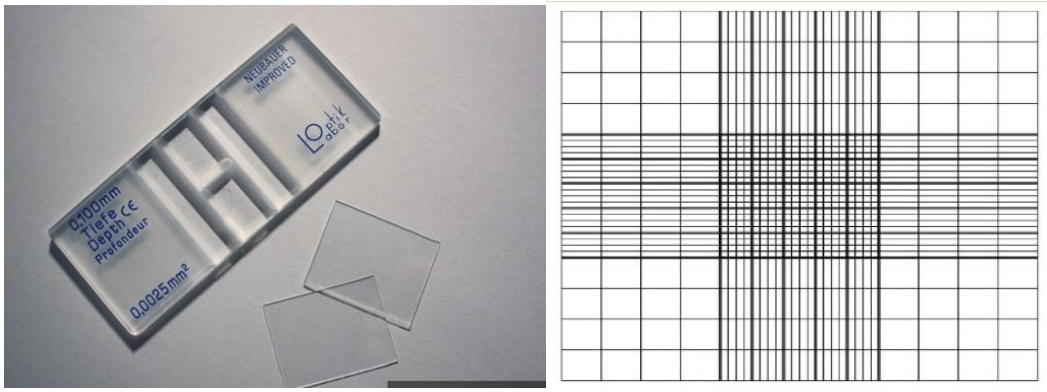


Figure 13 Neubauer improved counting chamber with gridlines shown

A 1:2 (v/v) dilution of 10µl sample of the cellular suspension was added to 20µl of trypan blue stain in a 0.5ml Eppendorf tube and vortexed for 10seconds. Then 10µl of this mixture was added to each of two chambers on the haemocytometer and five large squares counted on each. The total number from the ten squares was divided by 2 to get the average number of cells in five squares. Then the cell count was calculated with the formula outlined in Equation 1

Equation 1

$N = (n \times 10^4) \times$ (dilution volume of total cell suspension) x (Dilution volume of trypan blue)

Equation 2 Haemocytometer count

$N = c \times (4000/s) \times D$

n= number of cells/mm3

c=number of cells counted in given number of squares

s=number of small squares counted (5 squares contain 80 small squares)

D=dilution rate eg: 1:2

Equation 3 Population/Cell Doubling time (DT)

Population doublings = (log(end cell/start cell) / log(2)) x 24hours.

End cell = number of harvested cells

Start cell = number of seeded cells

End cell / start cell can also be referred to as the fold change in growth

For most of the experiments cells from passage four (P4) were used, thus the characterisation of the population doubling times at this passage was necessary for calculating number of cells present at certain time points during the experiments. Number of P4 cells present after T= hours of incubation. Number of P4 cells present after T= hours of incubation.

Equation 4 **Cell numbers at T=X hours**

Number of cells present after N generations = 2^number of generations x Initial number of cells seeded

2.2.6 CRYOGENIC PREPARATION AND PROTOCOL.

2.2.6.1 **Trypsinisation**

To trypsinise the cells an aliquot of 0.05% trypsin EDTA in PBS free off magnesium and calcium was placed in the incubator to warm to 37°C. The flasks containing cellular monolayers at 60-80% confluence, were taken into the sterile fumehood. The cell supernatants were poured off into a beaker then 10ml of sterile PBS was used to wash the flask before pouring off, this step was repeated twice. 5ml of trypsin was added to each flask and it was swirled for 30seconds to contact the whole surface. Then the excess trypsin was poured off and flasks placed in the incubator for 2minutes. After 2minutes the flasks were placed on the microscope to view the cells, the flasks were gently swirled to detach the cells. If cells were still adhered another the flasks were placed back into the incubator for another minute. To stop the enzymatic process, 5ml of culture media containing 10% FBS was added to the flask and the whole content from the flask was retrieved with a Pasteur

pipette and put into a15ml conical tube. This tube was then gently centrifuged at 250g for 5minutes. The cell supernatant was pipetted off and 1ml of warm basal culture media was added and the cell pellet was gently re-suspended, and cells could be counted as per section 2.2.5.1.

2.2.6.2 **Cryopreservation**

After the cell count had been established and the desired number of cells passaged, the cellular suspension was again centrifuged at 250g for 5minutes at RT. The supernatant was removed, and the cell pellet re-suspended in 1ml of cryogenic media at 4°C. Standard cryogenic media consisted of; 75% DMEM (low glucose) with 25% FBS and 5% DMSO.

The dilution factor was calculated for each sample and cryogenic media added to achieve a final concentration of $1x10^6$ cell/ml. A 1ml aliquot was added to each 1ml cryogenic vial (Greiner, Mediray). Vials were then placed in a Mr Frosty controlled freezing container (ThermoFisher Scientific) to freeze at the rate of -1°C per minute in the -70°C Freezer.

2.2.6.3 **Thawing procedure of cryogenic samples for cell culture**

Mesenchymal stromal cell samples were stored in cryogenic vials in -70°C freezer. They were removed from the freezer and held with forceps in a warm (37°C) water bath until just melted during 30-60s. The vials were then wiped with paper towels to dry and with ethanol to disinfect the vials. The cellular suspension containing $1x10^6$ cells in 1ml of cryogenic media (75% DMEM (low glucose) (12320-032, Gibco, Lifetechnologies), 20% FBS (1009-148, Gibco, Lifetechnologies) and 5% DMSO (Sigma) was removed from the vial with a 3ml sterile transfer pipette and put in a 15ml conical tube. Warm (37°C) DMEM (lg) wash media was dripped in slowly for the first 3ml then rapidly up to 10ml. The samples were mixed and centrifuged at 250g in an Eppendorf table centrifuge (5810 R, ThermoScientific), for 5minutes at RT. The supernatant was tipped off in a waste beaker and the cell pellet was gently re-suspended in 1ml of warm culture media (DMEM (low glucose (1g/L)) (ThermoFisher), 10% FBS (ThermoFisher), 1% Antibiotic-antimycotic solution (Sigma), gentamicin 0.05mg/ml (ProPharma) and Fungizone 0.01%

(Sigma). A 10µl sample of the cellular suspension was removed with a 10µl pipette and added to 20µl of trypan blue stain in a 0.5ml Eppendorf tube and vortexed for 10seconds. Then 10µl of this mixture was added to each of two chambers on the haemocytometer and viewed on an inverted Leica DMRD microscope at 10x and cell number counted. The cells at a seeding density of $5x10^3$ live cells/cm^2 were then added to either a T25 (in 10ml media) or T75 (20ml of media) flask and cultured for 36-72hours before an active growth phase/log phase was established. At an estimated confluency of 60-80% the flasks were then trypsinised to passage the cells or to cryopreserve the cells for later use in the experiments.

2.2.7 MYCOPLASMA DETECTION

2.2.7.1 **DNA extraction**

Cryogenically preserved cells were thawed at RT and centrifuged at 200g for 5 minutes 4°C in a benchtop centrifuge (Eppendorf). The supernatant was removed, and the cell pellet was re-suspended in a 200µl volume Digestion buffer containing 10µl of 10mg/ml Proteinase-K. The samples were then digested overnight in a 600rpm shaking thermomixer at 65°C. The following day the samples were cooled to RT and vortexed vigorously for 20 seconds. In the fume hood, 200µl of chloroform was added in order to remove protein from the sample. The tubes were mixed by vortexing vigorously for 20 seconds and finally incubated on a rotating mixing wheel for 5minutes at RT. The sample was centrifuged at 10,000g for 5minutes at 4°C. The top aqueous layer was transferred to a new sterile 1.5ml Eppendorf tube. Two volumes of ice-cold ethanol (100%) and one volume 2:1v/v of 3M Sodium Acetate (NaOAc) was added to precipitate the DNA. The tube was placed at -20°C for a minimum of one hour. The tubes were removed then centrifuged at 16,000g for 20 minutes at 4°C. The supernatant was removed carefully and the pellet was washed by the addition of 1ml ethanol (70%). The samples were centrifuged at 16,000g for 15min at 4°C. The supernatant was removed by pipetting and discarded, the tube was left to airdry to ensure no remaining ethanol present. The DNA was re-suspended in 100µl 10mM TE-buffer (pH 8) and stored on ice. The DNA concentration and quality was measured using the Nanodrop™ spectrophotometer.

2.2.7.2 Determination of sample DNA concentration using NanoDrop™ spectrophotometry

The NanoDrop spectrophotometer (NanoDrop™ 2000, ThermoScientific) was used as outlined by the manufacturer. Briefly, the Nanodrop™was cleaned using 5µl milliQ water and tissue paper. A blank was determined by placing 1µl of TE-buffer on the detector. Each sample was then added in a 1µl drop onto the detector and analysed. The detector was wiped carefully between each sample. A graph was obtained with the concentration and purity data displayed following the recording of the absorbance (A) readings at A_{260}, A_{280} and A_{280}, respectively. A minimum value of 50ng/µl is desired for the PCR analysis and if the values were higher than 50ng/µl a dilution was performed accordingly.

2.2.7.3 Polymerase Chain Reaction (PCR)

The PCR method used was developed by Uphoff and Drexler (2004). Primers were sourced from the University of Waikato University Inventory as described by Mayall (2015). Briefly, a 5µm primer mix of degenerate primers (Table 5) that target the Mycoplasma DNA was prepared and then a PCR mastermix with i-Taq™ (iNtRON Biotechnology, Ngaio Diagnostics Ltd, New Zealand) was prepared Table 5. Positive (POS) and negative (NEG) controls were included;

Primer	Nucleotide sequence (5' to 3')	Orientation
LMP13	CGCCTGAGTAGTACGTwCGC	FORWARD PRIMER*
LMP14	TGCCTGrGTAGTATGCTCGC	FORWARD PRIMER*
LMP15	CrCCTGAGTAGTATGCTCGC	FORWARD PRIMER*
LMP16	CGCCTGGGTAGTACATTCGC	FORWARD PRIMER
LMP17	GCGGTGTGTACAArACCCGA	REVERSE PRIMER*
LMP18	GCGGTGTGTACAAACCCCGA	REVERSE PRIMER

Table 5 Primers Mycoplasma PCR. The molarities of primers containing accessory symbols (w or r)* are reduced by 50%, therefore is adjusted for accordingly in Table 6)

Table 6 depicts the primer concentration and volume used to prepare a 5µm Primer and the PCR reaction was planned and set up as follows in Table 7.

Primer	Concentration	Volume
LMP13	100µm	20µl
LMP14	100µm	20µl
LMP15	100µm	20µl
LMP16	100µm	10µl
LMP17	100µm	20µl
LMP18	100µm	10µl
Sterile MQ H_2O		100µl

Table 6 Primers Mastermix for Mycoplasma detection

SAMPLE ID (lane 1-8)	**2x PCR Mix (i-Taq™) (µl)**	**5µM Primer Mix (µl)**	**POS Control (µl)**	**NEG Control (µl)**	**Internal DNA (µl)**	**Test Cell DNA (µl)**	**H_2O (µl)**
NEGATIVE Control	12.5	1		1			11.5
NEGATIVE Control plus Internal DNA	12.5	1		1	1		10.5
POSITIVE Control	12.5	1	1				10.5
POSITIVE Control plus Internal DNA	12.5	1	1		1		9.5
Sample 1	12.5	1				1	10.5
Sample 1 plus Internal DNA	12.5	1			1	1	9.5
Sample 2	12.5	1				1	10.5
Sample 2 plus Internal DNA	12.5	1			1	1	9.5

Table 7 PCR Reaction for Mycoplasma detection

Eight PCR tubes were labelled with numbers 1-8 and a 10µl volume was pipetted into the tubes in the sterile fume hood. The PCR tubes were placed in the thermocycler (BioRad) using the conditions outlined in Table 8.

94°C	2min	
35 cycles of:	94°C	4sec
	65°C	8sec
	72°C	16sec plus 1 sec extension time during each cycle
72°C	7min	

Table 8 PCR thermocycler conditions.

After completion of the thermocycle protocol the samples were allowed to cool at RT before agarose gel electrophoresis.

2.2.7.4 **Agarose gel preparation**

A 1.3% TAE agarose gel was prepared in a 250 ml Erlenmeyer flask using 40ml of TAE buffer and 0.52grams of agarose (HyAgarose 9012-36-6), Hydragene). The flask with its content was weighed and the weight recorded, then placed in a microwave oven on medium high for two minutes. If crystals of agarose could be seen, the mixture was then heated in one minute intervals until the crystals fully dissolved and the mixture was clear. The flask was re-weighed and de-ionised water was added to compensate for evaporative loss during heating. The agarose was cooled to 55-60ºC and then 2ul of Redsafe (21141, iNtRON) stain was added to allow visualisation of the DNA under UV light. The mixture was poured into a level gel tray and allowed to set at RT.

2.2.7.5 **Agarose Gel Electrophoresis**

The agarose gel was lifted from the mould and placed in TAE buffer in the electrophoresis tank (Owl Separation Systems). TAE buffer was added to completely immerse the gel. Two microliters of a 100 base pair (bp) ladder (PCR DNA ladder, M106R, Genescript, USA) was loaded in well 0 and after this 10µl of each sample (1-8) was loaded into corresponding well. The i-Taq has loading dye already added. The agarose gel was run at 90V, 500mA for 30minutes at RT. Following electrophoresis, the gel was lifted from the tank and placed in the trans-illuminator (Aplgen Omega Lum G Gel Documentation System, GMI) and viewed and photographed under UV light. Mycoplasma positive samples are expected to show a band at 502-520bp. All samples containing the internal control DNA should

show a band at 986bp. The internal control demonstrates that the PCR conditions are working.

2.2.8 TRILINEAGE DIFFERENTIATION

The trilineage differentiation potential was assessed for dermal fibroblasts, bone marrow and umbilical matrix derived cell lines. We assessed the differentiation potential of all cell populations at P4, as this was the passage where we had obtained sufficient number of cells and previous studies had used passages between P2 and P5, the decision was made to use cells all at the same passage to eliminate variability. This was achievable except for the commercial DF cell line, which was purchased at P13 and in order to multiply numbers the passage we worked with was at P16. The differentiation potential was assessed using a semi-quantitative measuring scoring system, scale 0-9.

Adipogenesis was confirmed at 14 days by using Oil Red staining of lipid droplets within the cytoplasm and a nuclear counterstain of haematoxylin eosin (H&E, Sigma). Chondrogenic differentiation was confirmed either by i.) fixation of monolayers grown in various culture conditions at different time points and staining with Alcian Blue, which highlights deposits of glycosaminoglycan's (GAGs) or ii.) by the harvest of cartilage pellets at Day 21 in chondrogenic induced cultures and subsequent fixation and cryostat preparation of pellets for histology and Alcian blue staining to visualize GAGs, lacunae formation and chondrocytes within the pellet matrix, counter staining with H&E. Osteogenic differentiation was confirmed between day 14-21 by staining of the monolayer with Alizarin Red to visualise calcium deposits.

2.2.8.1 **Adipogenic differentiation**

A commercial kit (Stempro® Adipogenic Differentiation Kit, A10410-01 Gibco) was used according to manufactures instruction and as per Barberini et al.(2014) and Carvalho et al. (2013). Actively growing cells were trypsinised as per standard protocol, centrifuged with the supernatant discarded by decanting. The cells were re-suspended in 1ml basal culture media and counted manually with a live/dead stain (trypan blue) and a Neubauer haemocytometer. Cells were plated $1x10^4$ cells per cm in 24-well plates (Nunc Delta, ThermoFisher) in 1ml basal culture media.

The cells were allowed to attach and multiply for 48hours until 60-70% confluent. Spent media was removed with a 3ml transfer pipette and discarded and 1ml of new basal culture media was applied to the control wells and also 1ml of the StemPro Adipogenic differentiation media was added to duplicate wells on the same plate, The plates were observed every 24hours for confluence and morphology and photos were taken at 2, 7 or 14days. Media was changed every three days. After 7, 14 or 21days the plates were removed from the incubator and media removed by a 3ml pipette. Each well was washed with PBS three times, then 1ml of 4% paraformaldehyde (PFA) was applied for 20minutes at RT to fixate the cells. The PFA was removed and the wells were rinsed with 1ml PBS twice followed by 1ml of 60% isopropanol for 5minutes. The plate was then inverted to pour the excess off prior to Oil Red-O staining.

Oil Red-O stain was freshly prepared according to a protocol derived from Lonza, 2011. Briefly, in the fume hood, 150mg Oil-Red-O powder added to 50ml 99% isopropanol. Thirty millilitres of this solution was mixed with 20ml deionized water and allowed to stand for 10minutes at RT, before filtered through filter paper into a clean beaker. One millilitre of the Oil-Red-O stain was added to each well and incubated for 5minutes at RT. Tap water was slowly rinsed down the centre of the plate to allow each well to be washed without direct pressure, ensuring the monolayer was kept intact. The wells were counterstained with 1ml of Haematoxylin for one minute at RT and then the tap water rinse step was repeated. Plates were viewed in a brightfield microscope (Olympus IX71) and photos were taken at 10x and 40x magnification. Each cell line was assessed for number and size of lipid vesicles and given a semi- quantitative adipogenic score, ranging from zero to nine (Table 9).

Percentage of differentiated cells		Number of lipid vesicles Within cytoplasm		Size of lipid vesicles	
0	0-25	0	0	0	0
1	25-50	1	0-5	1	Small <1/3 of nucleus
2	50-75	2	5-10	2	Medium =1/3 of nucleus
3	75-100	3	>10	3	Large >1/3of nucleus

Table 9 Semi-quantitative Adipogenic scoring system (0-9). Adapted from Redmond-Hubbard (2014).

2.2.8.2 **Chondrogenic differentiation**

The protocol used for chondrogenic differentiation was based on three different methodologies. Firstly, a commercially available Stempro® Chondrogenic Differentiation Kit (A10069-01, Gibco) was used as per manufactures instruction. A highly concentrated cell suspension was made containing $1.6x10^7$ cells per ml. For the micromass culture system; a 5µl drop of this solution ($8.5x10^4$ cells) was placed in the centre of the well in a 24well plate and incubated in a high humidity chamber inside the incubator (37°C 5% CO_2) for two hours, then basal culture media was added to the control wells and chondrogenic differentiation media in duplicate wells. Then incubated at 37°C in 5% CO_2 and grown for 14-21 days with regular media change every 3-4days. The cells were observed every 24hours for confluence and morphology and photos were taken at 48hours, 7, 14 and 21days.

Secondly, the micromass culture system published by Pilz et al. (2010) was adapted as follows. The differentiation was carried out in 96 well round-bottomed plates (Nunc, ThermoFisher). The wells were seeded with 8.5×10^5 cells in 5µl drops of basal culture medium placed in the centre of the well. The micromass droplets were incubated at two hours at 37˚C in 5% CO_2; 180µl of StemPro® chondrogenic medium was pipetted into the wells and culture continued for 21days with regular media change every 3-4days.

Finally, a pellet culture system described by Redmond Hubbard (2014) was carried out in parallel. A cell suspension containing $2.0x10^5$ cells were transferred to a 15ml tube and washed twice in PBS by centrifuging at 150g for 3minutes, after the second wash the supernatant was completely removed and 500µl of the StemPro chondrogenic media was applied to completely cover the cell pellet. The tubes were placed in a rack in the incubator (37°C 5% CO_2) and grown for 21 days with regular media change every 3-4days.

Using the micromass and pellet culture protocol it was not possible to view the cells on the microscope during the differentiation process. However, the media was inspected regularly for colour and cloudiness that would indicate contamination.

To confirm chondrogenic differentiation two staining methods were used;

1.) Staining of cells in a 24 well plate

After 7, 14 and 21 days respectively one third of the samples were fixed and evaluated. Each well of the 24-well plate were washed three times with PBS, then 4% PFA was applied for 20minutes at RT to fix the cells. One millilitre of Alcian Blue was applied overnight in the dark at RT to stain the monolayer in the wells. The wells underwent multiple washes with PBS until no dye residue was remaining. The monolayer was viewed on an inverted microscope (Olympus IX71)) at 10x and 40x magnification and photos were taken.

2.) Staining of cryopreserved sections

After 21 days the resulting cartilage pellets were harvested from the 96-well plate or 15ml tube and evaluated by histology. Briefly; pellets were detached and lifted from the wells or the 15ml tubes by use of a 21g needle and a fine tipped disposable pipette. The pellets were fixed in 1ml of 4% PFA for 1hour, then dehydrated three times in 400µl 30% sucrose solution before being incubated in 400µl of 30% sucrose overnight at 4°C. The following day 2/3 of the sucrose solution was removed and two drops of OCT (Jung tissue freezing medium, ThermoFisher) was added. The tube was incubated over night at 4°C to ensure penetration of OCT into the pellet. Half a millilitre of Alcian blue stain was applied to each pellet for 30minutes to stain the surface layer to make visualisation easier during the cryostat

sectioning step. Each cryomould was half filled with OCT and a pellet was placed in the centre of the mould and covered with more OCT. The moulds were snap frozen with freezing spray (Surgipath, Frostbite freezing spray) and sectioned at 4μm on a cryostat (Leica, CM1850). The sections were placed onto Surgipath Apex microscope slides and air-dried overnight. The slides were placed in a 3% Acetic acid wash for 3min, then placed in 1% Alcian Blue stain overnight and rinsed in distilled water for 1min or until clear. A nucleic acid counterstain (H&E, Sigma Aldrich) was applied for 1 minute then rinsed with tapwater. After being dipped briefly in alcohol to dehydrate then dipped in xylene, the slides were allowed to air dry. Using xylene mounting medium (Organo/Limonene Mount, #O8015 Sigma Aldrich) and glass coverslips the slides were viewed at 10x and 40x magnification on the microscope (Zeiss, Axiovert 40 CFL) to evaluate staining of glycosaminoglycans (GAGs) and the formation of lacunae. An adapted version of a semi-quantitative scoring system developed by Redmond-Hubbard (2014) was used to assess structural changes to the pelleted cells and the intensity of Alcian Blue staining Table 10.

Pellet shape and structure		**Lacunae formation**		**Staining intensity of pellet**	
0	No tertiary structure	0	No gaps	0	No colour change
1	Soft, irregular	1	< 10% gaps	1	Light blue
2	Round, Medium hardness	2	10-50% gaps	2	Medium blue
3	Round, Hard pellet	3	>50 % gaps	3	Intense blue

Table 10 Semi-quantitative Chondrogenic scoring system (0-9). Adapted from Redmond-Hubbard (2014)

2.2.8.3 **Osteogenic differentiation**

A commercial kit (Stempro Osteogenic Differentiation Kit, A10069-01, Gibco) was used according to manufacturer's instruction and as per Spaas 2012. Cells were plated at $5x10^3$ cells per cm in 24-well plates (Nunc, ThermoFisher) in 1.0ml basal culture media and allowed to attach and multiply for 48hours until 60-70% confluent. Spent media was removed and discarded. One millilitre of new basal

culture media was applied to the control wells and 1.0ml osteogenic differentiation media to triplicate wells on the same plate. The cells were observed and scored every 24hours for confluence and morphology; number of clusters and pellets, and photos were taken at 2, 7, 14 and 21 days. The media was changed every 3days. After 7, 14 and 21 days the plates were removed from the incubator and media was removed and discarded. Each well was washed with 1ml of PBS three times. Then 4% PFA was applied for 20minutes at RT to fixate the cells. Alizarin red (pH 4.2) was applied for 45minutes in the dark at RT to stain the monolayer. The wells were given a thorough wash with tap water until no stain residue was remaining. The monolayer was viewed on an inverted Olympus IX71 microscope at 10-40X magnification and photos were taken. An adapted version of a semi-quantitative scoring system devised by Redmond-Hubbard (2014) was used to assess structural changes to the pelleted cells and the intensity of Alizarin red staining (Table 11).

Percentage of differentiated cells		**Area of staining**		**Stain intensity**	
0	<5%	0	Small 0-25µm	0	No stained cells
1	10-25%	1	Small/medium 25-50µm	1	Light red
2	25-50%	2	Medium 50-75µm	2	Red
3	>50%	3	Large 75-100µm	3	Bright red

Table 11 Semi-quantitative Osteogenic scoring system (0-9) adapted from Redmond-Hubbard (2014)

2.2.9 IMMUNOCYTOCHEMISTRY

-Use of fluorescent antibodies to determine CD marker expression

Equine mesenchymal stromal cells for the immunocytochemistry experiment were grown in sterile conditions with standard basal culture media (Chapter 2.2.3) on 13mm glass coverslips inserted into 24well plates (Nunc Delta, ThermoFisher) until estimated confluence of 80%. Fixation of cells was performed as described by Leica-Microsystems (http://www.leica-microsystems.com/science-lab/how-to-prepare-your-specimen-for-immunofluorescence-microscopy). Chilled, (-20°C)

100% Methanol (MeOH) was gently added to the wells and incubated at -20°C for 20minute to fix the cells. The fixation solution was aspirated and discarded and then pre-chilled (-20°C) then 80% Acetone was added for one minute to permeabilise the cells. The wells were then washed twice with PBS before adding 1ml of PHEM buffer (60mM PIPES, 25mM HEPES, 10mM EGTA and 2mM $MgCl_2$) to each well, for storage. Before the next step the PHEM buffer was removed and the coverslips with the fixed cellular monolayer were removed manually with forceps from the wells. The coverslips were placed on a glass microscope slide with the monolayer facing up. An aliquot of 20µl of the primary monoclonal antibody diluted (1:40) in Sodium azide (PBSNaAz) solution, (see Table 12 for antibodies used), was applied to the coverslips and incubated at 4°C overnight. No primary antibody was applied to the negative control, just the PBSNaAz solution. After overnight incubation the coverslips were washed three times with PBS. Then 20µl of the secondary antibody conjugated (1:200) to a fluorophore (rat anti-mouse FITC) was applied to the coverslips and incubated for 30minutes at 37°C in the dark. After this the coverslips were washed with PBS three times and allowed to air dry. Before mounting them on glass slides in glycerol mounting media. The slides were kept covered or in the dark during this procedure to avoid bleaching of the fluorescence. They were viewed immediately on a Zeiss Axiostar phase contrast microscope fitted with a fluorescence power pack. If fluorescence was observed, photos were taken within 1hour of completion of staining on an Olympus microscope fitted with a fluorescence pack/blue light laser (488nm) at 40x magnification. The cells were observed in bright field and blue light fluorescence and photos of each view were taken. The staining intensity was given a subjective score of 0-4 with 0 being no fluorescence observed and 3 being very intense fluorescence.

Primary Antibody	**Supplier**	**Code**	**Clone**
CD105 reactive against horse	Abacus	SEMCA1557	clone SN6
CD29 mouse anti-human	Norrie	303001	clone TS2/16
CD44 rat anti-mouse/human	Norrie	103001	clone IM7
CD45 mouse anti-human	Abacus	SEMCA87A	clone F10-89-4
MHC-II mouse anti-human	Abacus	SEMCA1085GA	clone CVS20

Table 12 Primary non-conjugated antibodies used for Immunocytochemistry

The reported immunophenotype for equine MSC is: $CD29^+$, $CD44^+$ and $CD90^+$ and $CD45^-$ and MHC-II $^-$, respectively. Equine peripheral blood mononuclear cells at P-1, were used as positive control for CD45 and MHC-II monoclonal antibodies.

2.2.10 FLOW CYTOMETRY

-Method for determining presence of CD-markers on cell surface.

Mesenchymal stromal cell and dermal fibroblast samples stored in cryogenic vials (Greiner, MediRay) in liquid nitrogen vapour, were thawed in a 37°C water bath until the cellular suspension had just melted over 30-60s. Then the vials were dried with a paper towel and wiped with ethanol for disinfection. The cellular suspension containing $1x10^6$ cells in cryogenic media was removed from the vial with a 3ml sterile transfer pipette and added to a 15ml conical tube. Warm (37°C) DMEM (low glucose (1g/L)) wash media was dripped in slowly for the first 3ml then added rapidly up to 12ml. The samples were mixed by inversion and centrifuged at 250g for five minutes at 20°C. The supernatant was tipped off in a waste beaker and the cell pellet was gently re-suspended in 1ml of warm culture media (DMEM (low glucose (1g/L), Thermo Fisher), 10% FBS (Thermo Fisher), 1% Antibiotic-antimycotic solution (Sigma), gentamicin 0.05mg/ml (ProPharma) and Fungizone 0.01% (Sigma). The cells were set to equilibrate in a humidified incubator 37°C 5% CO_2. After one hour of incubation the cells were stained with trypan blue and counted manually using a haemocytometer. The live cell counts were recorded and dilution factors were applied to have the same concentration of cells per millilitre for analysis. The cells were centrifuged at 250g for 5minutes at 4°C to rapidly cool. Then the supernatant was tipped off and the cells re-suspended in FACS Buffer (PBS 1x and 1% FBS) to a concentration of 5 $x10^4$ cells per 10µl. An aliquot of 40µl ($2x10^5$cells) was used in the experiment. For each sample, 40µl was placed in two FACS tubes (round bottom 5ml glass tube); one unstained (negative control) and one sample, stained with 10µl of fluorescent antibody mastermix (Table 11.). The samples were incubated on ice in the dark for 30minutes, then washed twice in 1ml FACS Buffer and centrifuged 250g, 5min, 4°C. Two and a half microlitre of 4',6-diamidino-2-phenylindole (DAPI) nuclear stain (AbD Serotec, BUF061) was added and the cells were re-suspended in the residual volume of Fluorescence-

activated cell sorting (FACS) buffer (approx. 50µl) before vortexed and analysed on the BD SORP FACS Aria II cell sorter. The machine had initially been calibrated to determine a set voltage for each fluorophore. For each sample 100,000 events were recorded, and the dot plots were analysed for mean fluorescent intensity using FlowJo V7.6.5 analytical software (TreeStar, Ashland, USA).

An initial titration of antibodies was performed to determine the optimal concentration of each fluorophore. A mixture of mesenchymal stromal cells (ADSC, BMSC, UMBT and pBSC) was prepared and a 2-fold dilution series performed for each fluorophore. The optimal concentrations were determined and subsequently used in the main experiment. For the main experiment, a preparation of a fluorescent antibody mastermix was made (Table 13) to increase the speed of running the samples. An aliquot of 10µl of the mastermix was used per sample.

CD marker	**Code**	**Company**	**Clone**	**Fluorophore**	**Dilution**	**Mastermix**
CD29	303004	Mediray	TS2/16	PE	1/20	2.5µl
CD44	103030	Mediray	IM7	PE/CY7	1/80	0.625µl
CD90	202528	Mediray	OX-7	ALEXA700	1/80	0.25µl
CD45	SEMCA 87APCT	Abacus	F10-89-40	APC	1/20	5µl
MHC-II	SEMCA 1085F	Abacus	CVS20	FITC	1/80	1.25µl

Table 13 List of conjugated antibodies and dilutions used for the Mastermix for Flow cytometry

2.2.11 SYNOVIAL FLUID MODEL

2.2.11.1 **Set up of plates**

MSC from bone marrow, umbilical tissue and skin derived fibroblasts were utilised in this experiment. The MSCs were plated on 24-well plates (NuncDelta, ThermoFisher) at a seeding density of 20,000 cells per well = 11,300 cells per cm2 (Area per well = 1.76715 cm^2) in one millilitre of media. In the pilot experiment the cells were grown directly on the bottom of the plate, for the replications we used

sterilised (soaked in ethanol then flamed through Bunsen burner) 13mm glass coverslips inserted into the wells to allow removal for staining procedure.

The cells were assessed daily for attachment, morphology, growth pattern and degree of confluence. Photos of the cells were taken at 1, 2, 3 and 7days. The culture media was left unchanged for the duration of the experiment (Total 7days). This was done to assess cellular activity and how quickly they would metabolise the nutrients and to see if any resulting pH changes were caused by the cells themselves or the various concentrations of synovial fluid incorporated into the culture system. The cultures were assessed and the colour of the phenol red indicator in media was noted. On Day 0 and Day 7, the pH of the cell supernatant from each well was measured with pH indicator paper (Whatman, ThermoFisher).

For replication one and two; antibiotics and HEPES were added to the synovial fluid at identical concentration as the culture media, HEPES is a zwitterionic organic chemical buffering agent that helps minimise effects of change in carbon dioxide concentration produced by cellular respiration. Its' useful pH range is 2.5 to 3.5 or physiological pH 6.8 to 8.2.

To each ml of synovial fluid the following additives were included -: Antibiotic-antimycotic 0.001ml, Gentamicin 0.00125ml, Fungizone 0.0005ml, HEPES 5.96mg, the mixture was then vortexed for 20seconds. No signs of bacterial or fungal contamination was noted in the cultures.

A subjective estimation of confluence was performed by the same person in all experiments. A semi quantitative scoring system was devised by the author, where the number of cell clusters and their density (cluster or pellet) were recorded per well, was used to describe the change in morphology and growth pattern that was observed.

Trial	Treatment group: Cell source and sample number	Culture media composition
Pilot study	UMBT N=4	0% (control), 10%; 50% and 100% (no additives to the SF)
Rep 1	UMBT N=4	0% (control), 50% and 90% (added antibiotics, antifungal and HEPES to SF
Rep 2	UMBT N=4	0% (control), 50% and 90% (added antibiotics, antifungal and HEPES to SF
Mixed	Various N=4	0% (control), 6.25%, 12.5%, 25% and 50% (added antibiotics, antifungal and HEPES to SF
BMSC	BMSC N=4	0% (control), 50% (added antibiotics, antifungal and HEPES to SF
DF	DFs N=4	0% (control), 50% (added antibiotics, antifungal and HEPES to SF
UMBT	UMBT N=4	0% (control), 50% (added antibiotics, antifungal and HEPES to SF

Table 14 Different concentrations of allogenic synovial fluid evaluated

2.2.11.2 **Normal Synovial fluid experiment**

Various volumes of allogenic synovial fluid (SF) were obtained *ex vivo* from clinically normal joints by arthrocentesis. The joint was considered normal if the horse was free from lameness at the walk prior to euthanasia, and there were no clinical signs such as heat, pain or joint effusion present, that are commonly associated with joint disease (osteo-arthritis OA or degenerative joint disease DJD or septic joint disease SJD). The SF was collected *ex vivo* in a sterile manner at a local abattoir or on farm immediately after euthanasia. The hair was clipped over the carpal and stifle joints. Chlorhexidine surgical scrub was applied for a minimum of five minutes before the skin was wiped with methylated spirit. A sterile 10ml syringe and 21gg needle were used for arthrocentesis. Immediately after aspiration the SF was injected into sterile blood sample tubes containing no additives or anticoagulants (red top). Samples from different joints were mixed together to create one pooled synovial fluid sample per donor. The samples were kept at 4°C and processed within 4hours. At the laboratory the synovial fluid samples were centrifuged at 1000g for 10minutes to pellet any cellular content. The pellet was discarded, and the supernatant was removed and filtered (0.45µm Minisart, Sartorious stedim syringe filters) and frozen in 10ml aliquots in 15ml tubes at -70°C. An aliquot of each sample of synovial fluid was plated on 5% sheep blood

agar plates and placed in a 37°C incubator and visualised for any bacterial growth at 24hours and 48hours, if any growth was detected the sample would be discarded. An aliquot of each SF sample was also sent to an independent laboratory; New Zealand Veterinary Pathology, Hamilton and tested for Serum Amyloid A (SAA) and Total protein (TP) content.

2.2.11.3 **Normal Synovial fluid pilot trial**

The growth potentials of equine mesenchymal stromal cells from skin derived fibroblasts, bone marrow, umbilical cord perivascular tissue were determined in varying concentrations of equine allogenic synovial fluid (SF) obtained from apparently normal joints.

Normal synovial fluid samples were obtained *ex vivo* from aseptically prepared carpi or stifle joints as previously described in section 2.2.1. The synovial fluid samples were cryopreserved and kept at -70°C and thawed in RT before used in the experiments. Mesenchymal stromal cells for the synovial fluid pilot trial were grown in sterile conditions in duplicates on 24-well plates. Replications one and two were done with the cells grown on sterile 13mm glass coverslips inserted into the 24well plates. The basal culture medium was used in all experiments as described in Section 2.2.3 except that 5.96mg/ml of HEPES buffer 25mM, was added to the 50/50 SF media to help maintain physiological pH in the culture media.

2.2.11.4 **Inflammatory Synovial fluid**

The collected inflammatory synovial fluid (I-SF) samples were kept at 4°C and processed within 6 hours of collection. The samples were centrifuged at 1000g for 10min at 4°C, the cell pellet was discarded while the supernatant was retained, filtered through a 0.45µm syringe filter (16555, Minisart, Sartorious stedim), frozen, and stored at -70°C. An aliquot of each sample of I-SF was plated on sheep blood agar plates and placed in a 37°C incubator and visualised for any bacterial growth at 24hours and 48hours. If any growth was detected the sample was discarded.

The I-SF samples (N=12) were thawed at RT. Duplicate 0.5ml aliquots from each donor were placed in 1ml sterile Eppendorf tubes and again cryopreserved and kept at -70°C. The remainder I-SF samples were then pooled to make up a total volume of 50ml. An aliquot was taken from this pooled sample and tested for bacterial contamination on 5% sheep blood agar plates, the plates were visualised for any bacterial growth at 24hours and 48hours. Samples negative for any bacterial growth were sent to The New Zealand Veterinary Pathology (NZVP) laboratory for analysis of SAA and TP.

2.2.11.5 **Inflammatory Synovial Fluid Experiment**

The growth potentials of equine mesenchymal stromal cells and dermally derived fibroblasts were determined in 50% inflammatory allogenic synovial fluid mixed with 50% basal culture media (v/v).

Stromal cells from bone marrow, umbilical cord perivascular tissue and dermally derived fibroblasts were grown in sterile conditions in 24-well plates using basal culture medium (Section 2.2.3). The plates were kept at 37°C with 5% CO_2 in a humidified incubator (HealForce). The cells were assessed daily for attachment, growth pattern and degree of confluence as well as morphology. An estimation of confluence was performed by the same person in all experiments. The term confluence in cell culture is commonly used to describe the density of adherent cells and it is used as a measure of proliferation (Freshney 2010). It is combined with an estimated percentage, as an example 10% confluency means that 10% of the surface of the flask is covered with cells, 100% means that it is entirely covered. A record was also kept of the observed morphological change and the number of clusters as well as the number of pellets formed.

2.2.12 BIO ACTIVATION ASSAY

2.2.12.1 **Cytokine analysis by enzyme linked immunoassay (ELISA)**

A bio assay was set up to activate equine MSC and DFs with either TNFα or I-SF and compare with control cell populations grown in normal basal culture media. The outcome was to sample the cell supernatant and analyse for a panel of inflammatory and anti-inflammatory cytokines as listed in Table 15.

The plates were set up as Table 15 and cells were plated at a density of 20,000 cells per well in 1ml of media, allowed 36hours to attach and equilibrate to reach a state of active growth/log phase. The culture media was removed together with any unattached cells and cellular debris. The cells were exposed to one of three conditions;

1. Basal Culture Media (CM) control
2. Tumour Necrosis Factor alpha 25ng/ml (TNFα) in basal culture media
3. Inflammatory Synovial fluid 50% (I-SF) with 50% basal culture media (v/v) with 2X antibiotics/antifungals (1% Antibiotic-Antimycotic 1X, 0.05mg/ml Gentamicin and 0.01% Fungizone) and 25mM/L HEPES) to give the same final concentration as the basal culture media.

Time (hr)	**Control CM**	**Treatment 1 - TNFα**	**Treatment 2 - I-SF**	**Control CM**	**Treatment 1 -TNFα**	**Treatment 2 - I-SF**
24						
48						
72						
168						

Table 15 Layout of 24-well plate for activation experiment, one horse per plate, duplicate samples for each time point

The cell cultures were exposed to the activation conditions for 12hours, after this the activation agent and all supernatants were removed and discarded and the monolayers were washed three times with 1ml PBS. One millilitre of basal culture media was subsequently added to the wells and the plates were incubated as per normal cell culture protocol. The cell cultures were observed every 24hours and estimations of confluence and change in morphology were recorded and photographed. At each time point (24, 48, 72 and 168h) duplicate 1ml sample aliquots of the cell supernatant were collected into 2ml Eppendorf tubes and frozen at -70°C. These samples were later run on equine specific sandwich ELISAs (Genorise, USA) for testing of six selected cytokines, see Table 16.

After the initial sample aliquot was taken, new basal media was added to the well and cells were left growing for 21days, with medium changes every 3-4days, then cultures were terminated. Some plates had chondrogenic pellets and alcian blue stain was applied in situ and photographs were taken, other pellets were harvested at 21 days and processed for cryostat sectioning and staining as per the chondrogenic differentiation protocol, Chapter 2.2.8.2.

2.2.12.2 **Cytokine analysis - ELISA**

Equine specific ELISA kits for the following cytokines; IL-1b; IL-6; IL-10; TGF-β1, TNFα and PGE_2, were obtained from Genorise Scientific, USA.

Pro-inflammatory cytokines	**Anti-inflammatory cytokines**
Tumour necrosis factor alpha (TNFα)	Transforming growth factor beta 1 (TGF-β1)
Interleukin-1 beta (IL-1β)	Interleukin-10 (IL-10)
Interleukin-6 (IL-6)	Interleukin-6 (IL-6)
Prostaglandin E2 (PGE_2)	

Table 16 Selected cytokine panel

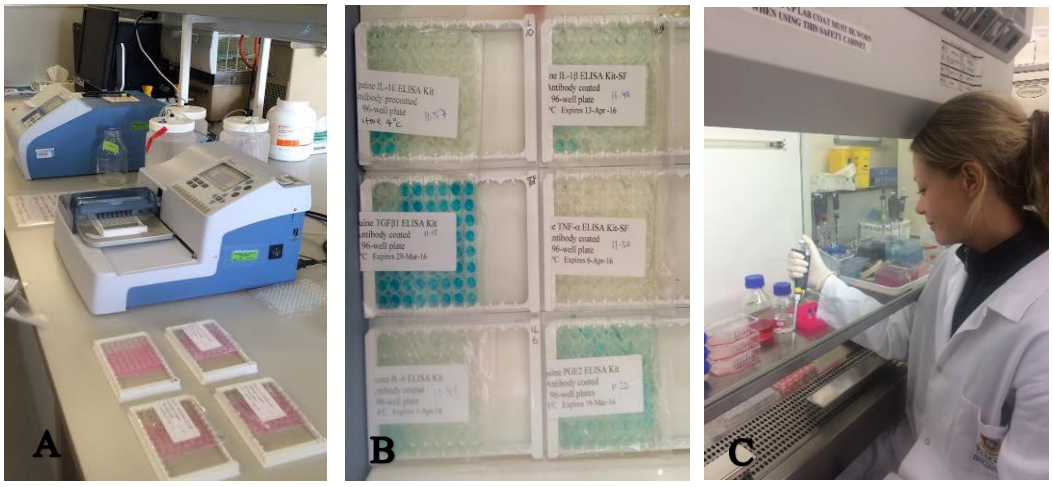


Figure 14 Overview of cytokine experiment setup. A). Microplate washer and reader set up, B) developed ELISA plates and C). Sterile preparation of samples for bio assay experiment

The reagents were prepared as per manufacturers' instructions and the assay procedure was followed exactly. The set up of plates and washer were set up as per

Figure 14. An automated plate washer (Wellwash Versa, Thermo Scientific) was used to wash plates between each step and the plates were read on a microplate reader at 450nm. The resulting data was saved as a Microsoft excel file. Cryogenically preserved 1ml aliquots of cell culture supernatant were thawed in tube racks at RT until just melted, then placed on ice to remain cool at 4°C. The ELISA strip plates were removed from 4°C and allowed to come to RT. A two-fold serial dilution of the standard of each cytokine was performed and 100µl of each known concentration was added to duplicate wells on the ELISA plate. The cell culture supernatant samples were vortexed (5sec) and 100µl was added to duplicate wells. The plate was covered and incubated at RT for two hours. Three washes were done through the automated plate washer and excess liquid was blotted away to ensure complete removal of the wash buffer. Then 100µl of detection antibody was added to each well and incubated for two hours at RT. The wash procedure was repeated. Then 100µl of working dilution of Conjugate was added and the plate was incubated in the dark for 20minutes. The wash procedure was repeated. Then 100µl of Substrate solution was added to all wells and incubated for 20minutes in the dark, after which 50µl of Stop solution was added and the absorbance was read immediately on the microplate reader and data recorded.

2.2.12.3 **Pilot trial; Temporal Study of one selected bone marrow and one dermal fibroblast cell line**

An initial validation experiment was performed using stromal cells from two different mesenchymal sources; BMSC (N=1 r=2) and DF (N=1 r=2). A temporal analysis of absorbance of all set time points (1, 2, 3 and 7days) was performed for each of those two donors. Plain culture media and media containing various concentrations of synovial fluid were also tested for confirmation of blank and background readings. This temporal analysis was done with the aim to identify an optimal time point where the remainder samples could be compared. The 24hour time point was selected for the continuing experiment.

The second part of the experiment (ELISA2 and 3) was performed with three cell sources; ELISA 2 BMSC (N=4) and DF (N=4) and ELISA3 UMBT (N=8) and DF (N=4). The samples were collected at T=24hours and run in duplicates. A standard

curve of known concentrations was prepared in GenStat 64-bit Release 18.1 and plotted for each cytokine.

2.2.12.4 **Bio Activation Assay Run1**

-Activated BMSC vs. DF

The first experiment evaluated six cytokines; IL-1b; IL-6; IL-10; TGF-β1, TNFα and PGE_2. Cryopreserved cell supernatant samples from eight horses from two different sources (BMSC (N=4) and DFs (N=4)) obtained from the Activation experiment were used. The samples were analysed at time point T=24h. The ELISAs were run as per manufacturers' instruction as previously described in section and all samples were run as duplicates. The absorbance data was analysed on Minitab and GenStat 64-bit statistical software.

2.2.12.5 **Bio Activation Assay Run2**

-Activated UMBT vs. DFs

The second experiment evaluated the following cytokines; IL-10; TGF-β1, TNFα and PGE_2. Cryopreserved cell supernatant samples from twelve horses from two different sources (UMBT (N=8) and DFs (N=4)) obtained from the Activation experiment were used. The samples were analysed at time point T=24hours. The same dermal fibroblast cell lines (N=4) as tested previously in experiment 1 were tested again. The ELISAs were performed as per manufacturers' instruction (Genorise Scientific, USA). The assay was repeated twice and each sample was run in duplicates. A standard curve was created and had a general logistic fitted:

Equation 5 Fitted logistic ELISA Standard curve

$$Y= A + C/ (1 + e^{-B(X-M)})$$

(Y= absorbance and X= predicted concentration)

The predicted concentrations of each cytokine produced by each cell line were calculated and graphed. Data were expressed as mean ± SD of the secreted factor. Then using population growth data the cytokine production per 10^4 cells were calculated and graphed.

2.2.12.6 **Bio Activation Assay Run3;**

-Activated BMSC vs. UMBT vs. DFs

The third experiment evaluated only IL-10. Cryopreserved cell supernatant samples from three different sources; DFs (N=4), BMSC (N=5) and UMBT (N=8) obtained from the Bio Activation assay experiment were used. The samples were analysed at time point T=24hours. The ELISAs were performed as per manufacturers' instruction (Genorise Scientific, USA). The assay was repeated twice and each sample was run in duplicates. A standard curve was created and had
a Michaelis- Menten function fitted:

Equation 6 Fitted logistic ELISA Standard curve

$$Y = \theta_1 X / \theta_2 + X$$

(Y= absorbance and X= predicted concentration)
The predicted concentrations of each cytokine produced by each cell line were calculated and graphed. Data were expressed as mean ± SD of the secreted factor. Then using population growth data, the cytokine production per 10^4 cells was calculated and graphed.

2.3 STATISTICAL ANALYSIS

A number of different experiment were performed and a repeated measures model that recognised multiple observations as belonging to the same horse was used to test for differences in proliferation, differentiation as well as cytokine production after activation, between treatment groups and time points. Numerical data and residuals were graphed as histograms for each subset, to visualise the distribution of data to assess frequency distribution and normality. An a priori analysis of sample size was done with G* Power calculator, 3.1.9.2 for Windows XP, (HH University of Dusseldorf, Germany).

Experiment 1 Population doubling times; GraphPad Prism software version 7.02 for Windows (GraphPad Software Inc, La Jolla, CA, USA) was used in the statistical analyses. The cell populations from all five tissue sources were counted at seeding and again at 80% confluence at passage 4. Duplicates samples were counted per cell line. The residuals of means for each tissue source were plotted in histograms to view the frequency distribution and the data was confirmed to be normal Gaussian distribution at 95% confidence interval (Appendix 4). The

D'Agostino & Pearson normality test also confirmed the data to be normally distributed (alpha= 0.05). One-way ANOVAs were performed to test the inter-group variability of means. The data is presented as Means ± SD

Experiment 2 Flow Cytometry

FlowJo V7.6.5 analytical software (TreeStar, Ashland, USA) was used for statistical analysis of flow cytometry data. Quadrant markers to determine marker expression were set according to negative control stains, including ‘‘fluorescence minus one’’ (FMO) controls that used all antibodies except that to the marker in question. Each run recorded 100,000 events. The data for each cell line is presented as dotplots and individual histograms for each CD marker.

Experiment 3 Trilineage Differentiation; The GraphPad Prism software version 7.02 for Windows (GraphPad Software Inc, La Jolla, CA, USA) was used in the statistical analyses. Cell populations from three tissue sources were induced to undergo differentiation in two separate replications of the experiment. Subjective scoring using a semi-quantitative scale, was performed on triplicate samples from each cell populations. The residuals of means for each differentiation potential (adipo, chondro and osteogenic) were plotted in histograms to view the frequency distribution and the data was confirmed to be normally distributed at 95% confidence interval (Appendix 4). The D'Agostino & Pearson normality test also confirmed the data to be normally distributed (alpha=0.05). One-way ANOVAs were performed to test the inter-group variability of means. The data is presented as Means ± SD.

Experiment 4 Synovial Fluid Model

The GraphPad Prism software version 7.02 for Windows (GraphPad Software Inc, La Jolla, CA, USA) was used in the statistical analyses. Three culture conditions were applied. A temporal analysis was performed at three different time points (24, 48 168h), for data collected within source (intra-source) for the control and two culture conditions. A subjective estimate of the percentage of confluence was recorded on duplicate samples per treatment and cell line. The mean for each cell line was added to the source data and graphed as Means ± min-max. For each time

point a paired t-test was run to compare the means within source for control vs each culture condition.

Experiment 5 Bio Activation Assay

GenStat 64-bit Release 18.1 (PC/Windows 7, VSN International Limited, Hemel Hempstead, United Kingdom), was used for the generation of standards curves and fitted equations, as well as one-way ANOVA analysis using absorbance and predicted concentrations as response variables. Student Newman Keuls was used as a post-hoc test to distinguish means between treatment groups. GraphPad Prism software version 7.02 for Windows (GraphPad Software Inc, La Jolla, CA, USA) was also used to calculate and graph concentrations per 10^4 cells. The data was plotted in histograms to confirm a normal Gaussian distribution (Appendix 4). Student paired t-test were performed for inter-source data to compare the effect of each of the two activation groups vs control per cytokine. The data is expressed as the Mean ± Standard Deviation.

CHAPTER 3: RESULTS

3.1.1 EXPERIMENT 1: MORPHOLOGICAL STUDY

H0: The hypothesis is that stromal cells from adipose, bone marrow, peripheral blood and umbilical cord tissue have equivalent morphological characteristics as dermally derived fibroblasts

Cell lines from five equine sources were used during this study; adipose (ADSC; N=4), bone marrow (BMSC; N=6), peripheral blood (pBSC N=4), umbilical cord matrix (UMBT; N=8) and skin dermis (DF; N=4). All cell lines showed a fibroblast-like morphology and plastic adherence. The cells were spindle shaped, slender and elongated. After seeding in plastic culture flasks, the cells formed distinct colonies and with increasing time in culture a monolayer. Figure 15 below shows a representation of each cell source. For DFs and BMSC this phenotype was maintained across all the passages, P0 to P6, (P16 for the commercial DF cell line). From passage P2 to P6, there was a small difference noted in the morphology of the UMBT cell lines; cells appeared slightly smaller and acquired a more triangular to polygonal shape with shorter cytoplasmic extensions compared to the elongated swirls of the DF and BSMC cells. Still they were identified as a mesenchymal phenotype.

When cultures were left more than two weeks without passaging a mesenchymal-epithelial transition (MET) was observed in the DF cell lines. Figure 16 shows examples of the cellular morphology after two weeks in culture of two DF cell lines and two BMSC cell lines. The cells comprising the two DF monolayers are polygonal in shape with more regular dimensions and grow attached to the substrate in discrete patches, here in a monolayer. This is typical for an epithelial phenotype. The BMSC cells still has a mesenchymal morphology although cells are slightly more expanded than a monolayer that has had less time in culture.

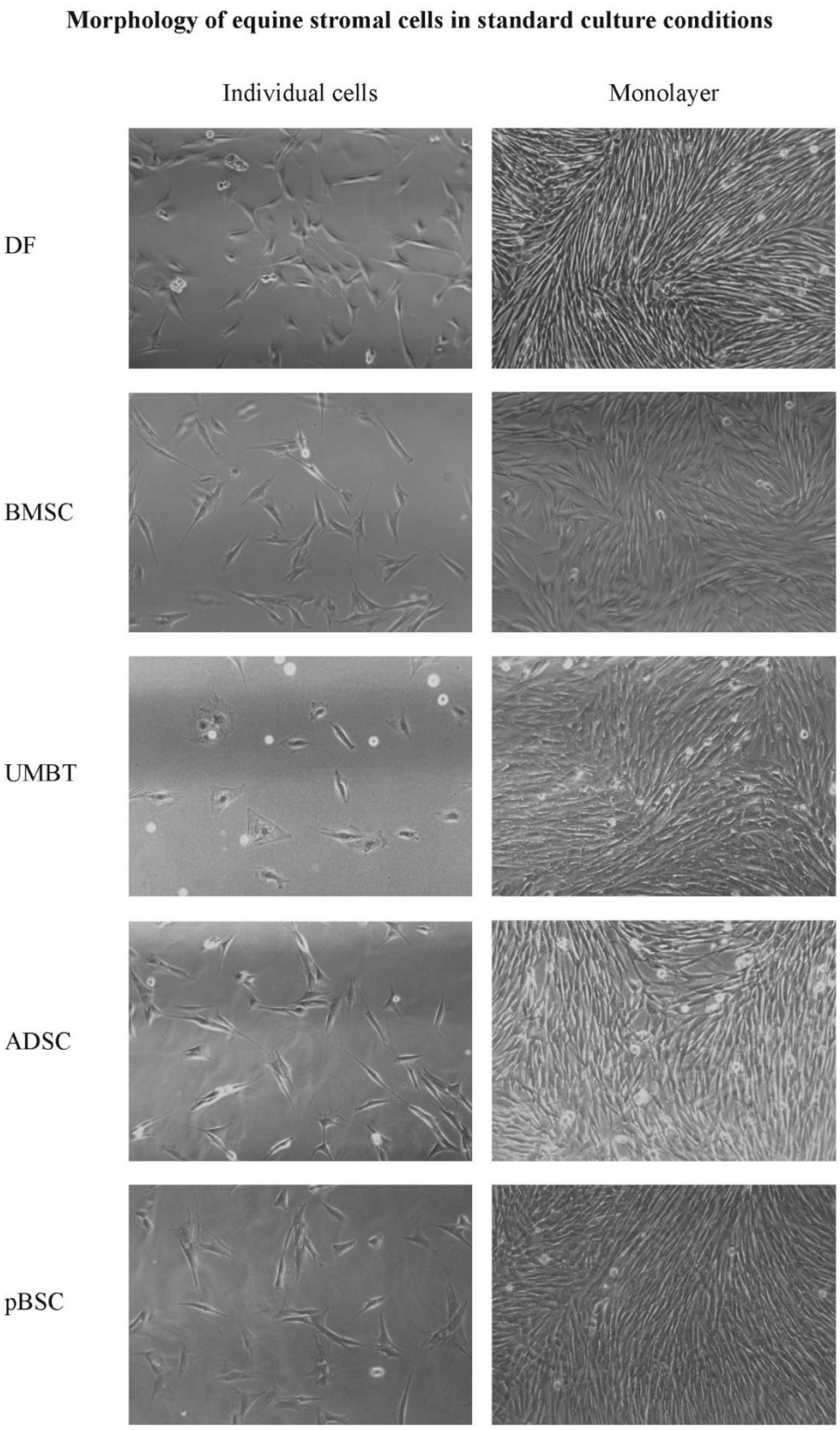


Figure 15 Representation of selected stromal cell lines and dermal derived fibroblasts displaying both individual cell morphology and monolayer formation

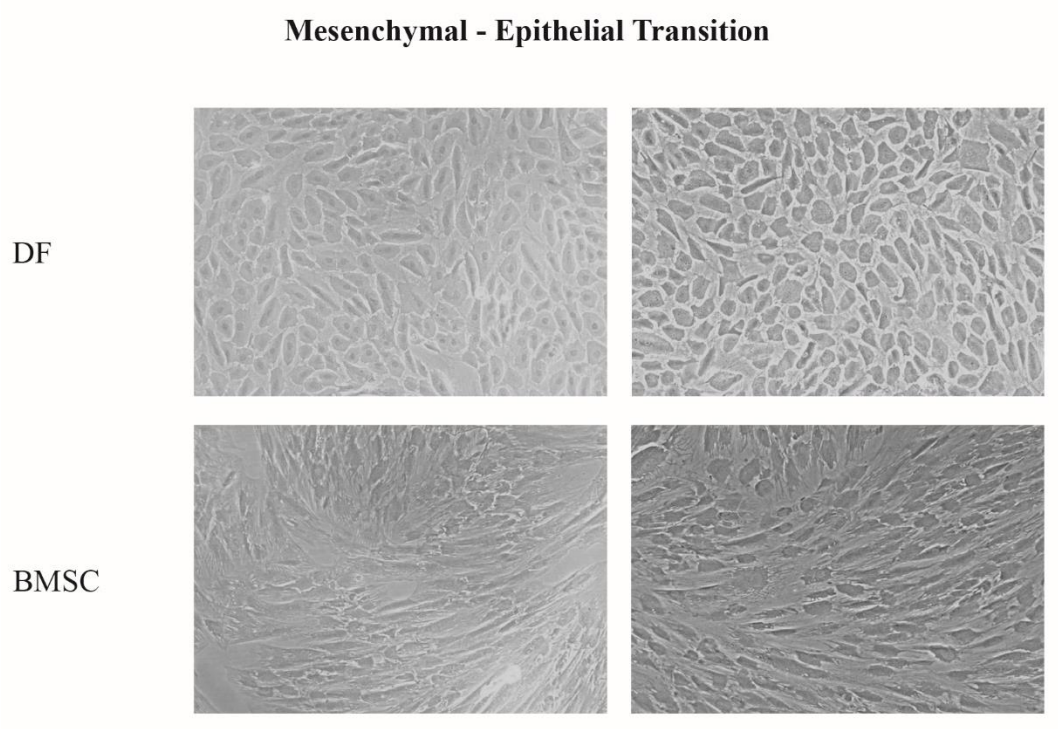


Figure 16 Mesenchymal-epithelial transition of two DF and two BMSC cell lines at P4 after >2 weeks in culture with no passaging of cells

3.1.1.1 Population Doubling Times

H0: The hypothesis is that stromal cells from adipose, bone marrow, peripheral blood and umbilical cord tissue have equivalent population dynamics as measured by population doubling times, as dermally derived fibroblasts

Table 17 below represents the Mean ± SD and SEM of the populations doubling times for each cell source. There were no statistical significant differences (P=0.78) in population doubling time between the five tissue sources noted at P4.

Population Doubling Times (hours) at P4	**BMSC (N=6)**	**UMBT (N=8)**	**DF (N=4)**	**ADSC (N=2)**	**PBSC (N=4)**
Mean	26.70	33.32	30.18	31.70	25.61
Std. Deviation	8.58	10.13	18.20	6.08	16.20
Std. Error of Mean	3.24	3.38	9.10	4.30	8.10

Table 17 Mean of Population Doubling times for all tissue sources at P4

Figure 17 below is a graphical representation of the data in Table 17 showing the Mean ± standard deviation of hours taken for cell populations from each tissue source to double in numbers.

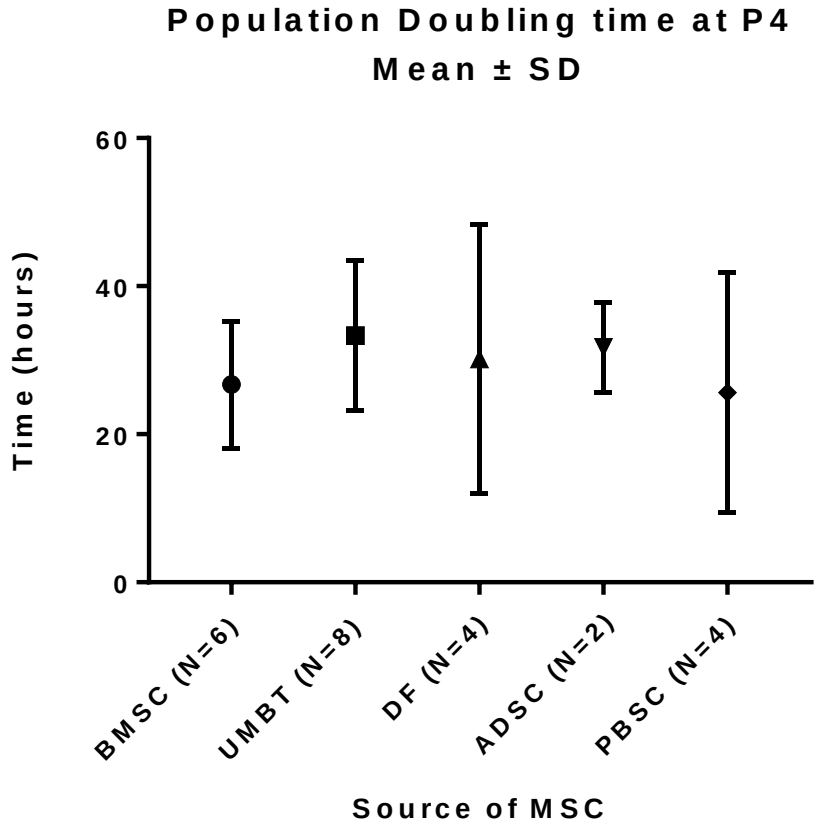


Figure 17 Population Doubling times (hours) per tissue source at passage 4 expressed as Mean ± SD. There was no significant inter-source difference (P=0.78) for population doubling times

The exponential growth could also be assessed for each tissue source over a 72 hour time period as displayed in Figure 18. The growth curves were calculated using the population doubling means from each tissue source over the time period of 72 hours post seeding, since the experiments were carried out during this time point (24hours post activation agent removal).

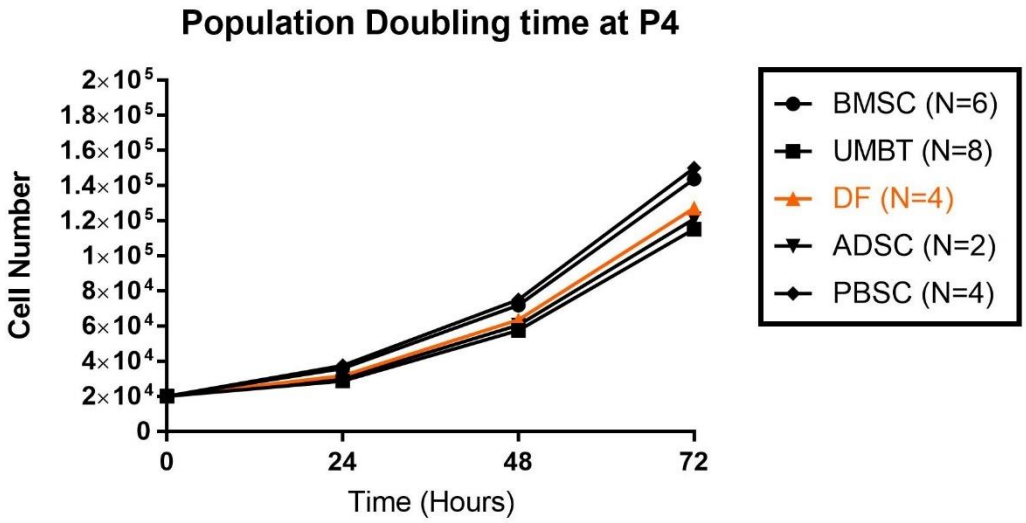


Figure 18 Exponential growth of all MSC and DF populations at passage 4

3.1.1.2 **Mycoplasma PCR**

All cell samples submitted for mycoplasma testing had excellent DNA recovery. The DNA concentrations of the samples as measured with NanoDrop, (Nanodrop 1000 V3.7, Thermo Scientific) were in the range of 113.8 -889.8ng/µl (N=12). Figure 19 shows the electrophoresis results of the Mycoplasma PCR reaction.

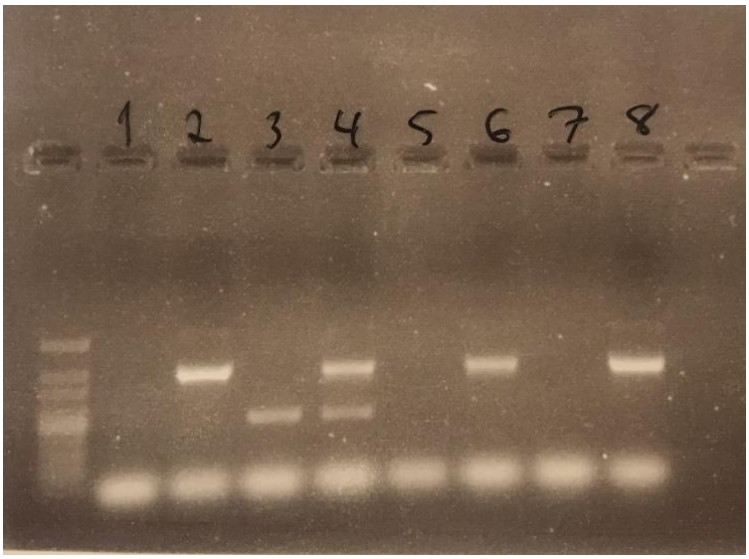


Figure 19 Electrophoresis gel Mycoplasma negative BMSC and DF (lane 5 and 7)

Lane 1 represents the negative control and thus indicates that there is no contamination but primer dimers are evident. The band for the Internal DNA is seen in lane 2 and the positive control (*Mycoplasma*) in lane 3, with the two combined

(Internal DNA plus positive control) seen in lane 4. The two samples (BMSC and DF) are in lanes 5 and 7, showing no bands, thus, no *Mycoplasma* contamination was detected. All the cell lines used in this experiment were found to be negative for *Mycoplasma* species.

3.1.2 EXPERIMENT 2: IMMUNOPHENOTYPING OF MSC & DFS

3.1.2.1 Immunocytochemistry (ICC)

H0: The hypothesis is that stromal cells from adipose and bone marrow have the same qualitative expression of cell surface markers, Positive for; CD29, CD44, CD90 and Negative for; CD45, MHC-II, as dermally derived fibroblasts

The optimal dilution for these selected primary monoclonal antibodies was determined to 1:20-1:40 and for the secondary fluorescent labelled antibody the optimal dilution was 1:50-1:100. Subjective staining scores displayed in Table 18.

	Primary antibody dilution series					
Antibody	1:20	1:40	1:80	1:160	1:320	Negative control
CD29	1.5	1.5	0.5	0.5	0.0	0.0
MHC-II	0.2	0.0	0.0	0.0	0.0	1.5 (MNC)
	Secondary antibody dilution series					
Antibody	1:50	1:100	1:200	1:400	Negative control	Positive control
CD29	1.5	1.0	0.5	0.5	0.0	
MHC-II	0.5	0.5	0.0	0.0	0.0	1.5 (MNC)

Table 18 Subjective staining score of Primary and secondary monoclonal antibody dilution series. Mean (N=2, triplicate samples). Subjective staining score ranged from 0 (no fluorescence observed) to 3 (intense green signal).

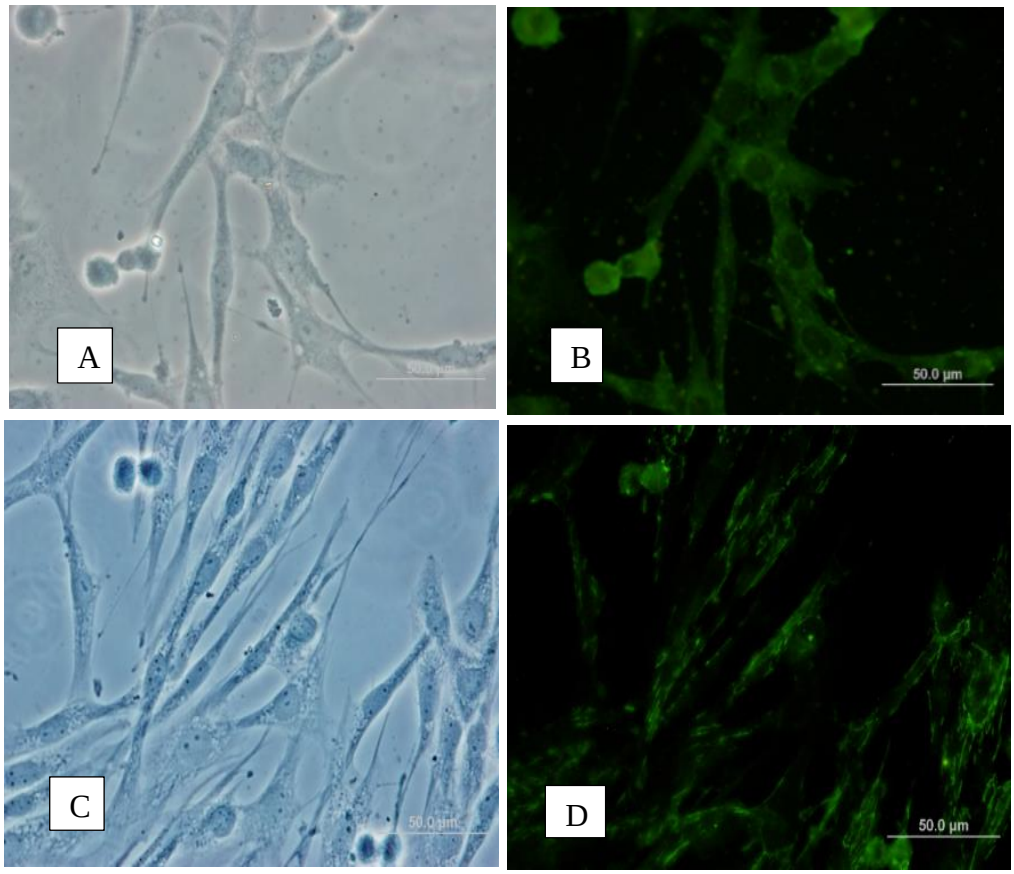


Figure 20 Morphology and cell surface marker expression for two equine cell types. Dermal fibroblasts: A.) confocal microscopy at 40X magnification B.) Positive staining for CD29, secondary antibody rat anti-mouse FITC at 490nm. Adipose stromal cells C.) Confocal microscopy at 40X magnification D.) Positive staining for CD29, secondary antibody rat anti-mouse FITC at 490nm. Scale bar = 50 μM.

Positive immunofluorescence for CD29 was shown in equine ADSC (N=2), BMSC (N=2) and as well as in DF cell lines (N=2) as depicted in Figure 20. The fixed cells in the samples stained uniformly; if the sample was positive for stain, all of the cells stained. The same proportions applied for the negative marker MHC-II for which no staining was detected in either of the MSC or DF cell lines. However, the ICC results for MNC samples (positive control) showed positive results for both of the negative MSC markers; MHC-II and CD45, which confirms the antibodies are working. A more extensive investigation of the immunophenotypes of selected BMSC and DF cell lines were performed, and the results displayed in Figure 21. There was no difference in the morphological appearance of BMSC or DF populations and no apparent difference in distribution of CD markers on the cell

surface. Mononuclear cells were included as positive controls for the negative MSC markers; MHC-II and CD45.

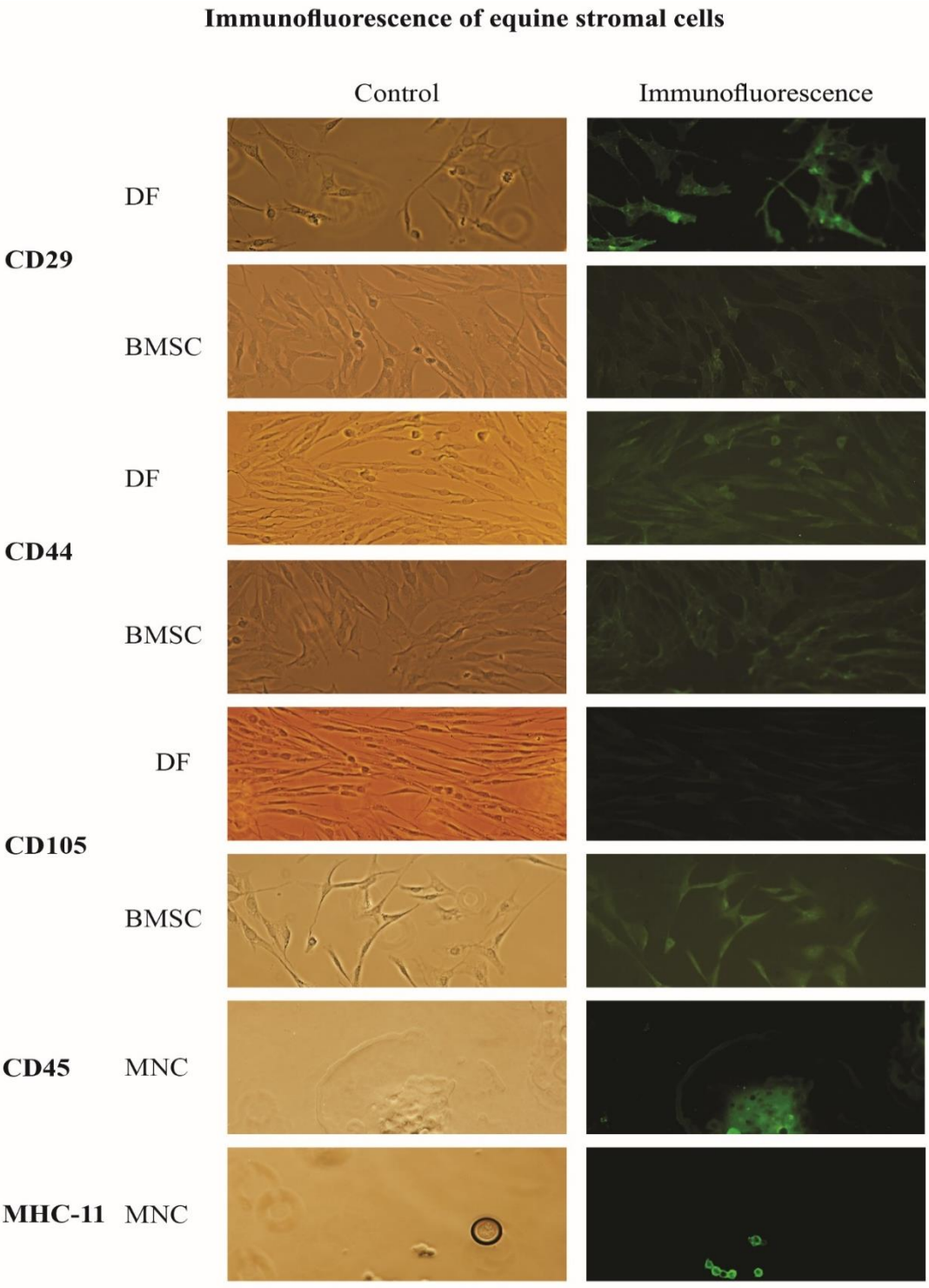


Figure 21 CD marker expression comparison for two equine cell types; DF and BMSC: confocal microscopy at 40x magnification, immunofluorescence viewed at 490nm.

3.1.2.2 **Flow cytometry**

H0: The hypothesis is that stromal cells from adipose and bone marrow have the same qualitative expression of cell surface markers, Positive for; CD29, CD44, CD90 and Negative for; CD45, MHC-II, as dermally derived fibroblasts

The CD marker analysis of the selected cell populations was performed with flow cytometry using the gating strategy shown in Figure 22. Top row of dot plots. The cells were first gated by Forward Scatter–A (FSC-A) and Sideways scatter–A (SCC-A) followed by doublet exclusion using FCS-A/FSC-H and SSC-A/SSC-H. Nonviable (DAPI+) cells were excluded by the use of DAPI staining.

The MSC cell populations were shown to have the following immunophenotype; $CD29^{+}$, $CD44^{+}$ and $CD90^{+}$ and $CD45^{-}$ and $MHC\text{-}II^{-}$. As displayed in Table 19.

Source	**Horses tested (N)**	**CD29**	**CD44**	**CD90**	**CD45**	**MHC-II**
BMSC	5	+	+	+	-	-
ADSC	3	+	+	+	-	-
pBSC	4	+	+	+	-	-
UMBT	4	+	+	Weak +	-	-
DF	4	+	+	+	-	-

Table 19 Flow cytometry results for MSC and DF, + Positive result, - Negative result

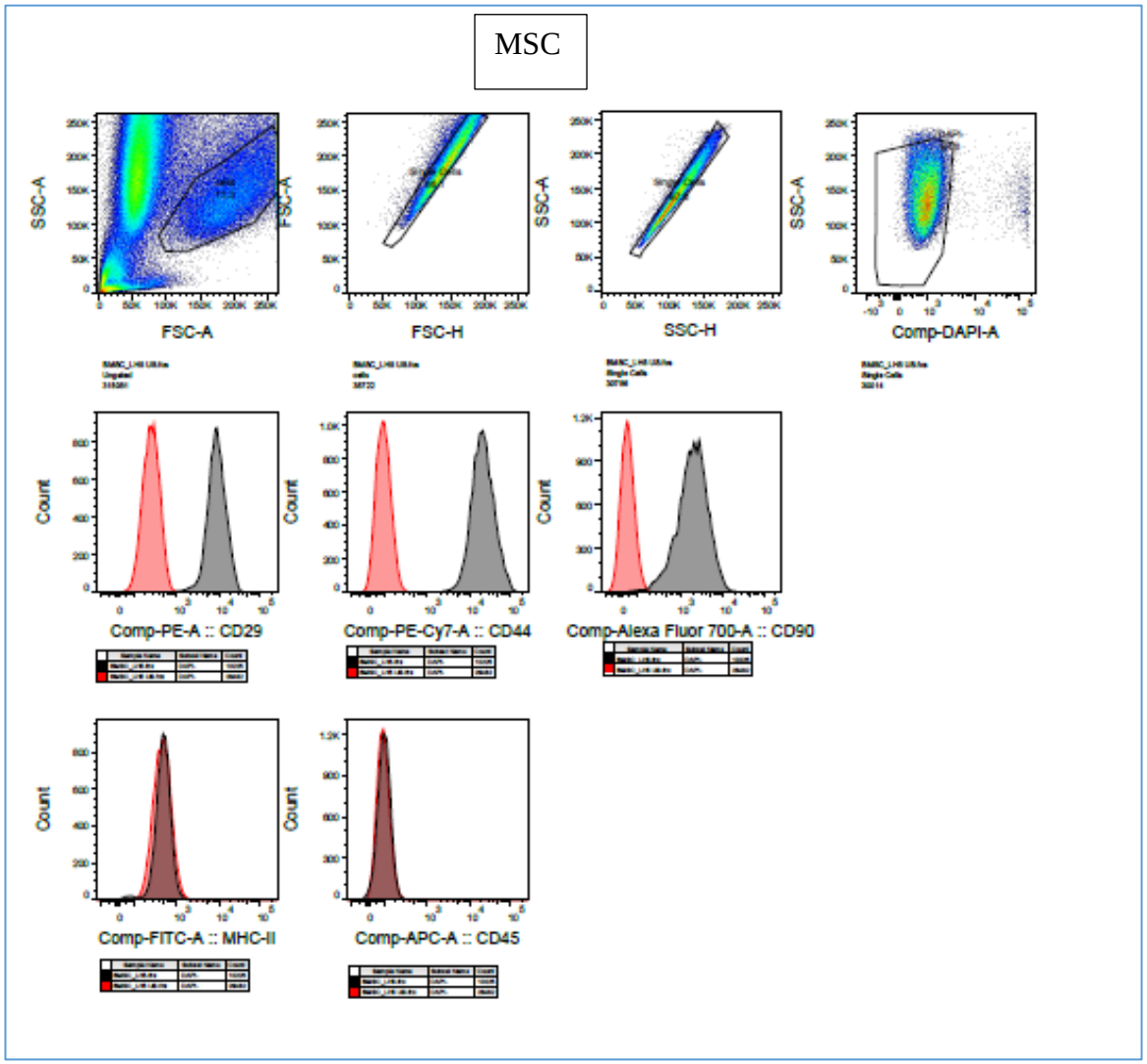


Figure 22 A representation of a typical mesenchymal stromal cell dot plots and histograms. Here a BMSC cell line is representing all of the stromal cell populations.

The results of this experiment demonstrated that equine stromal cells from adipose tissue, bone marrow and peripheral blood have the same immuno-phenotype as previously reported in the literature (DeSchauwer et al. 2011).

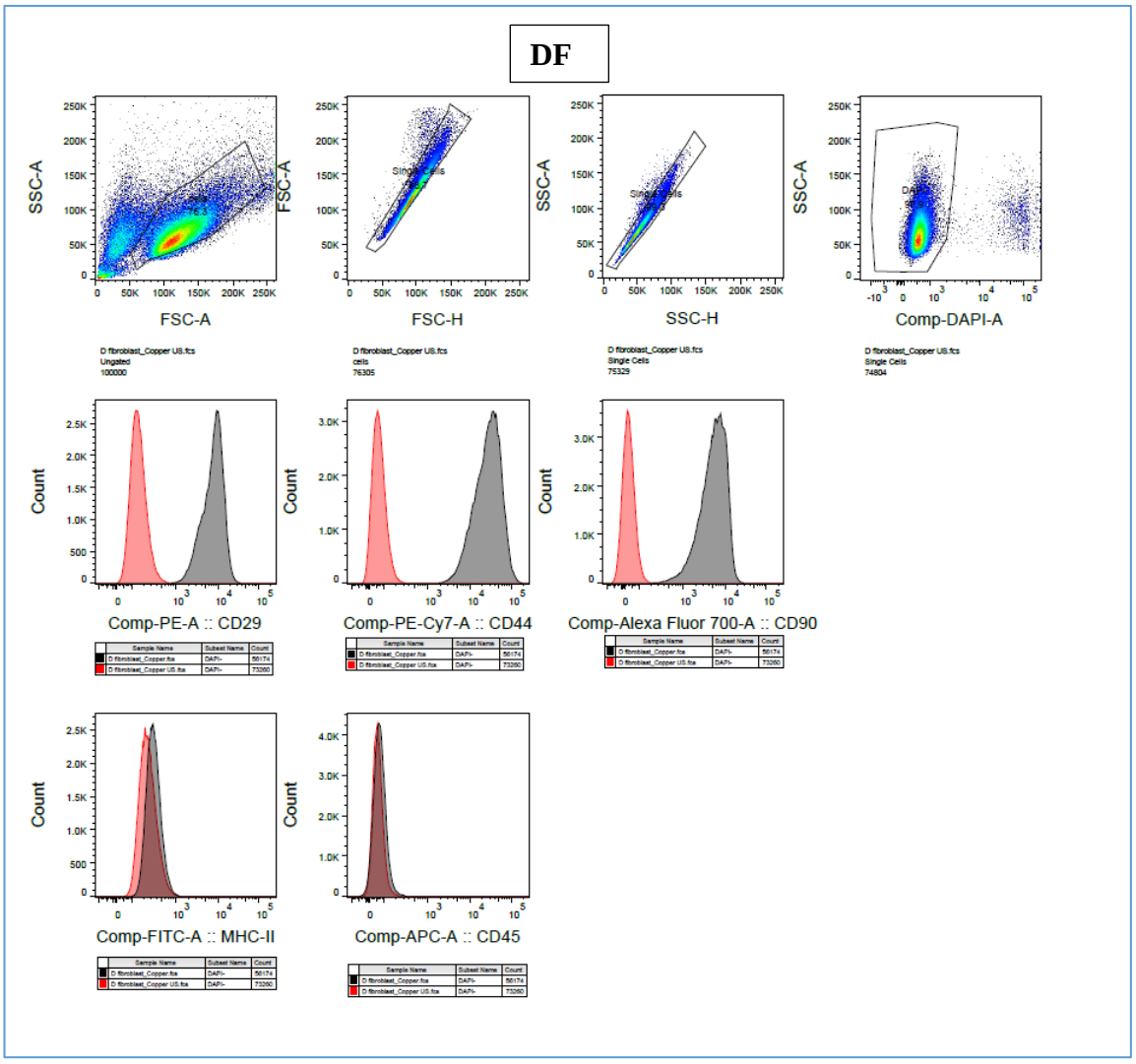


Figure 23 Typical dotplots and histograms obtained for DF cell populations

The equine dermally derived fibroblast cell lines (N=4) had the same immuno-phenotype as the MSC cell lines; $CD29^{+}$, $CD44^{+}$ and $CD90^{+}$ and $CD45^{-}$and $MHCII^{-}$. The positive immuno-phenotype noted for CD90 were not expressed as strongly on UMBT cells (N=4), as for other MSC, which will be discussed further. For a positive result the shift in intensity between negative control and a positive sample well separated as per the schematic below in Figure 24.

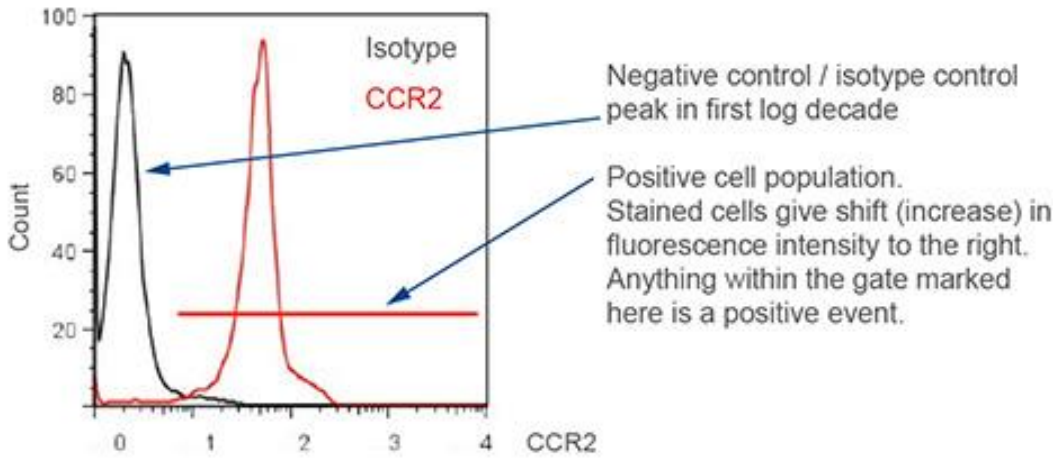


Figure 24 Illustration showing how to interpret the peaks obtained from flow cytometry data. Retrieved online on 2/2/17 http://www.abcam.com/protocols/introduction-to-flow-cytometry.

For UMBT cell populations labelled with CD90, the sample peaks were overlapping to a certain degree with the isotype control and although a positive result, it was not as clear demarcation in fluorescence intensity of these samples in comparison to the other MSC and DF cell populations (Figure 25, middle row, graph on right, CD90).

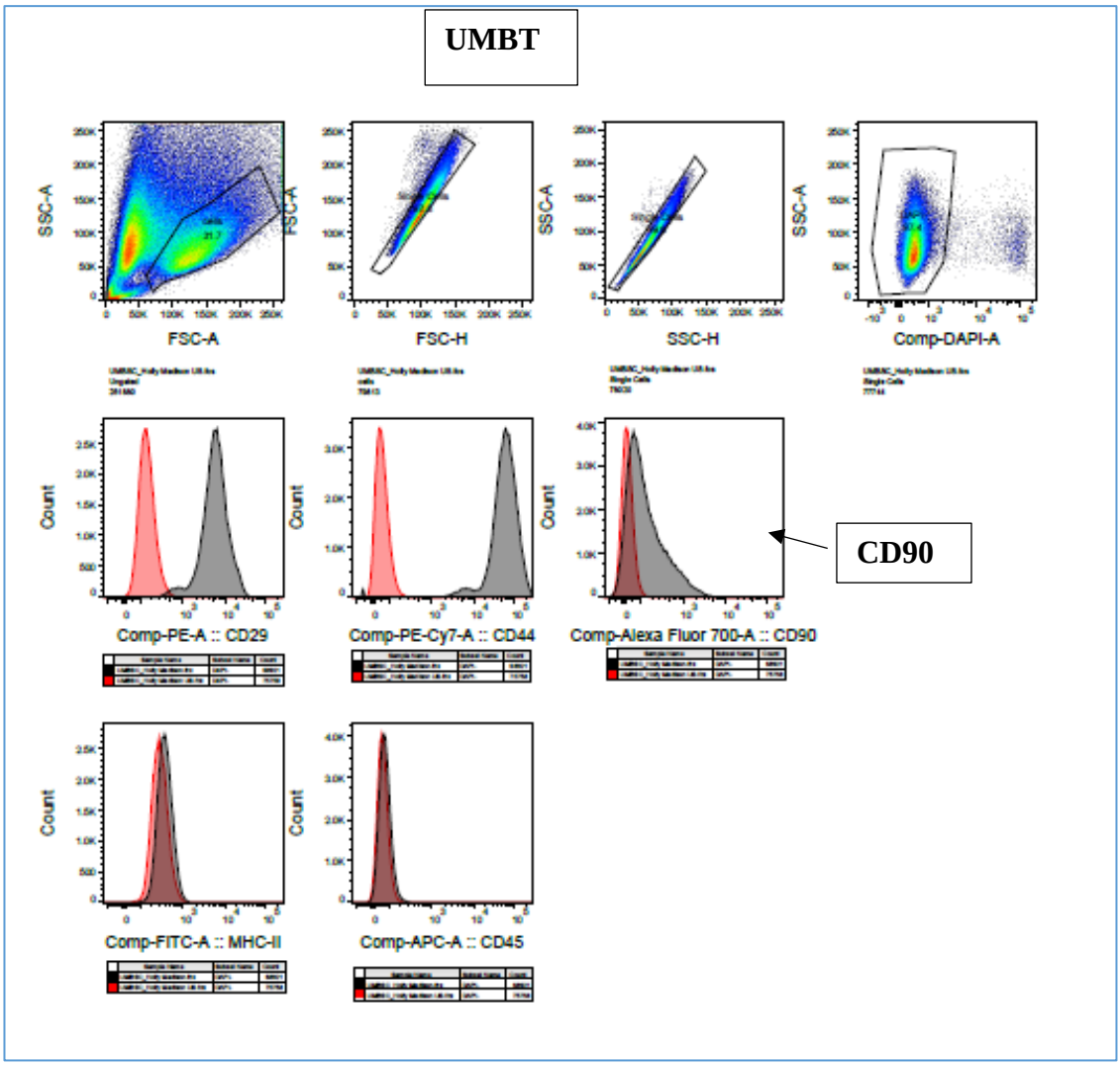


Figure 25 Typical dotplots and histograms obtained for UMBT cell populations, immune-phenotype; $CD29^{+}$, $CD44^{+}$ and $CD90^{+}$ and CD45 $^{-}$and MHC-II $^{-}$note for CD90 there is overlapping of the peaks for fluorescence intensity

Equine peripheral blood mononuclear cells (pMNC) were used as the positive control for the negative markers; CD45 and MHC-II. The flow cytometry results from these cell lines are displayed in Table 20. Thus, a positive result was expected for CD45 and MHC-II as shown with the previous immunocytochemistry results. However, only three out of four (75%) horses were positive for MHC-II and only one out of four horses (25%) was positive for CD45.

Source	Horse	CD29	CD44	CD90	CD45	MHC-II
pMNC	1	+	+	-	-	+
pMNC	2	+	+	-	-	+
pMNC	3	+	+	weak +	+	+
pMNC	4	+	+	+	-	-

Table 20 Flow cytometry results for equine peripheral blood mononuclear cells (P-1)

3.1.3 EXPERIMENT 3: TRILINEAGE DIFFERENTIATION POTENTIAL

H0: The hypothesis is that stromal cells from bone marrow and umbilical cord tissue have an equal potential for trilineage differentiation as dermally derived fibroblasts

All cell lines were observed to undergo differentiation under the defined induction protocols using commercial StemPro mesenchymal induction media. The isolated cell lines have the capacity to differentiate into adipocytes, chondrocytes and osteocytes, respectively. Figure 26 is a representative example of differentiation for each cell population. There was no significant difference between the cell sources for adipogenic differentiation scores ($P=0.5006$) and the range of Means were 5.1-5.3 (out of 9); BMSC=UMBT=DFs. However, both chondrogenic and osteogenic differentiation scores showed significant differences; $P<0.0001$ and $P=0.0096$ respectively, between the sources as presented in Figure 29.

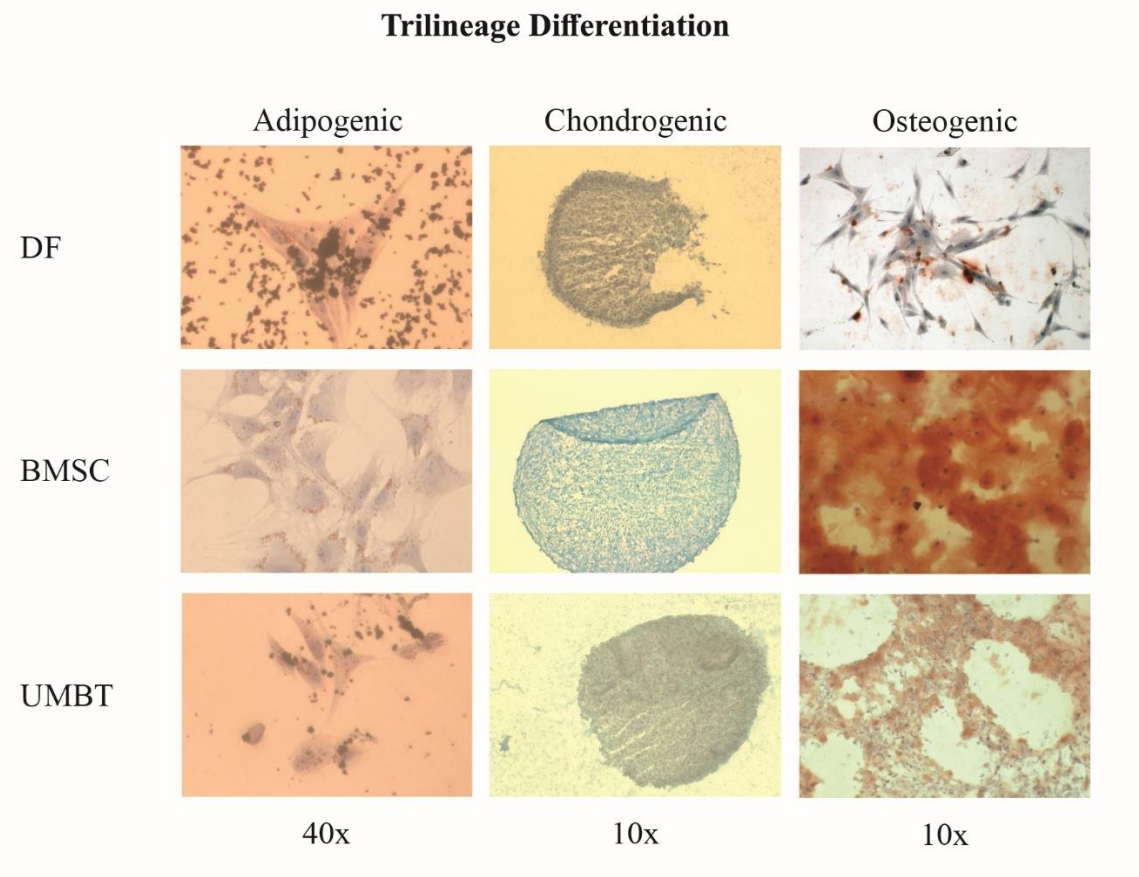


Figure 26 Representation of results obtained after trilineage differentiation of three different tissue sources, stained with Oil-Red-O (adipogenic), Alcian blue (chondrogenic) and Alizarin Red (osteogenic) as viewed on brightfield microscope (Olympus IX71) at 10x and 40x magnification

The first column in Figure 26 represents the adipogenic potential of each tissue source; DF, BMSC and UMBT. There has been a phenotypic change from a fibroblastic cell type into adipocytes and there are lipid globules, stained red with Oil Red-O, within the cell cytoplasm. The results showed that all the cell types investigated in this study, including dermal fibroblasts, have the potential to differentiate into adipocytes. The second column in Figure 26 represent chondrogenic differentiation potential of DF, BMSC and UMBT. The cells in a micromass culture system have formed a tertiary structure in the form of a firm cartilaginous pellet. The cells have a chondrogenic phenotype and visible lacunae have formed within the pellet. Chondrocytes produce GAGs and alcian blue stain is shown by blue coloration of the matrix. The third column is a representation of

osteogenic differentiation of DF, BMSC and UMBT. The cells formed aggregates and mineralised material, which stained red with Alizarin red stain.

Figure 27 shows the results obtained from trilineage differentiation for the commercial equine dermal fibroblast line (*Equus caballus* E.Derm (NBL6, CCL-57™ ATCC, USA). The cells were stained as described in previous section. This cell line was at passage 16 for the experiments and trilineage differentiation was confirmed even in this cell line, which is far later passage than most studies would ever perform differentiation experiments.

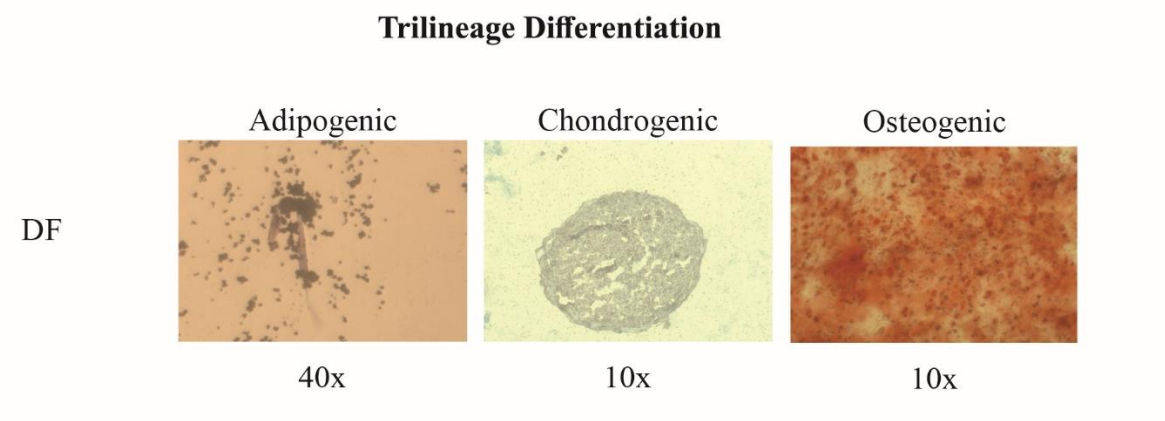


Figure 27 Representation of results obtained after trilineage differentiation of the commercial equine fibroblast cell line; *Equus caballus* E.Derm (NBL6, CCL-57, stained with Oil-Red-O (adipogenic), Alcian blue (chondrogenic) and Alizarin Red (osteogenic) as viewed on brightfield microscope (Olympus IX71) at 10x and 40x magnification.

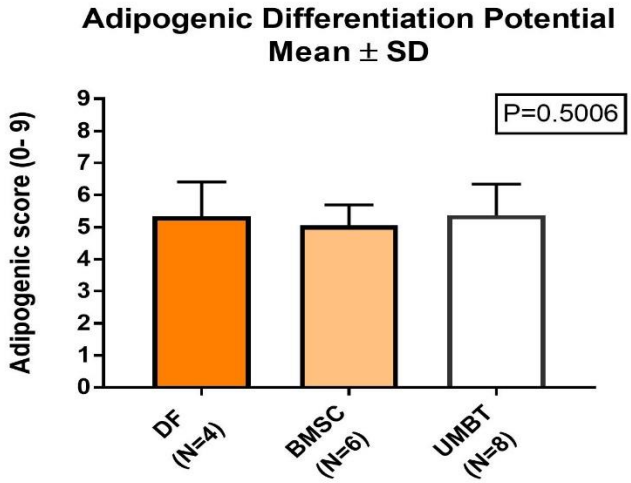
For chondrogenic induction three different culture systems were trialled. The micromass culture system in the round bottom 96-well plates was found to be the most successful method in our laboratory conditions. The cells formed a dense pellet, white to off-white in colour, which was attached to a cellular base layer at the bottom of the well. The shapes of the pellets were mainly spherical, and the size varied slightly between replications (intra-cell) and between cell lines (inter-source), range between 20µm-100µm. The StemPro media appeared cloudy white soon after induction (T=48-72h) of each culture, but no bacterial nor fungal contamination was detected. The conical tube culture system was unsuccessful in

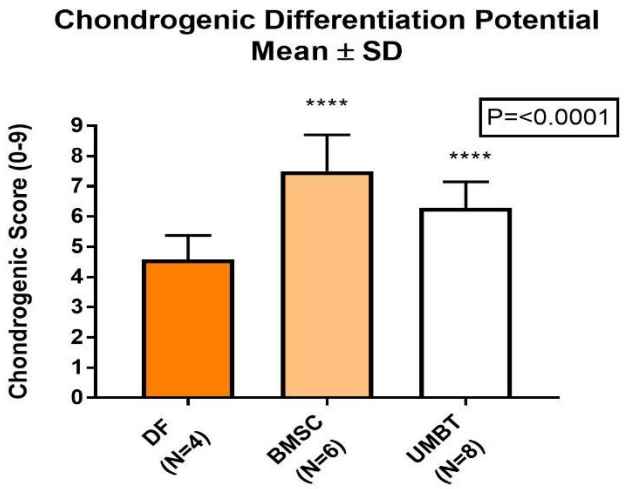
all of the attempts (r=6); the cells did not form a firm pellet structure and each culture media change resulted in disruption to the cells despite careful pipetting technique.

After 21days in the micromass culture system, the pellets were prepared using the cryostat sectioning technique and the sections were stained with Alcian blue to confirm the presence of GAGs, with a counter stain of H&E applied for visualisation of the cell nuclei. The initial run of samples only had Alcian blue stain applied and no H&E. As seen in Figure 27, the second column; BMSC, UMBT and DF cell lines were all able to differentiate into chondrocytes. The chondrogenic potential varied between the groups $P<0.001$. BMSC (7.5 ± 1.2 SD) and UMBT (6.3 ± 0.9 SD) showed a greater differentiation potential compared to DFs. The DF populations had a mean chondrogenic score of (4.6 ± 0.8 SD) and distinct pellets were formed, which stained positively with alcian blue, indicating formation of GAGs. The overall differentiation score was lower, nonetheless positive chondrogenic differentiation was confirmed for DFs in this culture system.

Osteogenic differentiation of the cells lines into osteoblasts with the associated calcium deposits produced by the cells were also observed. During osteogenic induction the cells formed monolayers that stained positive with Alizarin Red, see Figure 27, third column. There was a high rate of cellular death noted during the osteogenic incubation. The cultures were not able to be maintained for more than 10-14days before complete detachment of the cells from the culture plates were observed. No bacterial nor fungal contamination was observed as proven with negative culture on sheep blood agar, at 48hours. The osteogenic potential varied between the groups; multiple comparison ANOVA analysis revealed BMSC had a significantly higher osteogenic score ($P<0.01$) than the other cell sources; BMSC > UMBT > DF and the range of means were; 4.7-6.3 (out of 9).

Figure 28 shows the differentiation scores for adipo, chondro and osteogenic induction using commerical StemPro media by cell source. We also observed an induced chondrogenic phenotype in the synovial fluid trial and this is discussed further in this section.





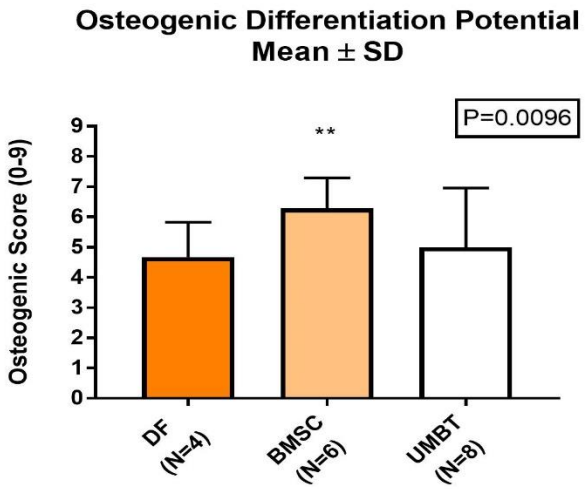


Figure 28 Semi-quantitative trilineage differentiation scores for adipogenic, chondrogenic and osteogenic potential for DF, BMSC and UMBT cell populations.

3.1.4 EXPERIMENT 4. SYNOVIAL FLUID MODEL

H0= The hypothesis is that stromal cells from bone marrow, umbilical cord tissue and dermally derived fibroblasts have the equivalent morphological characteristics when grown in standard culture conditions or culture containing 50% normal synovial fluid or culture containing 50% inflammatory synovial fluid

Mesenchymal stem cells from various sources (BMSC, UMBT) as well as dermally derived fibroblast (DF) have the ability to grow and proliferate in various concentrations of normal, healthy joint fluid for seven days.

3.1.4.1 **Pilot trial**

The initial pilot trial was performed with UMBT cells. Normal SF in concentrations 0%, 50% and 90% were used and the 0% group (CM) served as the internal control as depicted in Figure 29. The growth of cells in culture media supplemented with synovial fluid appeared growing more rapidly compared to control. The cells formed aggregations and the morphology of cells were more elongated. These observed effects served as reasoning for carrying out a follow-on experiment, which evaluated the effects of adding different concentrations of synovial fluid to the culture system.

Initially (T=24-48h), the UMBT cell lines grown in various concentrations of SF showed an increase in proliferation compared to control. With time >T=72h the growth rate slowed down and a phenotypic change was observed, the cells acquired a more elongated shape. A change in pH was also observed; SF containing media had a higher pH recorded at T=168h compared with control, Figure 30.

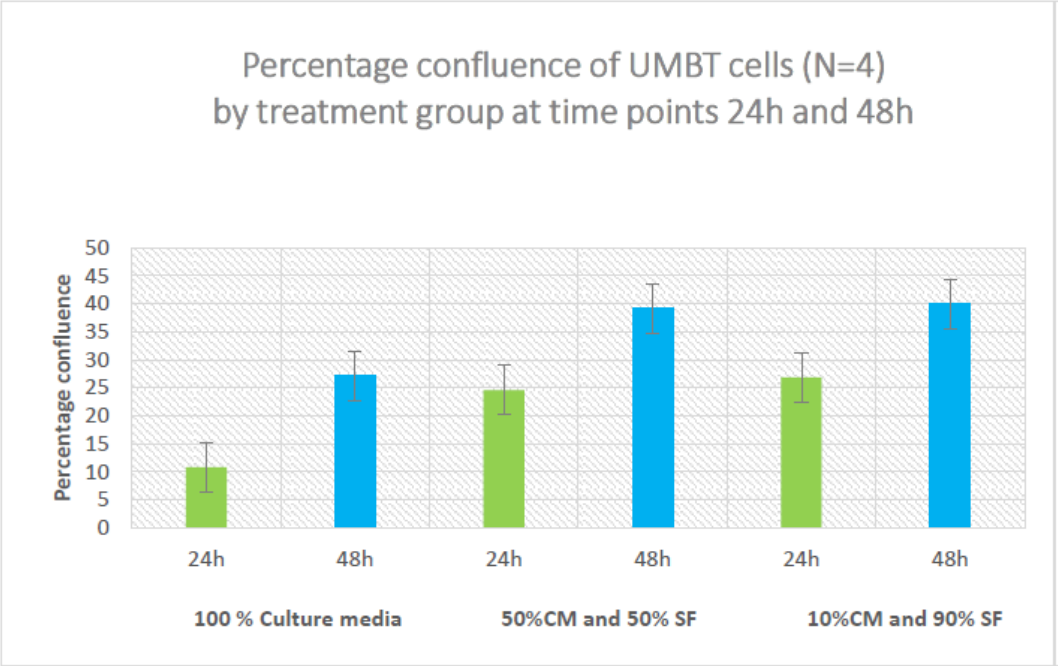


Figure 29 Pilot Trial. The effect of three different culture conditions on the percentage of confluence at T= 24hours and 48h of culture, (mean ± SD, (N=4, r=3) for treatment group CM and 50/50; (N=4 r=2) for treatment group 10%CM/90%SF)

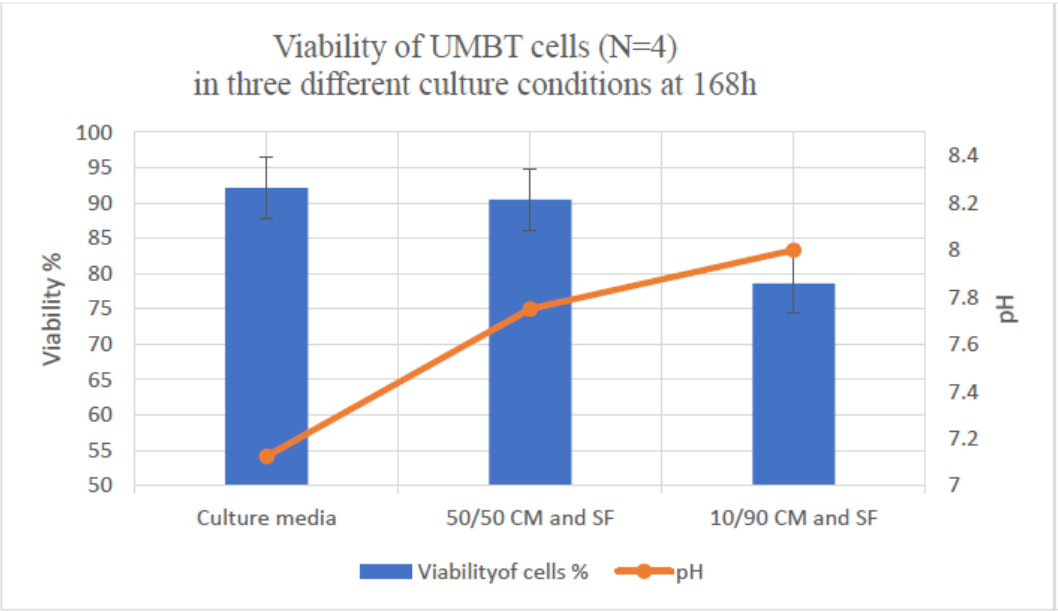


Figure 30 The effect of three different culture conditions on UMBT viability and pH of the cell supernatant after 168h of culture

The pH levels were measured at start of culture (T=0h); the pH of prepared and sterile filtered basal culture media was measured at 7.68, while pH of the normal synovial fluid (100%, pooled sample) was measured to 8.62 and 50% culture media and 50% Synovial fluid was measured to 8.1. However, the results still demonstrated a 12% variability in mean pH levels between the control group and treatment group 3. At T= 168hours pH levels were greater in treatment group 3 that had the higher concentration of SF added, Table 21 shows the mean pH levels. All groups (1-3) had a start pH level that were lower than the pH at T=168hours.

Group	Culture media composition	Initial pH	Mean pH at 168h (N=4, r=2)	% Difference in pH (T=168h /T=0h)
1	100% CM control	7.68	7.09	-8.7%
2	50% CM/ 50% SF	8.11	7.74	-5.6%
3	10% CM/ 90% SF	8.38	8.08	-3.6%

Table 21 pH levels and percentage difference between treatment groups.

For the main experiment, the effects of both i.) Normal SF and ii.) Inflammatory I-SF were evaluated. Samples of both normal and inflammatory synovial fluid were sent to an independent laboratory for Serum Amyloid-A (SAA) and Total Protein (TP) measurements. Results were:

SF Normal synovial fluid; SAA 2mg/L, TP 10g/L

I-SF Inflammatory synovial fluid; SAA 3mg/L, TP 12g/L.

An attempt to minimise the effects of pH was made by the addition of HEPES buffer to the synovial fluid enriched media, at same molarity (0.25mM) as the basal culture medium. At the end of the culture period T=168hours, no significant difference between the control (CM) of SF/ I-SF groups were noted (P=0.08). Figure 31 below shows the measured pH levels in control (CM) vs. SF and I-SF at T=168h.

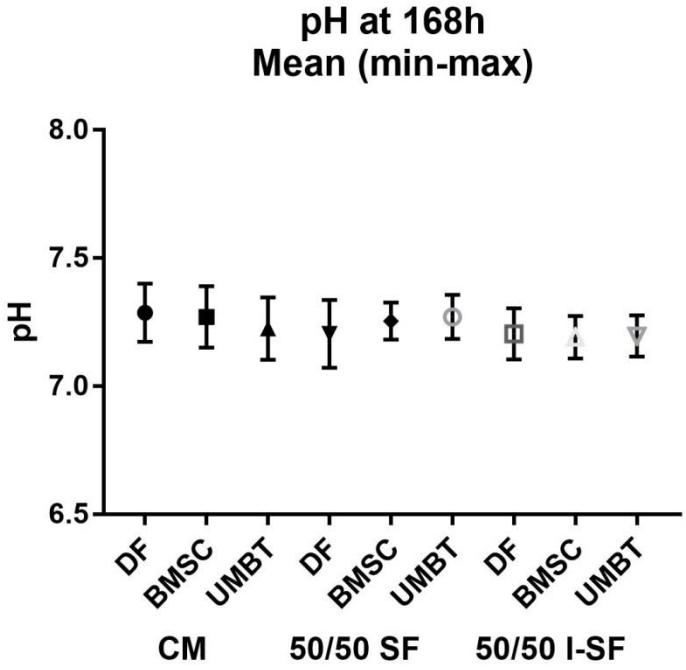


Figure 31 Synovial Fluid experiment; SF and I-SF compared to control. pH levels after buffering with HEPES (0.25mM) as measured at T= 168 hours for DF, BMSC and UMBT cell populations grown in three different culture conditions, P=0.08.

The collection of graphs below in Figure 32, are a temporal representation (T=24-168h) of the percentage of confluence reached by cell populations from the three different sources; DF, BMSC and UMBT. The normal synovial fluid experiment, Figure 34 A, B, C shows the results from cell populations grown in culture media (control) vs. 50% SF (treatment1). The Inflammatory synovial fluid experiment, Figure 34, D, E, F, shows the results from cell populations grown in culture media (control) vs. 50% I-SF (treatment2). A comparative analysis was made i.) within each source (intra-source; CM vs. 50/50 SF or I-SF) and ii.) between sources (inter-source; DF vs. BMSC vs. UMBT) at each of the time points. The data is presented as Means ± Min-Max. P values presented on the graphs are intra-source correlations from paired t-tests.

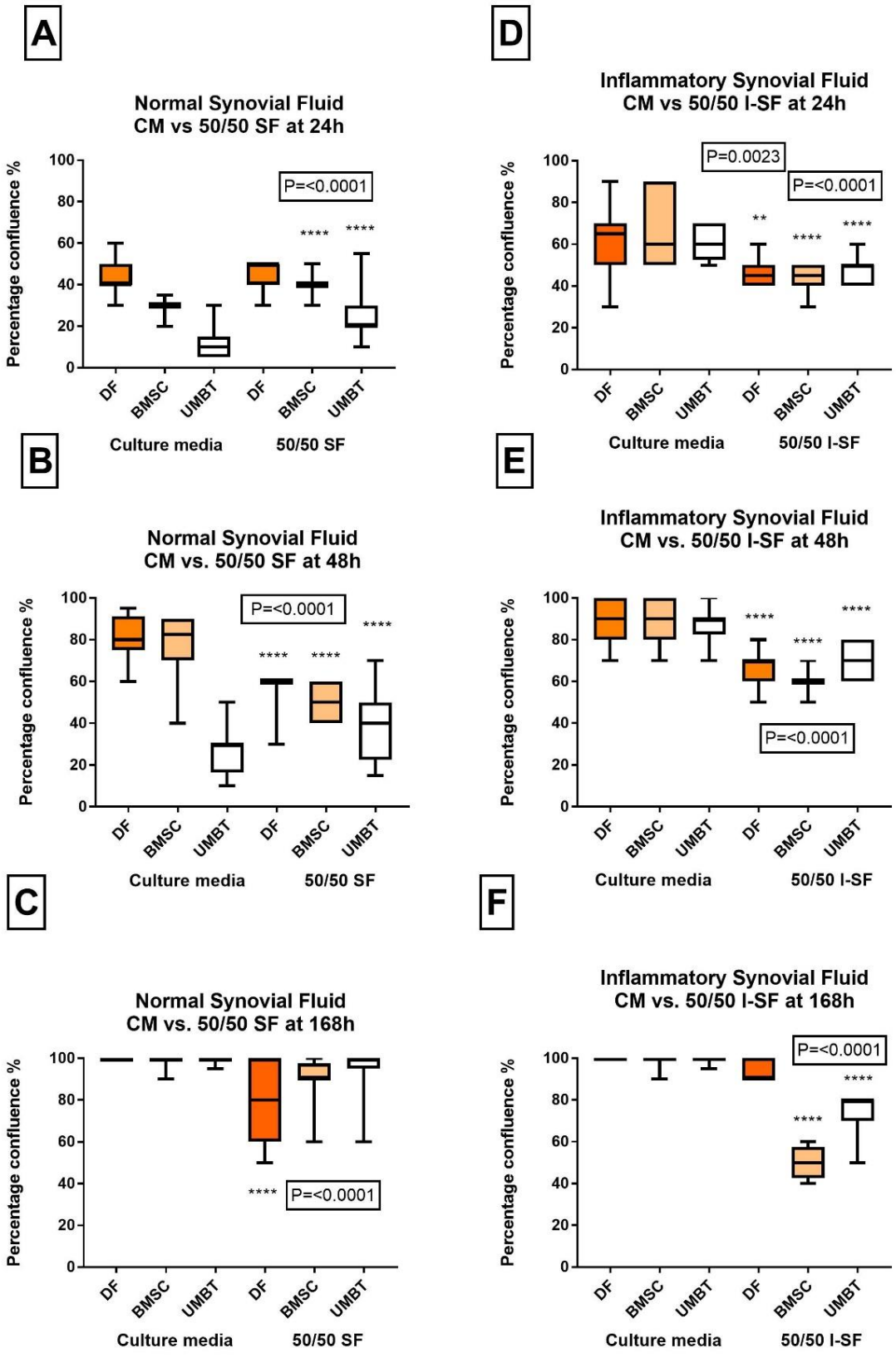


Figure 32 Synovial Fluid Experiment; Growth characteristics as expressed in percentage of confluence (%) Control vs. SF vs I-SF. ****P < 0.0001. **P < 0.0023.

Analysis of this data was undertaken using two separate subgroups:

- Intra-sample variation between control and treatment, and
- Inter-source variation between sources.

The results for the Normal Synovial fluid experiment Figure 32 A, B, C, showed that dermal fibroblasts have similar growth rates in the treatment group (50% SF) compared to control (CM) at T=24h. However, at T=48 and 168h the growth rate was reduced in the 50% SF group ($P<0.001$) compared to control (CM). This correlates with the phenotypic change where the cells aggregated to form clusters Figure 33, in the SF compared to the control group (CM) where cells formed a monolayer. With increasing time, the clusters became denser and formed a tertiary structure similar to the pellets seen during the chondrogenic induction protocol, Figure 34 A. The pellets were positive to alcian blue stain Figure 34 B indicating formation of GAGs and a change of the DF and MSC cell populations into a chondrogenic phenotype. This phenotypic change was noted at T=48h for the SF group.

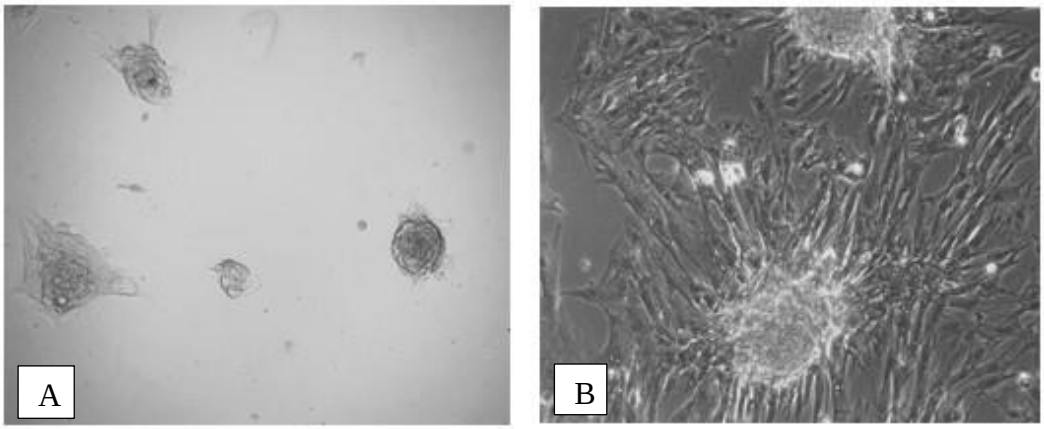


Figure 33 Phenotypic change cluster formation A.) StemPro Chondrogenic Culture Media B.) 50% I-SF culture media

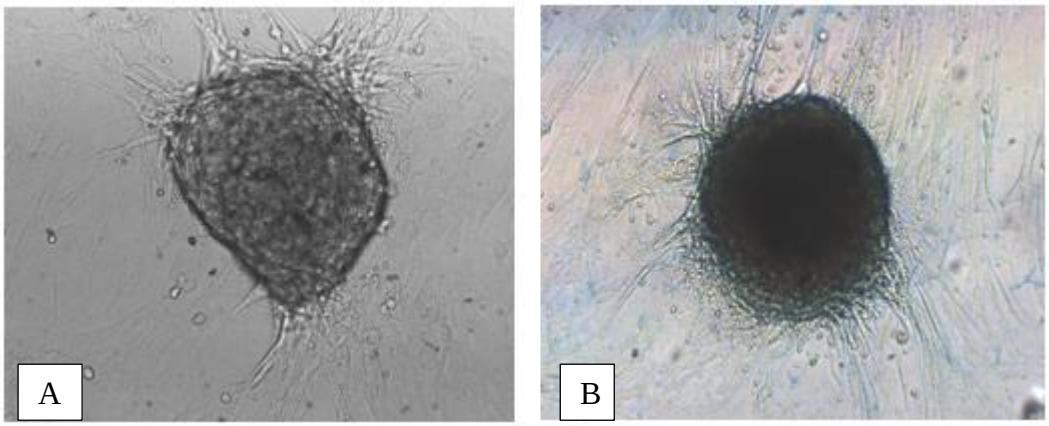


Figure 34 Phenotypic change dense cluster, A.) Pellet formation B.) Alcian blue positive

The two MSC populations, BMSC and UMBT initially showed an increased growth rate in the 50% SF group compared to control ($P<0.0001$), however at T=48h the confluence of BMSC populations were significantly less than control ($P<0.0001$), while the opposite was seen for UMBT, which expressed an increased growth rate in the 50% SF group. T=168h there were no difference between control and the SF group for BMSC and UMBT cell populations, however for DF there was a negative effect on growth rate ($P<0.0001$). This negative effect as measured in percentage of confluence correlated with a change in phenotype and induction of chondrogenesis. The results show that DF, BMSC and UMBT populations can survive well and proliferate in 50% normal allogeneic synovial fluid.

Figure 35 depicts phenotypic change during the synovial fluid experiment.

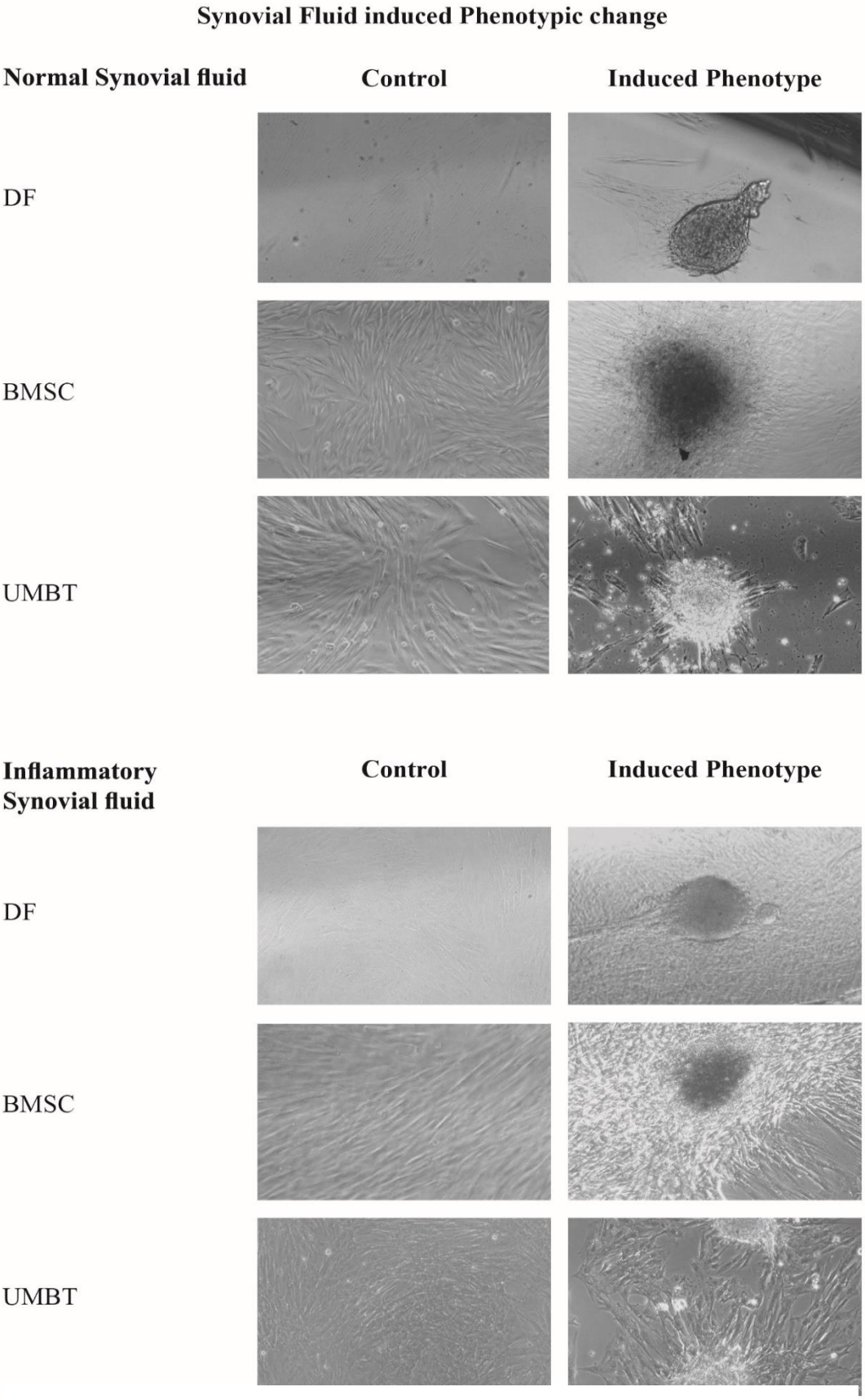


Figure 35 Synovial Fluid experiment. Phenotypic change noted in the SF and I-SF groups compared to control (CM) by cell source.

During the Inflammatory Synovial Fluid Experiment, the cell populations showed more consistent performance, Figure 36; Inflammatory synovial fluid graphs. Percentage confluence were decreased in the 50% I-SF groups compared to controls, DF ($P=0.0023$) and BMSC and UMBT ($P<0.0001$) at T=24. However, at T=168h the opposite was seen to the Normal Synovial Fluid Experiment; DF populations had the same percentage of confluence (near 100%) in the 50% I-SF group as compared to control (CM) vs. BMSC and UMBT that now showed a decrease in proliferation ($P<0.0001$) in the 50% I-SF group. The negative effect seen on confluence correlated with change in phenotype and induction of chondrogenesis. A phenotypic change was noted earlier (T=24h) in the I-SF experiment and the number of clusters and tertiary pellets were increased compared to the SF group, except for one group; BMSC at T=168 the number of clusters were increased in the SF group compared to the I-SF group. As the time in culture progressed (T48-168h), the clusters became denser and formed a tertiary structure similar to the pellets we see in the chondrogenic induction protocol. These pellets were positive to alcian blue staining, indicating a change of the cell populations into a chondrogenic phenotype. Thus, cellular viability and proliferation rates (as measured as percentage confluence) were only mildly affected with both normal and inflammatory allogeneic synovial fluid incorporated into the culture system and chondrogenesis was observed in all of the studied equine cell populations.

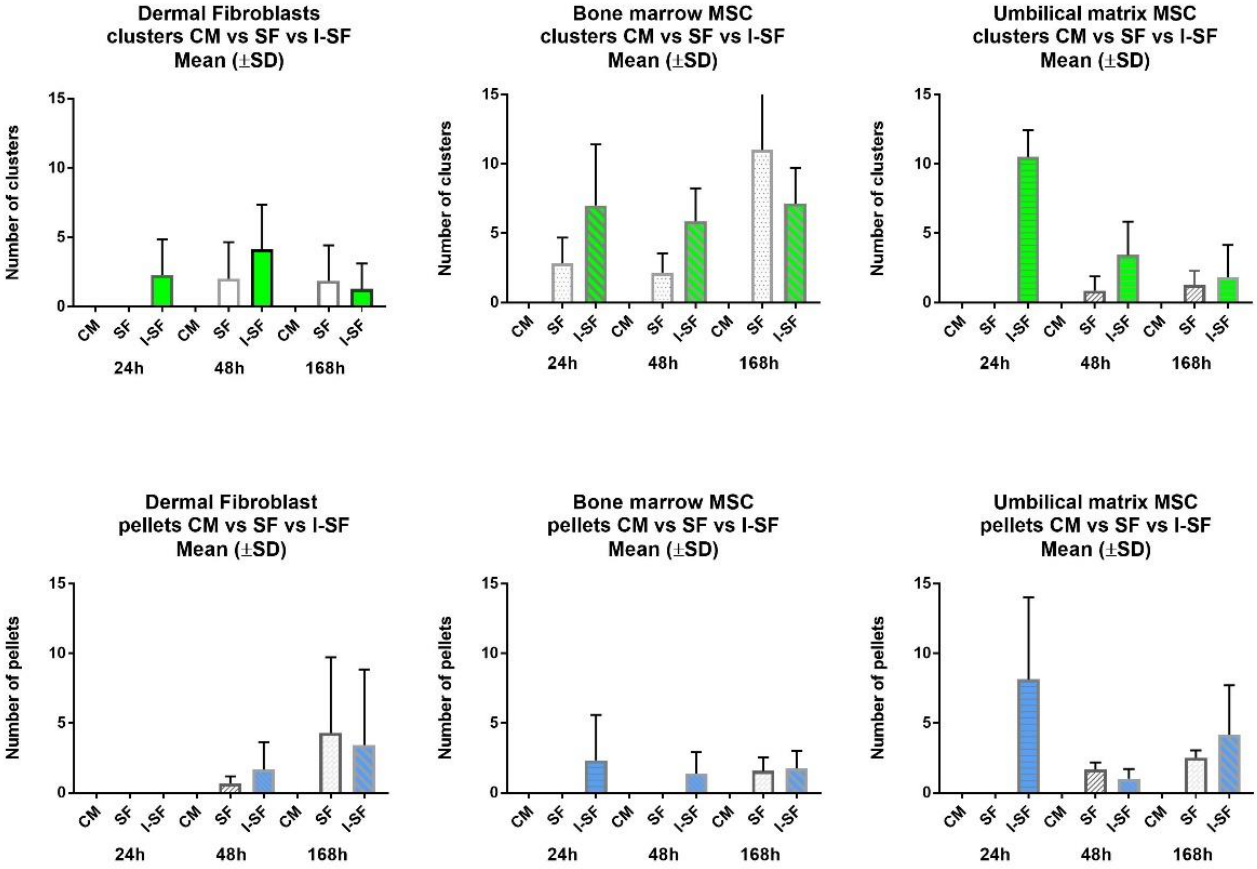


Figure 36 Synovial Fluid Experiment; Phenotypic change in Control (CM) vs. SF vs. I-SF groups at T=24, 48 and 168hours.

3.1.5 EXPERIMENT 5: BIO ACTIVATION ASSAY

3.1.5.1 **Cytokine Analysis by Enzyme linked immunoassay (ELISA)**

H0: The hypothesis is that activated stromal cells from bone marrow and umbilical cord tissue have equivalent cytokine production as activated dermally derived fibroblasts

H0: The hypothesis is that activation with TNF alpha of stromal cells from bone marrow or umbilical tissue or dermal tissue derived fibroblasts equals the effect of activation of the same cell populations with inflammatory synovial fluid,
as measured by the production of cytokines after activation

The results for PGE_2 showed that both the TNFα and I-SF activated groups produced less PGE_2 compared to the control group (CM). It was observed that for three of the ELISA plates; IL-1β, IL-6 and IL-10 there was only very small values of absorbance detected, that were not within the range of detection for the ELISA test kits. This was true for all of the samples from either of the three treatment groups (CM, TNFα and I-SF) Figure 37. It was deduced from this data that there was no detectable production of these cytokines from the tested BMSC and DF cell lines. The TNFα assay could not be validated in either of the two experiments. The initial run for IL-10 showed minimal absorbance from the DF and BMSC samples, however the next plate resulted in useful data.

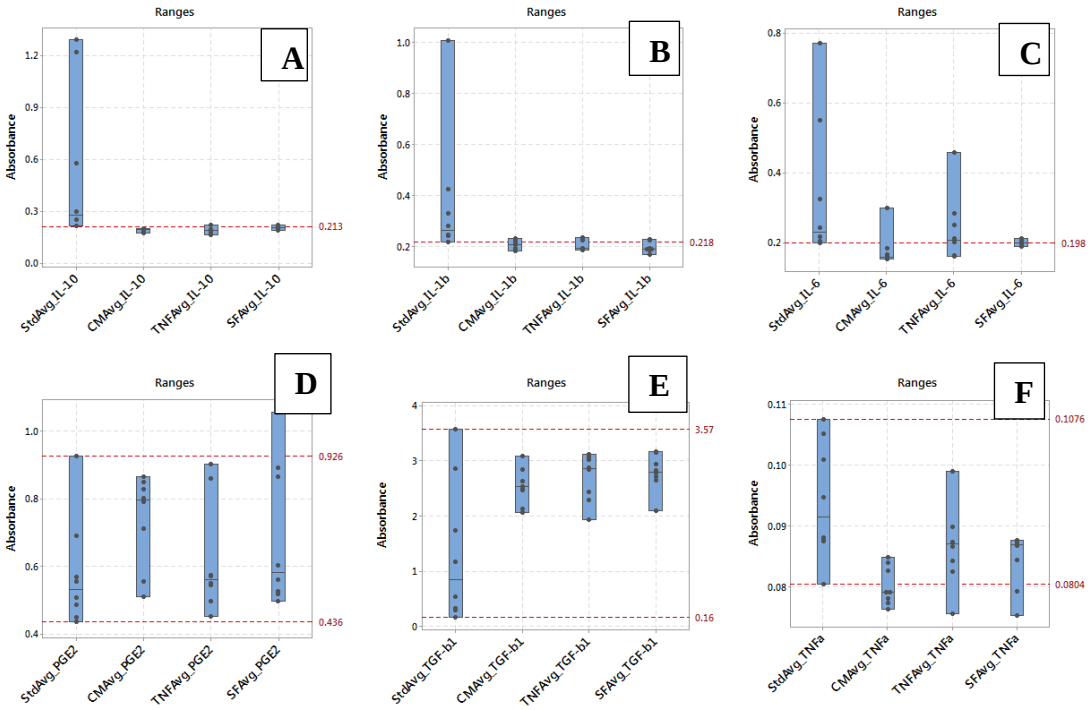


Figure 37 Schematic overview of range of absorbance obtained from initial run (Run1). A, B and C and F.) Samples were not within the range of detection within the standard curve for IL-10, IL-1b, IL-6 and TNFα. D and E.) Samples are within the range of detection of the standard curve and useful for analysis.

The culture media and synovial fluid samples were tested in duplicates for each cytokine. The results are presented in Table 22 below and shows the concentration in picogram/ml.

Sample	TGF-b1	PGE2	IL-10	TNFa
Culture Medium	95.27	0.83	22.47	3.87
Normal SF	254.68	1.11	28.89	6.03
Inflammatory SF	223.22	0.76	23.84	23.26
50/50 CM and I-SF	117.64	0.74	20.57	10.34

Table 22 ELISA result for media, concentration in picogram/ml. Numbers in red are below the limit of detection for the test kit.

The standard curves and the fitted logistics for three of the cytokines tested; TGF-β1 and PGE_2 and Il-10 as displayed below in Figures 38-40.

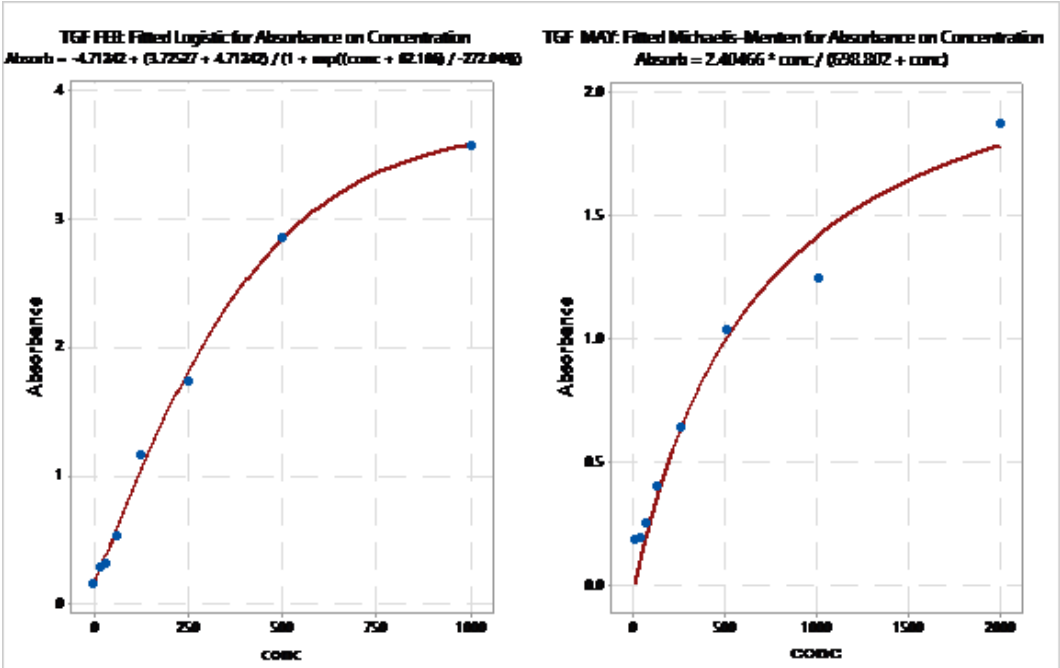


Figure 38 Standard curves for cytokine TGF-β1 for two different ELISA plates at two different time points February 2016 (Run) and May 2016 (Run2).

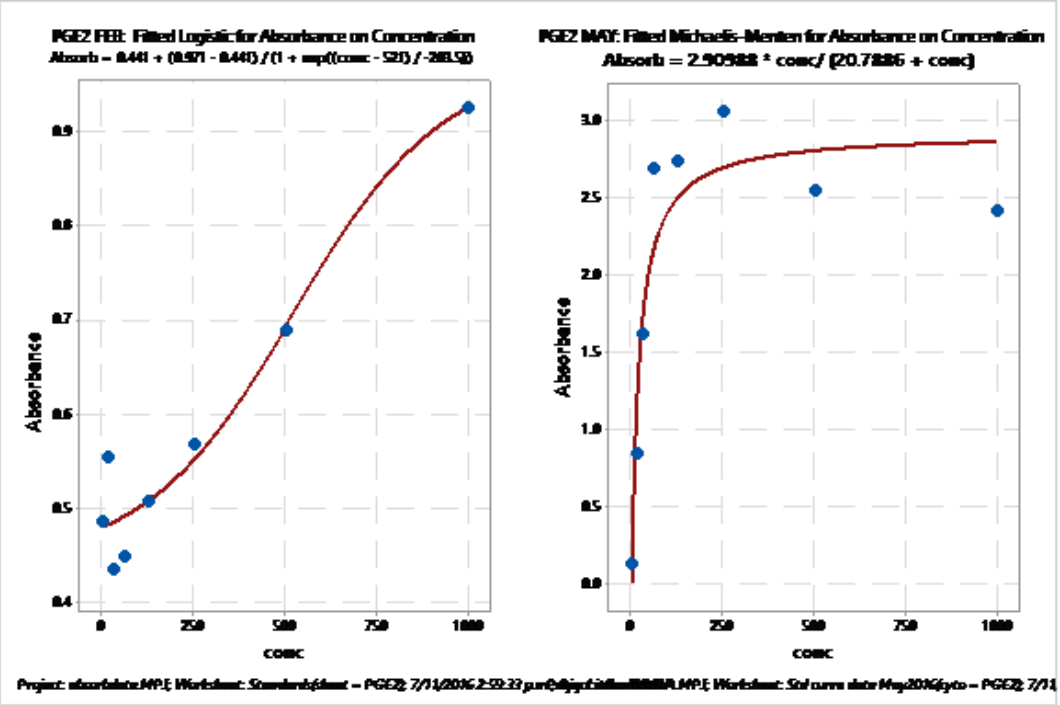


Figure 39 Fig Standard curves for cytokine PGE_2 for two different ELISA plates at two different time points February 2016 (Run) and May 2016 (Run2)

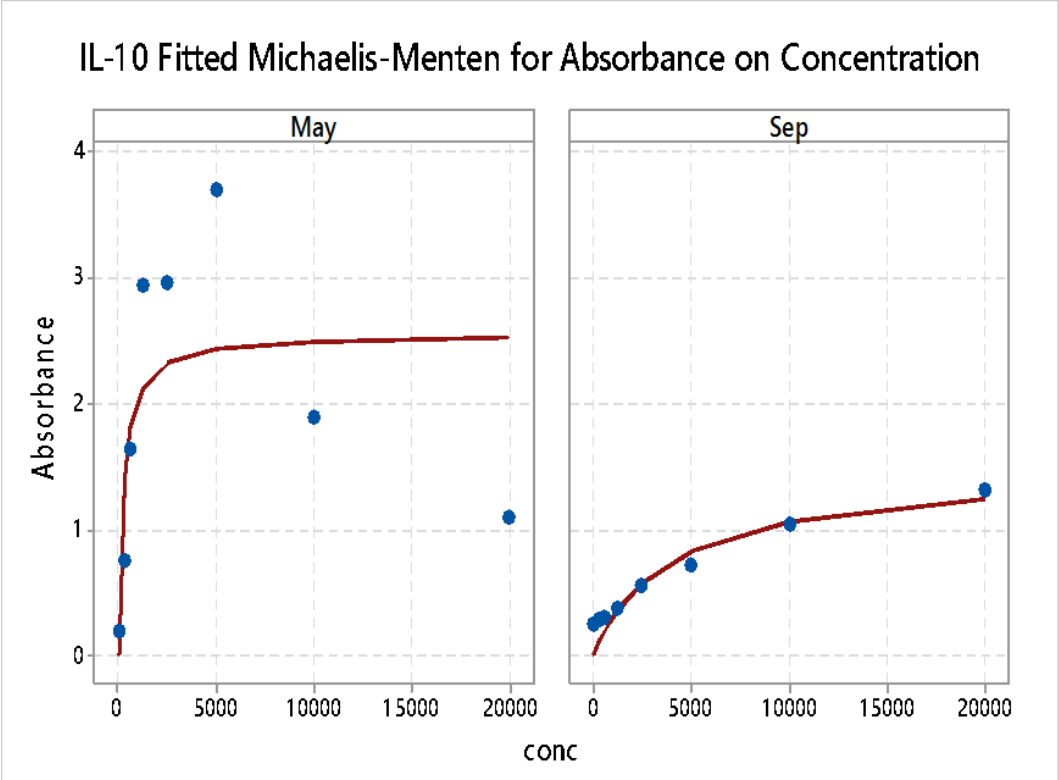


Figure 40 Standard curves for cytokine IL-10 for two different ELISA plates at two different time points May 2016 (Run2) and September 2016 (Run3)

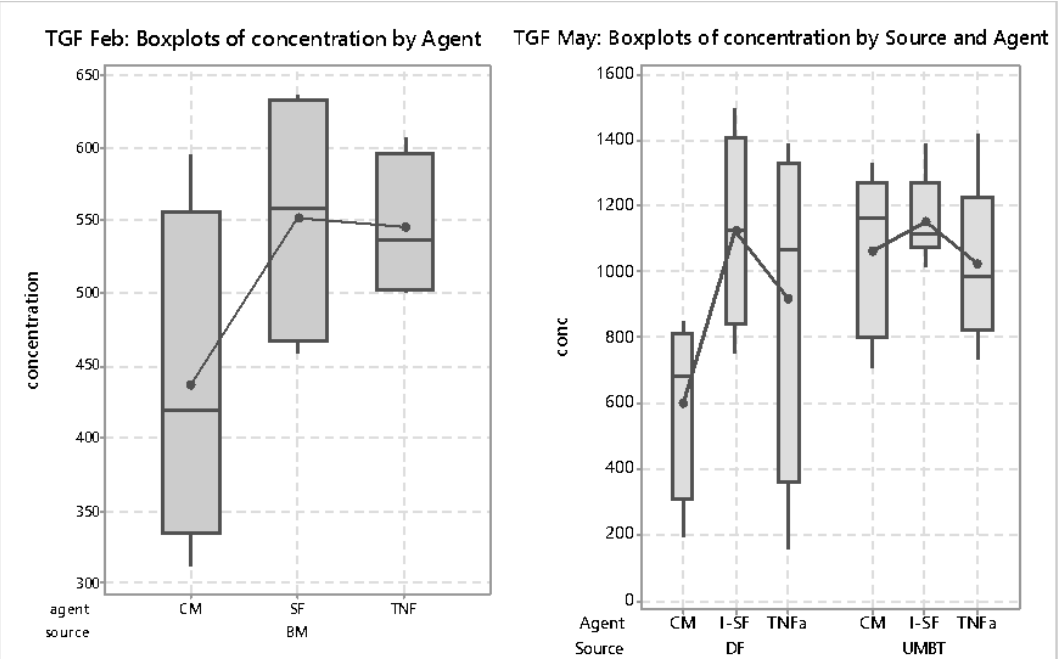


Figure 41 Concentration TGF-β1 pg/ml per source; (DF, BMSC and UMBT) after activation; TNFα or I-SF vs. control (CM) A.) Concentrations obtained for activated BMSC cells, range 340-630pg/ml B.) Concentrations obtained for activated DF and UMBT cells range, 200-1500pg/ml.

For TGF-β1 (Figure 41) the trend lines for all three sources; DF, BMSC and UMBT were the same; activation with either I-SF or TNFα caused an increase in TGF-β1 production compared to control (CM). However, when separating the source and activation agent no significance could be identified (P=0.06). The range of concentrations detected were 320-630pg/ml (Run1), vs. 180-1500pg/ml (Run2).

In contrast the PGE_2 concentrations varied greatly between the two runs (Figure 42); the range was 0-900pg/ml for BMSC and DFs (Run1) compared to 1.0-4.5pg/ml for UMBT (Run2). Thus, for UMBT (Run2), the PGE_2 concentrations were 100-fold less compared to results obtained at Run1. These concentrations are below the lower limit of sensitivity as the detection range for this PGE_2 ELISA test was 15-1000pg/ml. There were some negative results recorded for DF, which means that the concentrations of these samples were below that of the standards used. These samples were not included in the analysis. For BMSC; activated cells showed an increase in production of PGE_2 compared to control, No significant

difference between activation agents could be determined (I-SF=TNFα) for BMSC (P=0.4). For the DF cell populations, the control group (CM) produced higher concentrations of PGE_2 than each of the two activated groups (I-SF and TNFα) P=0.008. This is in opposite to the BMSC results.

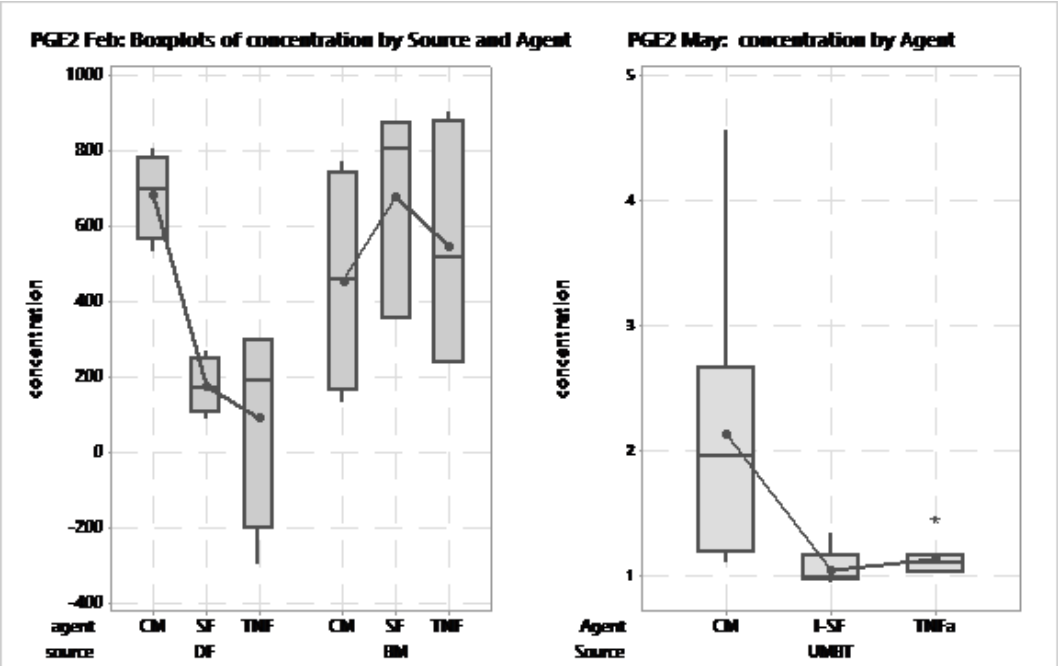


Figure 42 Concentration of PGE_2 pg/ml per source; DF, BMSC, UMBT after activation; TNFα or I-SF vs. Control (CM). A.) Concentrations obtained for activated DF and BMSC cell populations, range 0-900pg/ml B.) Concentrations obtained for activated UMBT cell populations, range 1.0-4.5pg/ml.

The result for IL-10 (Figure 43) when the three sources (DF, BMSC and UMBT) are analysed together in a one-way ANOVA, separating the sources and activation agents (I-SF and TNFα), significant results were obtained for DF and UMBT but not BMSC. DF results showed that activation with I-SF induced the cells to produce higher concentrations of IL-10 (I-SF>CM) (P=0.02) and the same for UMBT cells after activation with I-SF cells also produced more IL-10 compared to controls (I-SF>CM) (P=0.03). No significant result was obtained for BMSC. Nor for TNFα

activation groups when compared to control (P=0.57). However, the overall trend line for BMSC was similar to the other two groups, see Figure 43.

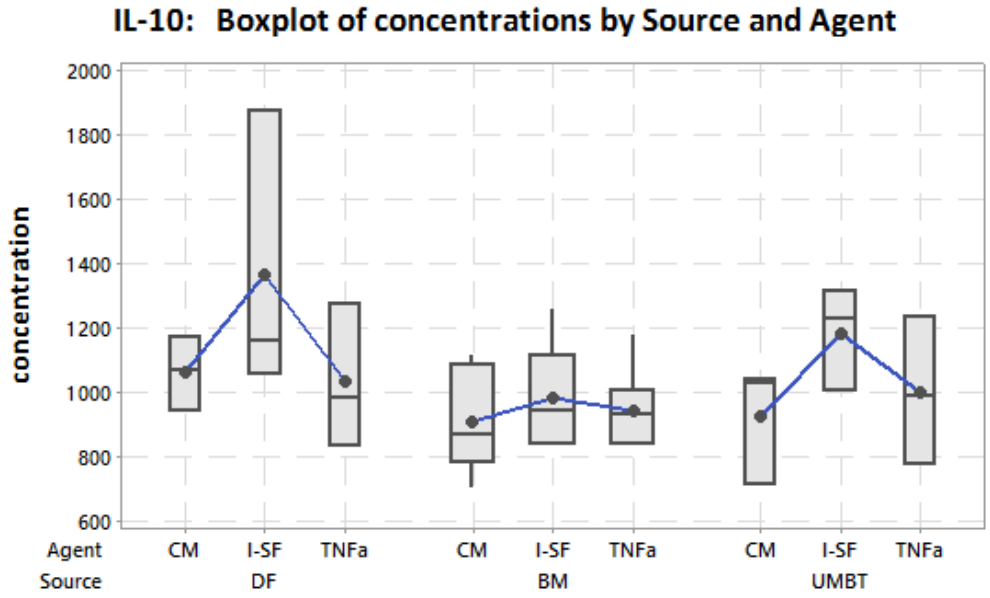


Figure 43 Figure Concentration of IL-10 in pg/ml per Source; DF, BMSC and UMBT after activation; TNFα or I-SF vs. control (CM), range 700-1900pg/ml

	DF			BMSC			UMBT		
TGF-β1	**CM**	**TNFα**	**I-SF**	**CM**	**TNFα**	**I-SF**	**CM**	**TNFα**	**I-SF**
Mean	500.0	658.9	781.1	436.6	544.9	552.2	1063.1	1024.0	1154.1
Std. Deviation	221.8	456.9	421.6	118.2	50.0	90.4	254.5	235.8	129.7
SEM	78.4	161.5	149.1	59.12	25.0	45.2	90.0	83.4	45.9
IL-10									
Mean	1061.0	1033.1	1364.2	906.5	944.1	981.3	927.4	997.9	1182.0
Std. Deviation	114.9	223.8	447.7	162.3	125.3	159.3	185.7	229.6	159.9
SEM	66.3	129.2	258.5	66.3	51.15	65.0	107.2	132.5	92.3
PGE_2		,							
Mean	681.6	242.7	173.1	452.4	542.6	712.6	2.1	1.1	1.1
Std. Deviation	114.4	107.5	74.6	308.3	355.1	242.1	1.1	0.1	0.1
SEM	57.2	53.8	37.3	154.1	177.5	121.3	0.4	0.1	0.1

Table 23 Cytokine concentration in pg/ml (Mean with SD and SEM) per source and activation agent. The UMBT cells produced PGE_2 concentrations that were below the detection range for the test kit (marked in red)

The bio assay activation experiments were performed with cells at P4. To estimate the amount of cytokine produced per 10^4 cells, the number of cells present after T= 60h of incubation was calculated with population doubling times from previous growth and culture characteristics experiment, Table 24.

Population doubling at P4 data	BMSC (N=6)	UMBT (N=8)	DF (N=4)
Number of cells present at T=60h (Mean)	$9.5x10^4$	$7.0x10^4$	$7.9x10^4$

Table 24 Population doubling times at T=60hours for BMSC, UMBT and DF at P4

The cytokine production per 10^4 cells are expressed as Mean ± SD. The assays were repeated twice and each sample was run in duplicates. The only significant intra-source result was found for DF cell populations for PGE_2 concentrations; cells in the control group (CM) produced more PGE_2 compared to activated groups; control (CM) >TNFα (P=0.0124) and control > I-SF (P=0.0027). The cytokine production per 10^4 cells expressed in picograms/ml, as represented below in Figure 44.

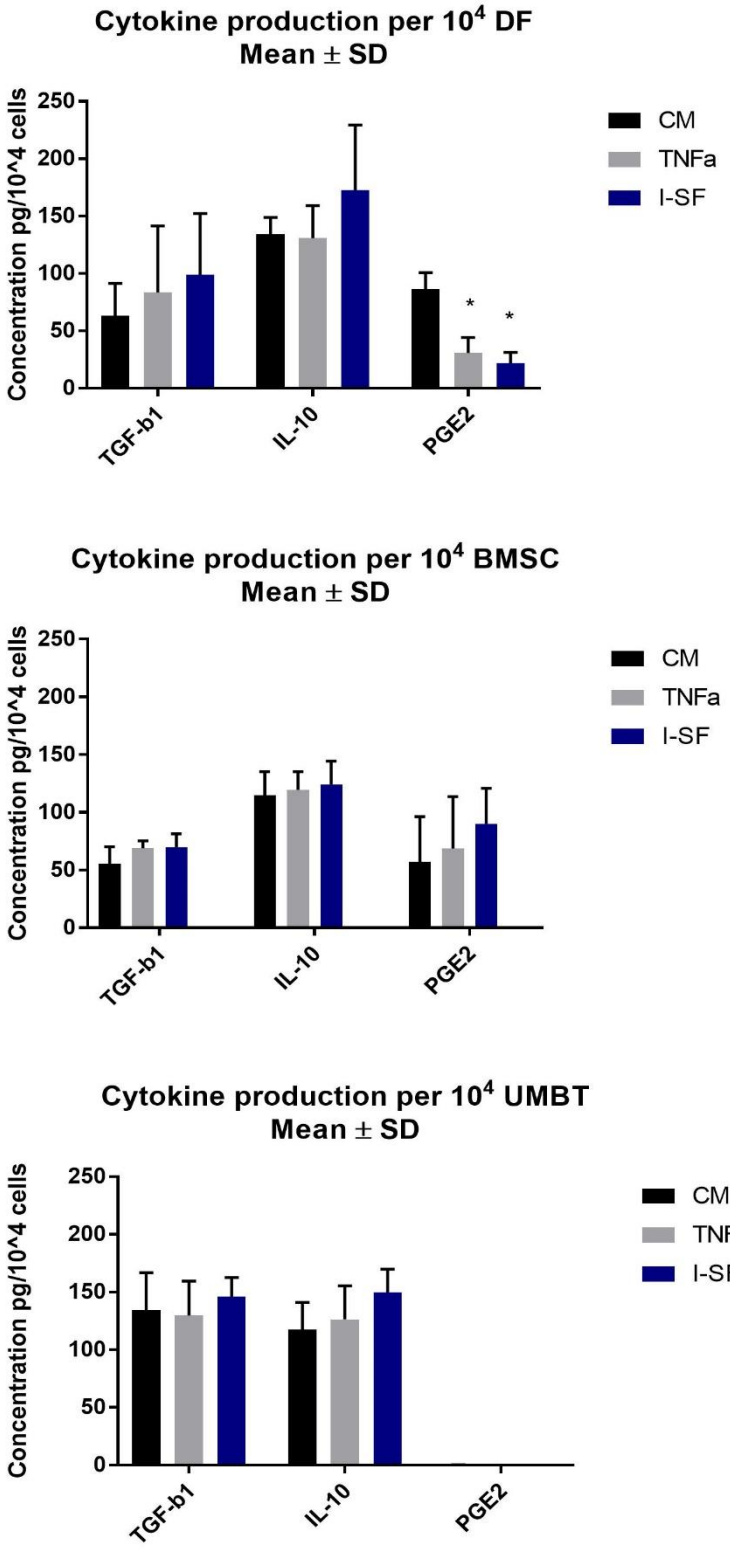


Figure 44 Cytokine production per 10^4 cells expressed in picograms/ml. per cytokine and source (DF, BMSC and UMBT) after activation agent (TNFα and I-SF) vs CM. *P < 0.05. **P < 0.005.

Because of the noticeable plate effect the concentrations of cytokine produced after activation could only be compared intra-source (DF vs BMSC vs UMBT) for IL-10, as all of the samples were run on the same plate at the same time, a non-significant intra-source result was obtained (P=0.811).

CHAPTER 4: DISCUSSION

Study design and sample collection

The selection of horses that the samples were collected from was biased with respect to sex. This was due to mares were the only available sex at Equibreed NZ, where the author was performing the research and could easily obtain samples. The samples obtained ex vivo were from horses that were destined for pet food. These horses were of mixed breed, sex and age. No attempt was made to match the samples for breed, sex or age. Carter-Arnold et al., (2014) showed that even when donors (N=6) were matched for age and gender there were donor-to-donor variability noted in both proliferation and differentiation ability of the cell populations. Schnabel et al., (2014) were biased using only TB horses and specified the age of the horse but not sex. Ranera et al., (2012) gave a range of age (8-12years) and range of weight (400-450kg) but did not mention breed or sex of the five horses in this study. Guest et al., (2010) used eight TB geldings at the age of 3-4years in an attempt to standardise these variables. In an ideal study design the donor horses would have been matched for breed, sex, age and tissues from all sources would have been collected from each donor. However, it is not possible to eliminate the effects of individual donors. An a priori sample size analysis revealed that the minimum number of donors required for a statistical power of 80% was 42 horses, when performing repeated measures within-between multiple group comparisons (one-way Anova).

The normal group of synovial fluid samples were collected *ex vivo*. An attempt was made to categorise the sample as normal by performing a clinical exam of the horse at walk to detect any lameness and palpation of the joints for any effusion. However, there could have been joints affected by disease that went undetected. The inflammatory synovial fluid samples were collected during arthroscopic surgery and they were confirmed to be abnormal by radiographs and intra operative visualisation of the joint. However, some developmental joint problems may have varying degree of inflammatory mediators present. The synovial fluid was analysed for SAA and TP, as well as for each cytokine that were used in the bio assay experiment. A more extensive analysis could have been carried out as there are

many more cytokines and growth factors present in synovial fluid. Synovial fluid from different animals and different joints were pooled to create a larger sample to eliminate donor and site variability. Other studies by Hegewald et al. (2004) used autologous and Boone (2013) allogenic synovial fluid. Boone (2013) used fluid from carpal and tarsal joints and pooled a total sample from 25horses. If using allogenic synovial fluid in an experiment it is necessary to pool a number of smaller samples together to get a uniform sample that can be used in the experiment.

To my knowledge no equine studies have fully addressed the relationship between fibroblasts and mesenchymal stem cells; such as morphology, presence of cell surface markers and potential for trilineage differentiation. Lack of clarification of the similarities and/or differences between these cell types confounds the evaluation of the efficacy of these cells in clinical situations. In human medicine there are recent studies that have acknowledged the relationship between mesenchymal stem cells and fibroblasts, Hematti (2012) and Ullrich et al., (2012) reviewed the literature to find little evidence on methods to distinguish the two cell populations from one another. This is in agreement with the present study, which showed that equine mesenchymal stromal cell populations from adipose, bone marrow and peripheral blood were indistinguishable from dermal fibroblasts with respect to; cellular morphology and culture characteristics *in vitro* and cell surface antigens (CD markers). During trilineage differentiation there were a significant difference in scores obtained for the chondro and osteogenic potential, but since dermal fibroblasts were shown to undergo trilineage differentiation this method cannot distinguish stromal cells from fibroblasts.

Morphological study and growth characteristics

H0: The hypothesis is that stromal cells from adipose, bone marrow, peripheral blood and umbilical cord tissue have equivalent morphological characteristics as dermally derived fibroblasts

The hypothesis was proven to be correct as no differences in morphology and growth characteristics using conventional culture methods could be detected between equine MSC and DF. However, when cultures were left at confluency for longer time; 2 weeks, there were differences in morphology noted.

A standardised culture medium was used for all experiments to reduce variability. It has been mentioned that serum origin and content; foetal bovine serum or equine serum, or serum free medium, can affect the growth of cells (Ho et al. 2008). The molecular profile and function of the cells may also change with passages and time in culture. For the experiments in this study, passage four was selected and all experiments were carried out with cells at this passage. This was done to eliminate passage number as a variable. This was especially true also for the immunophenotyping experiment as discussed later. Trypsinisation and cryopreservation of cells could potentially alter the biological function, the cells were all treated the same way and all cells used in experiments were cryopreserved and thawed.

-Standard culture conditions

Morphology and growth characteristics of the equine mesenchymal stromal cell and dermal fibroblast cell populations were shown to be indistinguishable under standard culture conditions. The typical spindle shaped morphology, plastic adherence and formation of monolayer are all typical characteristics of MSC and are well recorded in the equine literature. Dermally derived fibroblasts are also described to have the same characteristics (Blasi et al., 2012). The only study to our knowledge to record some data for an equine DF cell lines is Schnabel et al. (2013).

If cells were left at confluency for 2 weeks, dermal fibroblast cultures underwent mesenchymal to epithelial transition. This was not observed in BMSC cultures. It would've been an interesting follow up to passage the dermal fibroblast cells after this transition had occurred to see if stem characteristics and plasticity and even multipotency were preserved or if the cells had terminally differentiated into an epithelial phenotype.

Cells within certain epithelia appear to be plastic and thus able to move back and forth between epithelial and mesenchymal states via the processes of epithelial to mesenchymal or mesenchymal to epithelial transitions (Kalluri and Weinberg 2009). Three distinct subtypes of epithelial to mesenchymal differentiation are seen (Jordan et al. 2011); i.) Developmental EMT events occur in a temporally controlled

setting and contribute to embryonic morphogenesis and tissue remodelling during development ii.) The second EMT subtype is associated with wound healing and tissue regeneration in adult tissue and occurs in response to inflammation. Persistent inflammatory signals produce continuous activation of EMT resulting in organ fibrosis iii.) The third EMT subtype occurs during the progressive stages of neoplasia, whereby EMT in epithelial neoplastic cells produces cells that can invade other tissues and with increased metastatic capacity.

The observation of cells undergoing MET is likely to have occurred due to space constrictions and close cell to cell interactions as a reversal of the second EMT subtype. Mesenchymal to epithelial transition and the reverse thus seem to be an important function of stromal and fibroblastic cell populations as a response to changes in their environment *in vivo* and therefore a clear distinction between the two cell types would be difficult to make. Any given stromal cell population is likely to be heterogenous (Corradetti et al. 2011).Which means it contain both multipotent progenitor cells and more terminally differentiated fibroblastic type cells. The state which the cells are in are determined both by stage in cell cycle (Nakamura-Ishizu et al. 2014) and by the external stimuli from local tissue environment (Prockop 2013) and its' not a constant but a dynamic relationship. No equine studies to our knowledge have tried to evaluate or reported the degree of heterogenicity of their proposed stem cell populations. It seem plausible that each MSC cell line contain a mixed population of cells and morphology and growth characteristic studies fail to distinguish these cells apart.

Immunophenotyping by Immunocytochemistry and Flow cytometry

H0: The hypothesis is that stromal cells from adipose, bone marrow, peripheral blood and umbilical cord tissue have the same qualitative expression of cell surface markers, CD29, CD44, CD90 positive and CD45, MHC-II negative as dermally derived fibroblasts

This hypothesis was found to be true for this specific CD marker panel. It acknowledges the limitation of the number of cell surface markers included in the analysis. The two conventional methods evaluated for immunophenotyping were

immunocytochemistry and flow cytometry. The method of flow cytometry was far superior as both qualitative and quantitative results were obtained for a larger number of cells ($1x10^5$) as compared to immunocytochemistry where only a smaller number of cells could be subjectively evaluated.

When evaluating cell surface markers there are several factors that can contribute to variability in results (Ulrich et al. 2012); i.) The initial processing involve the use of proteolytic enzymes for adipose, umbilical and dermal tissue or a density gradient system for bone marrow and peripheral blood (Redmond Hubbard 2014; Sabatini et al. 2005), These enzymes and the *in vitro* culture conditions (method and media) can affect cell surface protein expression (Ulrich et al. 2012). In addition the specificity of antibodies or inappropriate or prolonged fixation can significantly diminish the antibody binding capability (Williams et al. 1997). One of the main difficulties with ICC staining is to overcome specific or non-specific background and to optimise fixation methods and times. Time for the incubation of antibodies and optimisation of post-antibody wash buffers and wash times are all important for obtaining high quality immunostaining (Freshney 2010). All these factors were considered and trialled to produce satisfying immunofluorescence result for the equine MSC and DF in this present experiment. However, the initial protocol had to be modified several times before the process could be optimised for the selected antibodies. In addition, the presence of positive and negative controls for staining are essential for determining specificity. Equine peripheral blood mononuclear cells were used for the positive control for the two MSC negative markers; CD45 and MHC-II. A fluorescent nuclear stain (DAPI) could have been used to make visible the cellular morphology of the MSC, when the negative markers were evaluated (Xing et al. 2007). The immunofluorescence result obtained for the stromal cells for the negative markers without the nuclear stain was just a plain black field of view when the fluorescent light was applied. If DAPI had been used a photographic evidence could have been produced also of the two negative markers (CD45 and MHC-II). The results obtained were purely observational in the form of photos displaying the immunofluorescence of cells. For this method there were no objective measures of the intensity of the fluorescence nor quantification of the number of CD markers present on each cell. The production of a colorimetric scale

for intensity of fluorescence would have been useful. The ICC technique can still be useful to prove the presence of a certain CD marker on the cell surface, but as a method for distinguishing sub populations of stromal cells it was found not adequate.

Flow cytometry was shown to have several advantages over the immunocytochemistry method. It was more time-efficient and cryopreserved cells could be used without cell culturing steps and there were no fixation agents applied that could damage or alter the cell surface protein epitopes. Flow cytometry has the ability to define distinct cell populations by their size and granularity combined with the capacity to gate out dead cells and thus improved sensitivity (Brown and Wittwer 2000). The new flow cytometers can do multi-colour analysis, which allow for several antigens to be measured simultaneously (Baumgarth and Roederer 2000). More importantly, the data output is objective and thus a large number of cells can be processed, which produce a more accurate result compared to immunocytochemistry.

In this present flow cytometry study the same immunophenotype was determined for the equine DF cell lines as compared to adipose, bone marrow and peripheral blood stromal cells The flow cytometry obtained results for the mesenchymal stromal cell populations were in agreement with other equine studies (De Schauwer et al. 2011; De Schauwer et al. 2012; Dias et al. 2016; J. H. Spaas et al. 2013b), except for a small variance in the umbilical tissue derived stromal cells. In the umbilical tissue derived cells, the CD90 expression was a weak positive result. This was in contrary to previous studies by Iacono et al. (2012) and Carrade et al. (2012) where the umbilical cord cells showed a normal positive result for CD90 on flow cytometry. It could possibly be explained by the fact that our cells had been cryopreserved prior to flow cytometry. Dias et al. (2016) showed a reduction in CD90 expression after cryopreservation as compared to fresh isolated equine umbilical derived MSC. It suggests that the CD90 epitope on UMBT may be sensitive to the effects of cryopreservation. Immunocytochemistry could not be performed for CD90 due to lack of a suitable primary monoclonal antibody

sensitive to equine epitopes not being readily available commercially for this application.

This present study was not able to effectively distinguish equine mesenchymal stromal cells from adipose, bone marrow or peripheral blood from dermal fibroblasts with the methods of immunocytochemistry and flow cytometry. This is in agreement with Ulrich et al. (2012) who presented a short review of human mesenchymal stromal cells and fibroblasts where they discussed the inability of studies to establish a panel of markers to separate human mesenchymal stromal cells and dermal fibroblasts. They suggested Raman Spectroscopy as a highly sensitive method to discriminate between closely related cells.

Trilineage Differentiation

H0: The hypothesis is that stromal cells from bone marrow and umbilical cord tissue have an equal potential for trilineage differentiation as dermally derived fibroblasts

This hypothesis was rejected. Positive trilineage differentiation was observed in all cell populations of the three sources evaluated, (bone marrow, umbilical tissue and dermal fibroblast). The potential for differentiation however, varied between the sources.

The differentiation potentials of MSC is reported to decline with increasing number of passage (Baer and Geiger 2012). All of the cell lines in this study were shown to have the capacity to differentiate at P4, even the commercial equine dermal fibroblast line at P16. To our knowledge only Schnabel et al., 2014 have reported an attempt to induce trilineage differentiation of an equine dermally derived fibroblast cell line (N=1). They also used the StemPro® induction media, however they reported unsuccessful trilineage differentiation in this cell line. Given the donor effect (inter-horse) (Colleoni et al. 2009), which could confound results, using only one sample donor, seem to be inadequate for the Schnabel et al. (2013) study. Rohart et al. (2016) showed that most of the differences in human mesenchymal stem cells attributed to tissue source could be explained by donor genotype in an integrative secretome analysis. They also suggested that cultured

mesenchymal stem cells share a common phenotype that is intermediate between a quiescent perivascular progenitor and a differentiated cell type; the fibroblast. If mesenchymal stromal cells from multiple tissues from the same animal were used the "intra-horse" effect could be reduced. However, it was not practical to collect multiple tissue samples from each donor as some were client animals, some samples were cryopreserved stock from a previous study as well as the commercial cell line only had one tissue source collected from the donor horse. Some samples were collected ex vivo and although attempts were made to collect bone marrow samples ex vivo the author was unsuccessful. The umbilical cord samples were also a special category as peripheral blood could have been reasonably easily collected from mares and foals at time of birth, however no stud master would have agreed to collect bone marrow or adipose or skin dermis from newborn foals or mares. It is not possible to correct for differences in genotype between horses. An ideal study design would be to have samples from multiple sources from the same animal to eliminate genotype as a variable.

Colleoni et al. (2009) showed that for equine adipose and bone marrow there were a donor effect noted on cell yields obtained from raw material but no difference in differentiation potentials. Carter-Arnold et al. (2014) showed intra-donor variability despite matching horses for sex and age, of bone marrow derived cells for both proliferation rates and chondro- and osteogenic differentiation. These two studies did not perform any test for Mycoplasma contamination, which is another possible factor to consider. In the present study all of the cell lines were tested and proven free from Mycoplasma contamination. Mycoplasma contamination of cultures can extensively affect cell physiology and metabolism (Nikfarjam and Farzaneh 2011).

In the present study the bone marrow stromal cells were as expected superior at chondrogenic and osteogenic differentiation (Vidal. et al. 2008), while there were no significant inter-source difference noted for adipogenic differentiation. Youngstrom (2015) and also Vidal et al. (2011) discuss that some cells are pre-primed for a certain tissue type as they are already existing in a similar environment, for example bone marrow derived stromal cells would have a predisposition for osteogenic differentiation and tendon derived stromal cells would have a

predisposition for tenogenesis. A recent study by Zayed et al. (2017) compared BSMC and Synovial fluid derived MSC from the same donor to minimise the intra-horse effect. They used five horses in total with matched BMMSCs and SFMSCs, which were found to be similar in respect to cellular morphology, viability, and immunophenotype, but varied in their chondrogenic potential, and expression of the key chondrogenic proteins. They found there were considerable donor-to donor (inter-horse) variation.

Despite that some cell populations achieved higher differentiation scores, the method of trilineage differentiation is not selective to distinguish equine mesenchymal stromal cells from dermal fibroblasts. Trilineage differentiation has been a hallmark feature of identification of stem cells (Hematti 2012). However, the question can and should be asked regarding the usefulness of its application, as the results obtained after induction under supra-physiological conditions *in vitro* likely does not reflect the behaviour of the cell populations *in vivo* and the fact that fibroblasts can effectively undergo trilineage differentiation as shown in several human studies (Alt et al. 2011; Brohem et al. 2013; Sabatini et al. 2005). It is unknown if MSC after injection into a live animal undergoes the same process of differentiation as produced *in vitro.* The results by Guest et al. (2008) suggest the opposite as poor survival post 10days of injected BMSC was reported. This means that an alternative mechanism of action rather than differentiation of the injected cells and incorporation into the architecture of the neo-tissue need to be considered.

At present, mesenchymal stromal cells are mainly used to treat musculoskeletal diseases in equine veterinary medicine based on their ability to differentiate into various tissues of mesodermal origin (De Schauwer et al. 2013). De Schauwer et al. (2013) also points out that this is in marked contrast to human medicine, where mesenchymal stem cell therapies are primarily focused on immune-mediated, inflammatory, and ischemic diseases. It appears that the trilineage differentiation potential is irrelevant when considering cells for treatment. Since the *in vivo* effects seem to be produced by chemical messengers produced by the cells rather than the actual transformation and incorporation of cells into neotissue to repair the injured tissue.

Synovial fluid enriched culture conditions

H0= The hypothesis is that stromal cells from bone marrow, umbilical cord tissue and dermally derived fibroblasts have the equivalent morphological characteristics when grown in standard culture conditions or culture containing 50% normal synovial fluid or culture containing 50% inflammatory synovial fluid

This hypothesis was rejected. A difference in growth potential was noted between control and the two treatment groups; SF and I-SF. There was also a difference in phenotypic change noted between normal and inflammatory synovial fluid treatment, a faster induction of phenotypic change was noted for the I-SF group and more clusters of cells and pellets were formed on average independent of source of MSC or DF.

For the main experiment 50% concentration was selected based on results from (Hegewald et al. 2004), who studied the effects of autologous synovial fluid on equine bone marrow derived stromal cells. They found that chondrogenesis was induced with the addition of synovial fluid into the culture system. Boone (2013) was of the same conclusion that bone marrow derived stromal cells can survive and proliferate well in allogenic synovial fluid. In the present study a chondrogenic phenotype was noted as early as 24hours. One theory proposed would be that synovial fluid is more viscous than standard basal culture media, the cells were less free to move around before cellular attachment was achieved and consequently were more likely to form aggregates. This close cell to cell contact then likely induced the rapid change in phenotype due to more efficient cell communication and niche effects (Koch et al. 2008).

The bone marrow derived, and umbilical tissue derived stromal cells showed an initial increase in proliferation rates (as measured as percentage of confluence) in the normal synovial fluid group (SF) compared to matched controls (same cells grown in basal culture media, (CM)). Synovial fluid can be considered an ultrafiltrate of plasma (Blewis et al. 2007). It also contain high levels of hyaluronic acid, sulphated and non-sulphated glycosaminoglycans, proteolytic enzymes,

cytokines and growth factors. (McIlwraith 2005). The total composition of the pooled synovial fluid was not known, and was beyond the scope of this study to fully documnet the synovial fluid composition.

For the dermal fibroblast populations the percentage confluence between control (CM) and synovial fluid groups were the same at T=24h, then at T=48h the percentage confluence was decreased in the synovial fluid group. Overall the proliferation rates (measured as percentage of confluence) were affected by the addition of both normal synovial fluid and inflammatory synovial fluid to the culture systems. A morphologic change was induced that involved cluster formation and differentiation of the mesenchymal stromal cells and dermal fibroblasts towards a chondrogenic phenotype. The bone marrow and umbilical tissue stromal cells showed an earlier and more marked response; there were increased number of clusters and pellets formed, as compared to dermal fibroblasts.

The result that fibroblasts showed a lesser degree of phenotypic change could support the theory described by Covas et al. (2008) that a fibroblast is a more terminally differentiated version of a stem cell. As discussed previously in section 1.4.2, any given cell population show some heterogenicity; either from contamination of other cell types or the same type of cells that are at different stages of the cell cycle (Baer and Geiger 2012). In the present study the dermal fibroblast cell populations showed a lesser degree of phenotypic change compared to MSC in the synovial fluid experiments, as well as attaining significantly less chondrogenic and osteogenic scores during the trilineage differentiation experiment. Thus, it could be that dermal fibroblast cell populations have a higher proportion of cells that are terminally differentiated and therefore has a lesser multipotent potential as compared to bone marrow derived and umbilical tissue stromal cells. However, dermal fibroblasts populations do still express multipotency as shown in this experiment. Therefore, percentage confluence attained and phenotypic change (as quantified by number of clusters and pellets formed) induced by synovial fluid incorporation in culture system are not suitable as a measure to clearly define nor distinguish these cell populations from one another.

Activation Experiment

H0: The hypothesis is that activated equine stromal cells from bone marrow and umbilical cord tissue and dermally derived fibroblasts have equivalent cytokine production as the same cell populations grown in culture media.

This hypothesis was rejected. Activation of MSC and DF resulted in an increase in production of the selected cytokines. There was one exception; after activation DF cell populations decreased production of PGE_2 as compared to the control.

It was hypothesised that the functional behaviour of equine mesenchymal stromal cells versus dermal fibroblasts in standard culture conditions versus activated conditions may be a way to distinguish the cell populations apart. Blasi et al (2011) showed that functional bioassays were required to enable to distinguish between human mesenchymal stem cell and fibroblast cell lines. To our knowledge this the first study to attempt to clarify the functional similarities and/or differences between equine mesenchymal stromal cells and dermal fibroblasts.

H0: The hypothesis is that activation with TNF alpha of stromal cells from bone marrow or umbilical tissue or dermal tissue derived fibroblasts equals the effect of activation of the same cell populations with inflammatory synovial fluid,

The activation agents TNFα was selected based on Blasi et al. (2011). We hypothesised that inflammatory synovial fluid would be a biological substance that would activate MSC and DF in line with the discussion by Prockop (2013) on tissue products as natural inducers. Other activation agents mentioned in the literature are; IFN-γ (Carrade et al. 2012; Paterson et al. 2014) and phytohemagglutinin (PHA) (Carrade et al. 2012) and lipopolysaccharide (LPS) (Blasi et al. 2011). One limitation of this study was that we could not fully quantify all of the components of the pooled inflammatory synovial fluid as discussed previously. Future studies could compare the degree of activation exerted by the different *agents in vitro* and/or evaluate the *in vivo* effect of mesenchymal stromal cells injected intra-articular (activation) on change in cytokine concentrations in synovial fluid.

A panel of six cytokines were selected, based on the current literature on equine arthropathy (Bertone et al. 2001; Kamm et al. 2010; McIlwraith 2005) and tendon injuries (Hosaka et al. 2002). Enzyme linked immunosorbent assay was selected as the method for cytokine evaluation and equine specific ELISA kits were found to be commercially available from Genorise, USA. In contrast to an earlier study by Kamm et al. (2010) who used human ELISA kits or manufactured their own. Each kit had a specific detection range (picograms/ml) and standards supplied for manufacturing of a standard curve. It was surprising that during the initial experiment, results for absorbance could only be obtained for two of the secreted cytokines; TGF-β1 and PGE_2. The remainder four cytokines (Il-1β, IL-6, IL-10 and TNFα) were outside the range of detection. The selection of IL-1β can be questioned and the receptor antagonist protein, IRAP may have been more appropriate. Carrade et al. (2012) found IL-6 only to be produced by mesenchymal stromal cells (ADSC, BMSC, UMBT and UMBB) after activation with PHA or T-cells enriched PBMNC. Kamm et al. (2010) evaluated TNFα levels successfully with ELISA, their values were in the range of 10-40pg/ml. The Nori Equine TNFα kit used in the present study had a detection range of 31-2000pg/ml, hence the amount produced was too low to be interpreted with this kit. Kamm et al. (2013) did report a similar problem for IL-1β and MMP13. Despite the non-detectable results obtained for IL-10 during Run1, additional plates were acquired and in the following experiments results were produced within the correct range and within the limit of detection.

The result from this present study showed that for the anti-inflammatory cytokines; TGF-β1 was produced at higher levels after activation with I-SF compared to control (culture media) and compared to TNFα activation. This was significant for umbilical tissue stromal cells (P=0.03) and dermal fibroblasts (P=0.02) but not for bone marrow derived cells (P=0.57), the trend line was the same but non-significant. The results obtained in the present study for PGE_2 showed both TNFα and I-SF activated bone marrow stromal cells did increase their production of PGE_2, however the result was non-significant, P=0.40. For the dermal fibroblast cell populations, the control group (CM) produced higher concentrations of PGE_2 than each of the two activated groups, P=0.008. This was in opposite to the BMSC

results. The umbilical cord tissue cell population had concentration values that were below the lower limit of sensitivity for the test kit, so no result was recorded for this source. From a clinical point of view there is the potential to utilise this anti-inflammatory and immunomodulatory effect of mesenchymal stromal cells and dermal fibroblasts. If allogenic stromal cells and also dermal fibroblasts like our results show, are injected into an inflammatory environment in an injured equine synovial joints *(in vivo)*, in theory they should upregulate the IL-10 and TGF-β1 production, which could promote beneficial anti-inflammatory effects in the joint and the synovium. Prockop (2013) suggest that Prostaglandin E_2 plays a large role as part of a negative feedback loop regulating inflammation. The proposed mechanism of action is that TNFα activated stromal cells produce PGE_2 that drives resident macrophages with an M1 pro-inflammatory phenotype toward an M2 anti-inflammatory phenotype. In the present study activated bone marrow derived MSC increased their production of PGE_2 while dermal fibroblasts did not. Hence the conclusion is drawn that BSMC would be preferable to use as a therapy.

An a priori sample size analysis for the cytokine experiment revealed that the minimum number of samples required for a statistical power of 80% was 64 matched samples when performing paired t tests intra-source between control and the two activation agents (treatment groups). This number of samples was not achievable for this study.

The plate effect was noticeable and only for the IL-10 and TGF-β1 kits, were the absorbances recorded in a similar range for both replicates. For PGE_2 there were a 100-fold difference noted in absorbance for the UMBT cells and the concentrations obtained were below the detection range of the test kit, thus for UMBT it was not possible to analyse nor compare the results with the other two sources. A more accurate comparison across the three sources was obtained for IL-10 concentrations (Run3), as the plate effect was eliminated. The other two ELISAs for TGF-β1 and PGE_2, were run on two different plates on two separate occasions (Run1 and Run2). The results for TGF-β1 concentrations were still comparable, the range was 320-620pg/ml for BMSC (Run1) compared to 200-1400pg/ml for DF and UMBT (Run2). The large inter-test variability of these equine specific ELISA kits,

unfortunately make the results less reproducible so a different method of analysis should be considered for future cytokine evaluation.

Determination of the tissue source that produce the highest levels of cytokines associated with anti-inflammatory action and tissue repair could enable the selection of the best cell type for clinical applications. Ideally a more complete panel of cytokine and chemokines should be tested to further determine the differences between mesenchymal stromal cells and dermal fibroblasts. The *antibody array* method can simultaneously measure up to several hundreds of proteins (Hoogduijn et al. 2010). Compared to the ELISA method, antibody arrays are also highly cost-effective and consume less sample volumes. It can provide a broader perspective with the possibility to unveil other relevant molecules that may not have been previously considered. *Bead-based assays* confer savings in time and costs because they allow multiplexing, which is the simultaneous detection of multiple targets (Wagner and Freer 2009). Multiplexing allows for smaller sample volumes and high-throughput automation. However, the bead systems are not yet commercially available for equine specific cytokines. This signalling system by cytokine and chemokine expression is intriguing; MSC and DFs can influence the local tissue environment where they are applied. The plausible mechanism to explain the effects seen in tissues after MSC treatment, are that they provide regulatory function and immuno-modulation via secreted mediators; PGE_2, TGF-β1, IL-10, TSG-6 to control inflammation and aid in the repair and regeneration. Many equine musculo-skeletal injuries increase in severity due to excessive inflammation post the initial trauma. Mesenchymal stromal cells and dermal fibroblasts both acquired anti-inflammatory properties after activation (a simulated inflammatory environment), as shown in this study by the increased production of anti-inflammatory cytokines. Future research efforts should aim to characterise the full secretome of equine mesenchymal stromal cells and dermal fibroblasts when activated and applied in various clinical conditions. If their full potential could be evaluated, then treatments could ultimately be targeted to suit a specific inflammatory niche or type of injury.

Conclusion

It appears that the definition of equine mesenchymal stromal cells and dermal fibroblasts is purely an academic matter as suggested by Ulrich et al. (2012). Is a fibroblast a terminally differentiated form of a stromal cell? Or is it a dynamic process involving the mesenchymal to epithelial transition and the reverse process; epithelial to mesenchymal transitioning? Both which are commonly occurring in tissues as a response to the local environment. This combined with cell cycle events make it seem impossible to elucidate their relationship. It seems more important to define functionality of each cell population *in vitro* and *in vivo* to optimise the potential use for clinical applications. The goal of stem cell therapy in horses is to regenerate tissues that are inherently difficult to heal naturally with completely restored functionality. Any treatment which is able to restore the architecture of the damaged tissue and potentially reduce the time to recovery and rate of re-injury, would greatly help to reduce wastage of horses as well as overall costs incurred to the equine industry in New Zealand.

REFERENCES

Alt, Eckhard, et al. (2011), 'Fibroblasts share mesenchymal phenotypes with stem cells, but lack their differentiation and colony-forming potential', *Biology of the Cell,* 103 (4), 197-208.

Alves, Ana Liz Garcia, et al. (2001), 'Effects of beta-aminopropionitrile fumarate and exercise on equine tendon healing: Gross and histological aspects', *Journal of equine veterinary science,* 21 (7), 335-40.

Apostolakos, John, et al. (2014), 'The enthesis: a review of the tendon-to-bone insertion', *Muscles, Ligaments and Tendons Journal,* 4 (3), 333-42.

Baer, Patrick C and Geiger, Helmut (2012), 'Adipose-derived mesenchymal stromal/stem cells: tissue localization, characterization, and heterogeneity', *Stem cells international,* 2012.

Bailey, C. J., et al. (1997), 'Wastage in the Australian thoroughbred racing industry: a survey of Sydney trainers', *Aust Vet J,* 75 (1), 64-6.

Barberini, Danielle Jaqueta, et al. (2014), 'Equine mesenchymal stem cells from bone marrow, adipose tissue and umbilical cord: immunophenotypic characterization and differentiation potential', *Stem cell research & therapy,* 5 (1), 25.

Baumgarth, Nicole and Roederer, Mario (2000), 'A practical approach to multicolor flow cytometry for immunophenotyping', *Journal of immunological methods,* 243 (1), 77-97.

Becerra, Patricia, et al. (2013), 'Distribution of injected technetium99m-labeled mesenchymal stem cells in horses with naturally occurring tendinopathy', *Journal of Orthopaedic Research,* 31 (7), 1096-102.

Berg, Lise Charlotte, et al. (2008), 'Cartilage-derived retinoic acid-sensitive protein in equine synovial fluid from healthy and diseased joints', *Equine veterinary journal,* 40 (6), 553-57.

Bertone, AL, Palmer, JL, and Jones, J (2001), 'Synovial fluid cytokines and eicosanoids as markers of joint disease in horses', *Veterinary Surgery,* 30 (6), 528-38.

Blasi, Antonella, et al. (2011), 'Dermal fibroblasts display similar phenotypic and differentiation capacity to fat-derived mesenchymal stem cells, but differ in anti-inflammatory and angiogenic potential', *Vasc Cell,* 3 (5).

Blewis, ME, et al. (2007), 'A model of synovial fluid lubricant composition in normal and injured joints', *Eur Cell Mater,* 13, 26-39.

Boone, Lindsey Helms (2013), *Intra-articular administration of allogeneic equine bone marrow derived mesenchymal stem cells* (uga).

Borena, Bizunesh M, et al. (2015), 'Regenerative skin wound healing in mammals: state-of-the-art on growth factor and stem cell based treatments', *Cellular Physiology and Biochemistry,* 36 (1), 1-23.

Bosch, Gerco, et al. (2011), 'Computerised analysis of standardised ultrasonographic images to monitor the repair of surgically created core lesions in equine superficial digital flexor tendons following treatment with intratendinous platelet rich plasma or placebo', *The Veterinary Journal,* 187 (1), 92-98.

Bosch, Gerco, et al. (2010), 'Effects of platelet-rich plasma on the quality of repair of mechanically induced core lesions in equine superficial digital flexor

tendons: a placebo-controlled experimental study', *Journal of Orthopaedic Research,* 28 (2), 211-17.

Bouffi, Carine, et al. (2010), 'IL-6-dependent PGE2 secretion by mesenchymal stem cells inhibits local inflammation in experimental arthritis', *PloS one,* 5 (12), e14247.

Bourzac, Celine A, et al. (2014), 'Evaluation of ultrasmall superparamagnetic iron oxide contrast agent labeling of equine cord blood and bone marrow mesenchymal stromal cells', *American journal of veterinary research,* 75 (11), 1010-17.

Broeckx, S., et al. (2013), 'The influence of allogenic mesenchymal stem cells on the hematological status of horses', *J Stem Cell Res Ther,* 3 (2), 1-6.

Broeckx, S., et al. (2014a), 'Intravenous application of allogenic peripheral blood-derived mesenchymal stem cells: a safety assessment in 291 equine recipients', *Current stem cell research & therapy,* 9 (6), 452-57.

Broeckx, Sarah, et al. (2012), 'Tenogenesis of equine peripheral blood-derived mesenchymal stem cells: in vitro versus in vivo', *Journal of Tissue Science & Engineering,* 3.

Broeckx, Sarah, et al. (2014b), 'Regenerative therapies for equine degenerative joint disease: a preliminary study', *PloS one,* 9 (1), e85917.

Brohem, CA, et al. (2013), 'Comparison between fibroblasts and mesenchymal stem cells derived from dermal and adipose tissue', *International journal of cosmetic science,* 35 (5), 448-57.

Brown, Michael and Wittwer, Carl (2000), 'Flow cytometry: principles and clinical applications in hematology', *Clinical chemistry,* 46 (8), 1221-29.

Burk, Janina, et al. (2013a), 'Equine cellular therapy—from stall to bench to bedside?', *Cytometry Part A,* 83 (1), 103-13.

Burk, Janina, et al. (2013b), 'Growth and differentiation characteristics of equine mesenchymal stromal cells derived from different sources', *The Veterinary Journal,* 195 (1), 98-106.

Burry, Richard W. (2011), 'Controls for Immunocytochemistry: An Update', *Journal of Histochemistry and Cytochemistry,* 59 (1), 6-12.

Caplan, Arnold I (1991), 'Mesenchymal stem cells', *Journal of orthopaedic research,* 9 (5), 641-50.

Cardoso, T. C., et al. (2012), 'Isolation and characterization of Wharton's jelly-derived multipotent mesenchymal stromal cells obtained from bovine umbilical cord and maintained in a defined serum-free three-dimensional system', *BMC Biotechnol,* 12, 18.

Carrade, Danielle D, et al. (2012), 'Comparative analysis of the immunomodulatory properties of equine adult-derived mesenchymal stem cells', *Cell medicine,* 4 (1), 1.

Carrade, Danielle D, et al. (2011), 'Clinicopathologic findings following intra-articular injection of autologous and allogeneic placentally derived equine mesenchymal stem cells in horses', *Cytotherapy,* 13 (4), 419-30.

Carrade Holt, Danielle D, et al. (2014), 'Equine mesenchymal stem cells inhibit T cell proliferation through different mechanisms depending on tissue source', *Stem cells and development,* 23 (11), 1258-65.

Carter-Arnold, JL, et al. (2014), 'In vitro analysis of equine, bone marrow-derived mesenchymal stem cells demonstrates differences within age-and gender-matched horses', *Equine veterinary journal,* 46 (5), 589-95.

Carvalho, Armando de M, et al. (2013), 'Isolation and characterization of equine peripheral blood-derived multipotent mesenchymal stromal cells', *Pesquisa Veterinária Brasileira*, 33 (9), 1151-54.

Clegg, PD (2012), 'Musculoskeletal disease and injury, now and in the future. Part 2: Tendon and ligament injuries', *Equine Veterinary Journal*, 44 (3), 371-75.

Clegg, PD, Jones, MD, and Carter, SD (1998), 'The effect of drugs commonly used in the treatment of equine articular disorders on the activity of equine matrix metalloproteinase-2 and 9', *Journal of veterinary pharmacology and therapeutics*, 21, 406-13.

Colleoni, Silvia, et al. (2009), 'Isolation, growth and differentiation of equine mesenchymal stem cells: effect of donor, source, amount of tissue and supplementation with basic fibroblast growth factor', *Veterinary research communications*, 33 (8), 811-21.

Colville, T and Bassert, JM (2002), 'Clinical Anatomy & Physiology for Veterinary Technicians. Missouri: Mosby', (Inc).

Corradetti, Bruna, et al. (2011), 'Size-sieved subpopulations of mesenchymal stem cells from intervascular and perivascular equine umbilical cord matrix', *Cell proliferation*, 44 (4), 330-42.

Covas, Dimas T, et al. (2008), 'Multipotent mesenchymal stromal cells obtained from diverse human tissues share functional properties and gene-expression profile with CD146+ perivascular cells and fibroblasts', *Experimental hematology*, 36 (5), 642-54.

Dakin, S.G. (2016), 'A review of the healing processes in equine superficial digital flexor tendinopathy', *Equine Veterinary Education*.

De Grauw, JC, et al. (2014), 'In vivo effects of phenylbutazone on inflammation and cartilage-derived biomarkers in equine joints with acute synovitis', *The Veterinary Journal*, 201 (1), 51-56.

de Mattos Carvalho, Armando, et al. (2009), 'Isolation and immunophenotypic characterization of mesenchymal stem cells derived from equine species adipose tissue', *Veterinary immunology and immunopathology*, 132 (2), 303-06.

De Schauwer, Catharina, et al. (2011), 'Markers of stemness in equine mesenchymal stem cells: a plea for uniformity', *Theriogenology*, 75 (8), 1431-43.

De Schauwer, Catharina, et al. (2013), 'Mesenchymal stem cell therapy in horses: useful beyond orthopedic injuries?', *Veterinary Quarterly*, 33 (4), 234-41.

De Schauwer, Catharina, et al. (2012), 'In search for cross-reactivity to immunophenotype equine mesenchymal stromal cells by multicolor flow cytometry', *Cytometry part A*, 81 (4), 312-23.

De Schauwer, Catharina, et al. (2014), 'Characterization and profiling of immunomodulatory genes of equine mesenchymal stromal cells from non-invasive sources', *Stem cell research & therapy*, 5 (1), 6.

Dias, Marianne Camargos, et al. (2016), 'Intramuscular Transplantation of Allogeneic Mesenchymal Stromal Cells Derived from Equine Umbilical Cord', *International Journal of Stem Cells*, 9 (2), 239-49.

Dominici, M., et al. (2006), 'Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement', *Cytotherapy (Taylor & Francis Ltd)*, 8 (4), 315-17.

Dowling, BA, et al. (2000), 'Superficial digital flexor tendonitis in the horse', *Equine veterinary journal*, 32 (5), 369-78.

Duesterdieck-Zellmer, Katja F, et al. (2016), 'Ex vivo penetration of low-level laser light through equine skin and flexor tendons', *American journal of veterinary research,* 77 (9), 991-99.

Dunkel, B., et al. (2012), 'Stimulus-dependent release of tissue-regenerating factors by equine platelets', *Equine Veterinary Journal,* 44 (3), 346-54.

Dyson, PK, et al. (2008), 'Days lost from training by two-and three-year-old Thoroughbred horses: A survey of seven UK training yards', *Equine Veterinary Journal,* 40 (7), 650-57.

Eggenhofer, Elke, et al. (2014), 'The Life and Fate of Mesenchymal Stem Cells', *Frontiers in Immunology,* 5.

Feisst, Vaughan, et al. (2013), 'Characterization of mesenchymal progenitor cell populations directly derived from human dermis', *Stem cells and development,* 23 (6), 631-42.

Ferris, Dora J, et al. (2014), 'Clinical outcome after intra-articular administration of bone marrow derived mesenchymal stem cells in 33 horses with stifle injury', *Veterinary Surgery,* 43 (3), 255-65.

Fink, T. and Zachar, V. (2011), 'Adipogenic differentiation of human mesenchymal stem cells', *Mesenchymal Stem Cell Assays and Applications*, 243-51.

Freshney, R, Ian., (2010), 'Culture of animal cells', *Cell Proliferation,* 15 (2.3), 1.

Friedenstein, AJ, Chailakhyan, RK, and Gerasimov, UV (1987), 'Bone marrow osteogenic stem cells: in vitro cultivation and transplantation in diffusion chambers', *Cell proliferation,* 20 (3), 263-72.

Frisbie and Smith (2010), 'Clinical update on the use of mesenchymal stem cells in equine orthopaedics', *Equine Veterinary Journal,* 42 (1), 86-89.

Frisbie, et al. (2009), 'Evaluation of adipose-derived stromal vascular fraction or bone marrow-derived mesenchymal stem cells for treatment of osteoarthritis', *Journal of Orthopaedic Research,* 27 (12), 1675-80.

Godwin, E. E., et al. (2012), 'Implantation of bone marrow-derived mesenchymal stem cells demonstrates improved outcome in horses with overstrain injury of the superficial digital flexor tendon', *Equine Veterinary Journal,* 44 (1), 25-32.

Goodrich, Laurie R and Nixon, Alan J (2006), 'Medical treatment of osteoarthritis in the horse–a review', *The Veterinary Journal,* 171 (1), 51-69.

Grauw, JC de, et al. (2009), 'In vivo effects of meloxicam on inflammatory mediators, MMP activity and cartilage biomarkers in equine joints with acute synovitis', *Equine veterinary journal,* 41 (7), 693-99.

Greco, K, V,., et al. (2011), 'High density micromass cultures of a human chondrocyte cell line: A reliable assay system to reveal the modulatory functions of pharmacological agents', *Biochemical pharmacology,* 82 (12), 1919-29.

Guest, D.J., Smith, MRW, and Allen, WR (2008), 'Monitoring the fate of autologous and allogeneic mesenchymal progenitor cells injected into the superficial digital flexor tendon of horses: preliminary study', *Equine veterinary journal,* 40 (2), 178-81.

Guest, D.J., Smith, M. R. W., and Allen, W. R. (2010a), 'Equine embryonic stem-like cells and mesenchymal stromal cells have different survival rates and migration patterns following their injection into damaged superficial digital flexor tendon', *Equine Veterinary Journal,* 42 (7), 636-42.

Guest, D.J., Smith, MRW., and Allen, WR. (2010b), 'Equine embryonic stem-like cells and mesenchymal stromal cells have different survival rates and

migration patterns following their injection into damaged superficial digital flexor tendon', *Equine veterinary journal,* 42 (7), 636-42.

Gustafson, AL, ., et al. (2004), 'An ordered BAC contig map of the equine major histocompatibility complex', *Cytogenetic and genome research,* 102 (1-4), 189-95.

Hackett, Catherine H., et al. (2012), 'Comparison of Gene-Specific DNA Methylation Patterns in Equine Induced Pluripotent Stem Cell Lines with Cells Derived From Equine Adult and Fetal Tissues', *Stem Cells and Development,* 21 (10), 1803-11.

Hanson, Summer E, et al. (2010), 'Characterization of mesenchymal stem cells from human vocal fold fibroblasts', *The Laryngoscope,* 120 (3), 546-51.

Hegewald, AA, et al. (2004), 'Hyaluronic acid and autologous synovial fluid induce chondrogenic differentiation of equine mesenchymal stem cells: a preliminary study', *Tissue and Cell,* 36 (6), 431-38.

Hematti, Peiman (2012), 'Mesenchymal stromal cells and fibroblasts: a case of mistaken identity?', *Cytotherapy,* 14 (5), 516-21.

Herthel, Douglas J (2001), 'Enhanced suspensory ligament healing in 100 horses by stem cells and other bone marrow components', *AAEP proceedings* (47), 319-21.

Ho, A. D., Wagner, W., and Franke, W. (2008), 'Heterogeneity of mesenchymal stromal cell preparations', *Cytotherapy (Taylor & Francis Ltd),* 10 (4), 320-30.

Hoogduijn, Martin J, et al. (2010), 'The immunomodulatory properties of mesenchymal stem cells and their use for immunotherapy', *International immunopharmacology,* 10 (12), 1496-500.

Horwitz, E. M. and Dominici, M. (2008), 'How do mesenchymal stromal cells exert their therapeutic benefit?', *Cytotherapy (Taylor & Francis Ltd),* 10 (8), 771-74.

Hosaka, Yoshinao, et al. (2002), 'Localization of cytokines in tendinocytes of the superficial digital flexor tendon in the horse', *The Journal of veterinary medical science/the Japanese Society of Veterinary Science,* 64 (10), 945-47.

Hosaka, Yoshinao, et al. (2006), 'Effect of heat on synthesis of gelatinases and pro-inflammatory cytokines in equine tendinocytes', *Biomedical Research,* 27 (5), 233-41.

Hoynowski, Steven M, et al. (2007), 'Characterization and differentiation of equine umbilical cord-derived matrix cells', *Biochemical and biophysical research communications,* 362 (2), 347-53.

Hraha, T, H,., et al. (2011), 'Autologous conditioned serum: the comparative cytokine profiles of two commercial methods (IRAP and IRAP II) using equine blood', *Equine veterinary journal,* 43 (5), 516-21.

Hu, Alaine J and Bramlage, Larry R (2014), 'Racing performance of Thoroughbreds with superficial digital flexor tendonitis treated with desmotomy of the accessory ligament of the superficial digital flexor tendon: 332 cases (1989–2003)', *Journal of the American Veterinary Medical Association,* 244 (12), 1441-48.

Husby, Kirsty A (2016), 'In Vitro Evaluation of Therapeutic Laser Treatment on Equine Tendon Fibroblasts'.

I., Caplan. Arnold and Sorrell, J. Michael (2015), 'The MSC curtain that stops the immune system', *Immunology Letters,* 168 (2), 136-39.

Iacono, Eleonora, et al. (2012), 'Isolation, characterization and differentiation of mesenchymal stem cells from amniotic fluid, umbilical cord blood and Wharton's jelly in the horse', *Reproduction,* 143 (4), 455-68.

Jann, Henry W, et al. (2016), 'The Effects of a Crosslinked, Modified Hyaluronic Acid (xCMHA-S) Gel on Equine Tendon Healing', *Veterinary Surgery,* 45 (2), 231-39.

Jordan, Nicole Vincent, Johnson, Gary L, and Abell, Amy N (2011), 'Tracking the intermediate stages of epithelial-mesenchymal transition in epithelial stem cells and cancer', *Cell cycle,* 10 (17), 2865-73.

Kalluri, Raghu and Weinberg, Robert A (2009), 'The basics of epithelial-mesenchymal transition', *The Journal of clinical investigation,* 119 (6), 1420-28.

Kamm, JL, ., Nixon, AJ, and Witte, TH (2010), 'Cytokine and catabolic enzyme expression in synovium, synovial fluid and articular cartilage of naturally osteoarthritic equine carpi', *Equine veterinary journal,* 42 (8), 693-99.

Kidd, JA, Fuller, C, and Barr, ARS (2001), 'Osteoarthritis in the horse', *Equine Veterinary Education,* 13 (3), 160-68.

Koch, Berg, Lise C., and Betts, Dean H. (2008), 'Concepts for the clinical use of stem cells in equine medicine', *The Canadian Veterinary Journal,* 49 (10), 1009-17.

Koch, T. G., et al. (2007), 'Isolation of mesenchymal stem cells from equine umbilical cord blood', *BMC Biotechnol,* 7, 26.

Koenig, Toby J, et al. (2014), 'Treatment of Experimentally Induced Osteoarthritis in Horses Using an Intravenous Combination of Sodium Pentosan Polysulfate, N-Acetyl Glucosamine, and Sodium Hyaluronan', *Veterinary surgery,* 43 (5), 612-22.

Koerner, Jens, et al. (2006), 'Equine Peripheral Blood-Derived Progenitors in Comparison to Bone Marrow-Derived Mesenchymal Stem Cells', *Stem cells,* 24 (6), 1613-19.

Kol, A, et al. (2013), 'Autologous point-of-care cellular therapies variably induce equine mesenchymal stem cell migration, proliferation and cytokine expression', *Equine veterinary journal,* 45 (2), 193-98.

Kyurkchiev, Dobroslav, et al. (2014), 'Secretion of immunoregulatory cytokines by mesenchymal stem cells', *World journal of stem cells,* 6 (5), 552.

Lam, KH, et al. (2007), 'Descriptive analysis of retirement of Thoroughbred racehorses due to tendon injuries at the Hong Kong Jockey Club (1992–2004)', *Equine veterinary journal,* 39 (2), 143-48.

Lange-Consiglio, Anna, et al. (2011), 'In vitro studies of horse umbilical cord matrix-derived cells: from characterization to labeling for magnetic resonance imaging', *Open Tissue Eng Regen Med J,* 4, 120-33.

Lee, K, B,L,., et al. (2007), 'Injectable mesenchymal stem cell therapy for large cartilage defects—a porcine model', *Stem cells,* 25 (11), 2964-71.

Leijs, Maarten JC, et al. (2012), 'Effect of arthritic synovial fluids on the expression of immunomodulatory factors by mesenchymal stem cells: an explorative in vitro study'.

Li, Bin, et al. (2011), 'Evidence for Mesenchymal−Epithelial Transition Associated with Mouse Hepatic Stem Cell Differentiation', *PLoS ONE,* 6 (2), e17092.

Liu, Wei, et al. (2006), 'Repair of tendon defect with dermal fibroblast engineered tendon in a porcine model', *Tissue engineering,* 12 (4), 775-78.

Lorenz, Katrin, et al. (2008), 'Multilineage differentiation potential of human dermal skin-derived fibroblasts', *Experimental dermatology,* 17 (11), 925-32.

Lovati, et al. (2010), 'Characterization and differentiation of equine tendon-derived progenitor cells', *Journal of biological regulators and homeostatic agents,* 25 (2 Suppl), S75-84.

Lovati, Arianna Barbara, et al. (2011), 'Comparison of equine bone marrow-, umbilical cord matrix and amniotic fluid-derived progenitor cells', *Veterinary research communications,* 35 (2), 103-21.

Lui, PPY., et al. (2011), 'Tenogenic differentiation of stem cells for tendon repair—what is the current evidence?', *Journal of tissue engineering and regenerative medicine,* 5 (8), e144-e63.

Maia, Leandro, ., et al. (2013), 'Immunophenotypic, immunocytochemistry, ultrastructural, and cytogenetic characterization of mesenchymal stem cells from equine bone marrow', *Microscopy research and technique,* 76 (6), 618-24.

Marfe, G., et al. (2012), 'Blood derived stem cells: An ameliorative therapy in veterinary ophthalmology', *Journal of cellular physiology,* 227 (3), 1250-56.

Martinello, Tiziana, et al. (2009), 'Cryopreservation does not affect the stem characteristics of multipotent cells isolated from equine peripheral blood', *Tissue Engineering Part C: Methods,* 16 (4), 771-81.

Masters, John R and Stacey, Glyn N (2007), 'Changing medium and passaging cell lines', *Nature protocols,* 2 (9), 2276-84.

Mathon, Nicole F and Lloyd, Alison C (2001), 'Milestones in cell division: Cell senescence and cancer', *Nature Reviews Cancer,* 1 (3), 203-13.

McIlwraith (2005), 'Use of synovial fluid and serum biomarkers in equine bone and joint disease: a review', *Equine veterinary journal,* 37 (5), 473-82.

McIlwraith, Frisbie, DD, and Kawcak, CE (2012), 'The horse as a model of naturally occurring osteoarthritis', *Bone and Joint Research,* 1 (11), 297-309.

McIlwraith, et al. (2011), 'Evaluation of intra-articular mesenchymal stem cells to augment healing of microfractured chondral defects', *Arthroscopy: The Journal of Arthroscopic & Related Surgery,* 27 (11), 1552-61.

Mohanty, Niharika, ., et al. (2014), 'Immunophenotypic characterization and tenogenic differentiation of mesenchymal stromal cells isolated from equine umbilical cord blood', *In Vitro Cellular & Developmental Biology-Animal,* 50 (6), 538-48.

Nagy, Kristina, et al. (2011), 'Induced Pluripotent Stem Cell Lines Derived from Equine Fibroblasts', *Stem Cell Reviews and Reports,* 7 (3), 693-702.

Nakamura-Ishizu, Ayako, Takizawa, Hitoshi, and Suda, Toshio (2014), 'The analysis, roles and regulation of quiescence in hematopoietic stem cells', *Development,* 141 (24), 4656-66.

Neil, Kirsten M, ., Caron, John P, and Orth, Michael W (2005), 'The role of glucosamine and chondroitin sulfate in treatment for and prevention of osteoarthritis in animals', *Journal of the American Veterinary Medical Association,* 226 (7), 1079-88.

Nikfarjam, Laleh and Farzaneh, Parvaneh (2011), 'Prevention and detection of Mycoplasma contamination in cell culture', *Cell J (Yakhteh),* 13 (4).

Nixon, Alan J, Watts, Ashlee E, and Schnabel, Lauren V (2012), 'Cell-and gene-based approaches to tendon regeneration', *Journal of shoulder and elbow surgery,* 21 (2), 278-94.

O'Meara, B, et al. (2010), 'An investigation of the relationship between race performance and superficial digital flexor tendonitis in the Thoroughbred racehorse', *Equine veterinary journal,* 42 (4), 322-26.

O'Shea, John J, Ma, Averil, and Lipsky, Peter (2002), 'Cytokines and autoimmunity', *Nature Reviews Immunology,* 2 (1), 37-45.

Orford, Keith W and Scadden, David T (2008), 'Deconstructing stem cell self-renewal: genetic insights into cell-cycle regulation', *Nature Reviews Genetics,* 9 (2), 115-28.

Pacini, Simone, et al. (2007), 'Suspension of bone marrow-derived undifferentiated mesenchymal stromal cells for repair of superficial digital flexor tendon in race horses', *Tissue engineering,* 13 (12), 2949-55.

Paris, DBBP and Stout, TAE (2010), 'Equine embryos and embryonic stem cells: defining reliable markers of pluripotency', *Theriogenology,* 74 (4), 516-24.

Parker, R. A., Clegg, P. D., and Taylor, S. E. (2012), 'The in vitro effects of antibiotics on cell viability and gene expression of equine bone marrow-derived mesenchymal stromal cells', *Equine Veterinary Journal,* 44 (3), 355-60.

Paterson, Yasmin Z, et al. (2014), 'Equine mesenchymal stromal cells and embryo-derived stem cells are immune privileged in vitro', *Stem cell research & therapy,* 5 (4), 90.

Patterson-Kane, JC, Becker, DL, and Rich, T (2012), 'The pathogenesis of tendon microdamage in athletes: the horse as a natural model for basic cellular research', *Journal of comparative pathology,* 147 (2), 227-47.

Penny, Jasmine, et al. (2012), 'The biology of equine mesenchymal stem cells: phenotypic characterization, cell surface markers and multilineage differentiation', *Front Biosci,* 17, 892-908.

Pezzanite, Lynn M, et al. (2015), 'Equine allogeneic bone marrow-derived mesenchymal stromal cells elicit antibody responses in vivo', *Stem cell research & therapy,* 6 (1), 1.

Pilz, Gregor A, et al. (2010), 'Human term placenta-derived mesenchymal stromal cells are less prone to osteogenic differentiation than bone marrow-derived mesenchymal stromal cells', *Stem cells and development,* 20 (4), 635-46.

Pittenger, Mark F, et al. (1999), 'Multilineage potential of adult human mesenchymal stem cells', *science,* 284 (5411), 143-47.

Pool, RR (1993), 'Difficulties in definition of equine osteochondrosis; differentiation of developmental and acquired lesions', *Equine Veterinary Journal,* 25 (S16), 5-12.

Preffer, Frederic and Dombkowski, David (2009), 'Advances in complex multiparameter flow cytometry technology: Applications in stem cell research', *Cytometry Part B: Clinical Cytometry,* 76 (5), 295-314.

Prockop, Darwin J (2013), 'Concise review: two negative feedback loops place mesenchymal stem/stromal cells at the center of early regulators of inflammation', *Stem Cells,* 31 (10), 2042-46.

Pullen, Nicholas A, Ryan, Joan M, and Schiltz, Paul M (2016), 'Comparative efficacy of equine bone marrow derived stem cell culture protocols', (Am Assoc Immnol).

Radtke, Catherine, ., et al. (2013), 'Characterization and osteogenic potential of equine muscle tissue–and periosteal tissue–derived mesenchymal stem cells in comparison with bone marrow–and adipose tissue–derived mesenchymal stem cells', *American journal of veterinary research,* 74 (5), 790-800.

Ranera, B., et al. (2012), 'Comparative study of equine bone marrow and adipose tissue-derived mesenchymal stromal cells', *Equine Veterinary Journal,* 44 (1), 33-42.

Redmond Hubbard, Amy Louise (2014), 'A Comparison of Equine Adipose Tissue, Bone Marrow and Peripheral Blood as Sources of Mesenchymal Stem Cells'.

Reed, Sarah A and Johnson, Sally E (2008), 'Equine umbilical cord blood contains a population of stem cells that express Oct4 and differentiate into mesodermal and endodermal cell types', *Journal of cellular physiology,* 215 (2), 329-36.

Renzi, S., et al. (2013), 'Autologous bone marrow mesenchymal stromal cells for regeneration of injured equine ligaments and tendons: A clinical report', *Research in Veterinary Science,* 95 (1), 272-77.

Ricco, S., et al. (2013), 'Allogeneic adipose tissue-derived mesenchymal stem cells in combination with platelet rich plasma are safe and effective in the therapy of superficial digital flexor tendonitis in the horse', *Int J Immunopathol Pharmacol,* 26 (1 Suppl), 61-8.

Rich, F Ross (2014), 'Single-center study of 83 horses with suspensory injuries treated with adipose-derived stem and regenerative cells', *Stem Cell Discovery,* 2014.

Richardson, Lucy E., et al. (2007), 'Stem cells in veterinary medicine – attempts at regenerating equine tendon after injury', *Trends in Biotechnology,* 25 (9), 409-16.

Riggs, CM (2006), 'Osteochondral injury and joint disease in the athletic horse', *Equine Veterinary Education,* 18 (2), 100-12.

Robinson, J Paul and Roederer, Mario (2015), 'Flow cytometry strikes gold', *Science,* 350 (6262), 739-40.

Rohart, Florian, et al. (2016), 'A molecular classification of human mesenchymal stromal cells', *PeerJ,* 4, e1845.

Sabatini, Federica, et al. (2005), 'Human bronchial fibroblasts exhibit a mesenchymal stem cell phenotype and multilineage differentiating potentialities', *Laboratory Investigation,* 85 (8), 962-71.

Schnabel, Lauren V, et al. (2014), 'Equine bone marrow-derived mesenchymal stromal cells are heterogeneous in MHC class II expression and capable of inciting an immune response in vitro', *Stem Cell Res Ther,* 5 (1), 13.

Smith, RKW, et al. (2003), 'Isolation and implantation of autologous equine mesenchymal stem cells from bone marrow into the superficial digital flexor tendon as a potential novel treatment', *Equine veterinary journal,* 35 (1), 99-102.

Solchaga, Luis, A,., Penick, Kitsie J, and Welter, Jean F (2011), 'Chondrogenic differentiation of bone marrow-derived mesenchymal stem cells: tips and tricks', *Mesenchymal Stem Cell Assays and Applications*, 253-78.

Sole, A, et al. (2013), 'Distribution and persistence of technetium-99 hexamethyl propylene amine oxime-labelled bone marrow-derived mesenchymal stem cells in experimentally induced tendon lesions after intratendinous injection

and regional perfusion of the equine distal limb', *Equine veterinary journal*, 45 (6), 726-31.

Souza, Maria Verônica de (2016), 'Osteoarthritis in horses-Part 2: a review of the intra-articular use of corticosteroids as a method of treatment', *Brazilian Archives of Biology and Technology*, 59.

Spaas, ., et al. (2013a), 'The effects of equine peripheral blood stem cells on cutaneous wound healing: a clinical evaluation in four horses', *Clinical and experimental dermatology*, 38 (3), 280-84.

Spaas, Jan, et al. (2011), 'Purification and expansion of stem cells from equine peripheral blood, with clinical applications', *Vlaams Diergeneeskundig Tijdschrift*, 80 (2), 129-35.

Spaas, Jan H, et al. (2013b), 'Culture and characterisation of equine peripheral blood mesenchymal stromal cells', *The Veterinary Journal*, 195 (1), 107-13.

Spriet, Mathieu, et al. (2015), 'Scintigraphic tracking of mesenchymal stem cells after intravenous regional limb perfusion and subcutaneous administration in the standing horse', *Veterinary Surgery*, 44 (3), 273-80.

Strutz, Frank, et al. (1995), 'Identification and characterization of a fibroblast marker: FSP1', *The Journal of cell biology*, 130 (2), 393-405.

Tanner, J, V,., et al. (2012), 'Preliminary examination of wastage in Thoroughbred and Standardbred horses in New Zealand using training milestones', *Proceedings of the New Zealand Society of Animal Production*, 72, 172-74.

Tessier, Laurence, et al. (2015), 'Phenotypic and Immunomodulatory Properties of Equine Cord Blood-Derived Mesenchymal Stromal Cells', *PLoS One*, 10 (4).

Textor, Jamie, . (2011), 'Autologous biologic treatment for equine musculoskeletal injuries: platelet-rich plasma and IL-1 receptor antagonist protein', *Veterinary Clinics of North America: Equine Practice*, 27 (2), 275-98.

Thorpe, CT, Clegg, PD, and Birch, HL (2010), 'A review of tendon injury: why is the equine superficial digital flexor tendon most at risk?', *Equine veterinary journal*, 42 (2), 174-80.

Tnibar, Aziz, ., et al. (2015), 'An international multi-centre prospective study on the efficacy of an intraarticular polyacrylamide hydrogel in horses with osteoarthritis: a 24 months follow-up', *Acta Veterinaria Scandinavica*, 57 (1), 20.

Toupadakis, Chrisoula A, et al. (2010), 'Comparison of the osteogenic potential of equine mesenchymal stem cells from bone marrow, adipose tissue, umbilical cord blood, and umbilical cord tissue', *American journal of veterinary research*, 71 (10), 1237-45.

Trotter, GW and McIlwraith, CW (1996), 'Clinical features and diagnosis of equine joint disease', *McIlwraith, CW, Trotter GW Joint disease in the horse. WB Saunders. Philadelphia, USA*, 120-45.

Uccelli, Antonio and Rosbo, Nicole Kerlero (2015), 'The immunomodulatory function of mesenchymal stem cells: mode of action and pathways', *Annals of the New York Academy of Sciences*, 1351 (1), 114-26.

Ulrich, Christine, et al. (2012), 'Mesenchymal stromal cells and fibroblasts', *Tissue Sci. Eng*, 3, e109.

Uphoff, Cord C and Drexler, Hans G (2004), 'Detecting mycoplasma contamination in cell cultures by polymerase chain reaction', *Cancer Cell Culture: Methods and Protocols*, 319-26.

Van Loon, Vic JF, et al. (2014), 'Clinical follow-up of horses treated with allogeneic equine mesenchymal stem cells derived from umbilical cord blood for different tendon and ligament disorders', *Veterinary Quarterly,* 34 (2), 92-97.

Vidal, Martin A, et al. (2011), 'Evaluation of senescence in mesenchymal stem cells isolated from equine bone marrow, adipose tissue, and umbilical cord tissue', *Stem cells and development,* 21 (2), 273-83.

Vidal., et al. (2008), 'Comparison of Chondrogenic Potential in Equine Mesenchymal Stromal Cells Derived from Adipose Tissue and Bone Marrow', *Veterinary surgery : VS,* 37 (8), 713-24.

Violini, Stefania, et al. (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 (1), 1.

Violini, Stefania, et al. (2012), 'Isolation and differentiation potential of an equine amnion-derived stromal cell line', *Cytotechnology,* 64 (1), 1-7.

Wagner, Bettina and Freer, Heather (2009), 'Development of a bead-based multiplex assay for simultaneous quantification of cytokines in horses', *Veterinary immunology and immunopathology,* 127 (3), 242-48.

Wang, L., et al. (2015), 'Dynamic Expression Profiles of Marker Genes in Osteogenic Differentiation of Human Bone Marrow-derived Mesenchymal Stem Cells', *Chin Med Sci J,* 30 (2), 108-13.

Wayne, McIlwraith. C. (1982), 'Current concepts in equine degenerative joint disease', *Journal of the American Veterinary Medical Association,* 180 (3), 239-50.

Williams, JH, Mepham, BL, and Wright, DH (1997), 'Tissue preparation for immunocytochemistry', *Journal of clinical pathology,* 50 (5), 422-28.

Wilsher, S, Allen, WR, and Wood, JLN (2006), 'Factors associated with failure of Thoroughbred horses to train and race', *Equine Veterinary Journal,* 38 (2), 113-18.

Wilson, AM and Goodship, AE (1994), 'Exercise-induced hyperthermia as a possible mechanism for tendon degeneration', *Journal of biomechanics,* 27 (7), 899-905.

Xing, Yun, et al. (2007), 'Bioconjugated quantum dots for multiplexed and quantitative immunohistochemistry', *Nature Protocols,* 2 (5), 1152-65.

Yang, KG Auw, et al. (2006), 'The effect of synovial fluid from injured knee joints on in vitro chondrogenesis', *Tissue engineering,* 12 (10), 2957-64.

Youngstrom, Daniel Warren (2015), 'Mesenchymal Stem Cell Mechanobiology and Tendon Regeneration'.

Zafranskaya, M, et al. (2013), 'PGE2 Contributes to In vitro MSC-Mediated Inhibition of Non-Specific and Antigen-Specific T Cell Proliferation in MS Patients', *Scandinavian journal of immunology,* 78 (5), 455-62.

Zayed, Mohammed, et al. (2017), 'Donor-Matched Comparison of Chondrogenic Potential of Equine Bone Marrow- and Synovial Fluid-Derived Mesenchymal Stem Cells: Implications for Cartilage Tissue Regeneration', *Frontiers in Veterinary Science,* 3 (121).

APPENDIX 1: MEDIA, SOLUTIONS AND BUFFERS

Basal Culture Media for MSC – To make total volume of 300ml

Ingredients	Volume	Source
DMEM low glucose	355ml	LifeTechnologies
FBS	40ml	Gibco
Antibiotic-Antimycotic	4ml	Sigma Aldrich
Fungizone	400µl	Sigma Aldrich
Gentamicin	500µl	ProPharma

Filter sterilised 0.22µm stock ingredients

Cryogenic medium

Ingredients	Volume (20ml stock)	Source
75% DMEM	15ml	Thermofisher
20% FBS	4ml	Life Technologies/Gibco
5% DMSO	1ml	Thermofisher

Filter sterilised 0.22µm stock ingredients

Collagenase

Ingredients	Quantity/volume (10ml)	Source
Collagenase, Type 1A, 125 CDU/mg solid	0.02g	Sigma Aldrich
PBS	10ml	Thermofisher

Filter sterilised 0.22µm stock ingredients

2% Agarose gel

Ingredients	Quantity	Source
Agarose powder	.67g	Ngaio Diagnostics
1xTAE buffer	33ml	Waikato University

30% Sucrose solution 500ml

Ingredients	Quantity/volume 500ml	Source
Sucrose	150g	Sigma Aldrich
PBS 10x	50ml	Thermofisher
Sterile dH20	To 500ml	Waikato University

Filter sterilised 0.22 µm and stored at 4°C

50x TAE buffer 1000ml

Ingrediants	Quantity	Source
Tris	242g	Sigma Aldrich
glacial acetic acid	57.1ml	Sigma Aldrich
0.5M EDTA	100ml	Sigma Aldrich
dH20	To 1L	Waikato University

1 x TAE (20ml 50x TAE into 980 ml dH20)

1X PHEM buffer 100ml pH 6.9

Ingredients	Quantity	Source
60mM PIPES	1.81g	Sigma Aldrich
25mM HEPES	0.60g	Sigma Aldrich
0.5M EGTA	0.38g	Sigma Aldrich
2mM $MgCl_2$	0.02g	Sigma Aldrich

APPENDIX 2: CONSUMABLES, CHEMICALS, DRUGS

Consumables		
Product	**Supplier**	**Catalogue number**
Cell strainer - Green	Lifetechnologies	MILSLGV100RS
Cell strainer - Orange	Ray Lab, NZ	GR542070
Centifugation tubes 15ml	Interlab, NZ	KJ324
Centifugation tubes 50ml	Interlab, NZ	KJ326
Coverslips	Thermo Fisher Scientific	FSHMNJ-500-010G
Cryovial Greiner 1ml blue	Mediray, NZ	GR123279
Cryovials Greiner 2ml green	Mediray, NZ	GR126277
Falcon tube, 15ml sterile	Thermo Fisher Scientific	LBSCN8CT15
Falcon tube, 50ml sterile	Thermo Fisher Scientific	LBSCN8CT50
Filter papers	Thermo Fisher Scientific	LBS0001.070
Nunclon flasks 25cm	Thermo Fisher Scientific	NUN156340
Nunclon flasks 75cm	Thermo Fisher Scientific	NUN156499
Parafilm	Thermo Fisher Scientific	TS3800
Pipette bulbs	Thermo Fisher Scientific	C-P24820-60
Pipette tips 1ml	Interlab, NZ	LC1057-965
Pipette tips 200ul	Interlab, NZ	LC1059-965
Sterile disposable pipettes	Ray Lab, NZ	RL200CS01
Syringe filter - 22µm	Lifetechnologies	MILSLGV025RS
Syringe filter - 45µm	Minisart, Sartorious Stedim	16555
Surgipath Apex Cryostat Slides	Thermo Fisher Scientific	NC0884931
Surgipath® Frostbite® Spray	Leica Biosystems	3803100
0.5ml straws	Minitub, Australia	13408/0010
24 well plate nunclon	Thermo fisher Scientific	NUN142475

4 well plates nunclon	Thermo Fisher Scientific	NUN144444
6 well plate nunclon	Thermo Fisher Scientific	NUN145380
96 well plate round low cell binding surface	Thermo fisher Scientific	NUN145397
Chemicals general		
Acetic acid solution CH3COOH 0.1M	Sigma- Aldrich NZ	A9967
Acetone CH3COCH3	Sigma- Aldrich NZ	90872
Ethanol ETOH	Sigma- Aldrich NZ	E7023
Isoproponol	Sigma- Aldrich NZ	563935
Methanol MeOH	Sigma- Aldrich NZ	M1770
Paraformaldehyde	Sigma- Aldrich NZ	158127
Phosphate buffered saline 10x solution	Thermofisher	FSBBP399-1
Sodium Acetate NaOAc	Sigma- Aldrich NZ	S3272
Sodium azide 0.1M NaAz	Sigma- Aldrich NZ	8591
Sucrose C12H22O11	Biolab	AJA530-5KG
Cell Culture		
Preparation		
Collagenase type 1A	Sigma- Aldrich NZ	C9891-500MG
Lymphoprep	Mediray	N-1114545
Vigro Embryo flush media	Minitub, Australia	19982/6202
Culture		
Antibiotic - antimycotic	Lifetechnologies	15240-062
Dexamethasone	Sigma- Aldrich NZ	D8893-1MG
Dimethyl sulfoxide C2H6OS	Sigma- Aldrich NZ	D8418
DMEM low glucose, hepes, L-glut	Lifetechnologies	12320-032
Fetal bovine serum	Lifetechnologies	10091148
Fungizone (amphoterecin B)	Lifetechnologies	15290-026
Gentamicin 80mg in 2ml	ProPharma	
Glutamax	Thermo Fisher Scientific	35050061
HEPES sodium salt C8H17N2O4S	Sigma- Aldrich NZ	H3784
Trypan blue 0.2um filtered Hyclone	Thermo Fisher Scientific	HYCSV30084.01

Trypsin-EDTA	Lifetechnologies	25200-056
Mycoplasma PCR		
HyAgarose	Hydragene	9012-36-6
I-Taq, 2 PCR mastermix iNtRON Biotechnology	Ngaio Diagnostics	25027
PCR DNA ladder	Genescript	M106R
Proteinase K recombinant PCR grade	Sigma-Aldrich, NZ	EO0491
Redsafe, iNtRON Biotechnology	Ngaio Diagnostics	21141
Antibodies		
Immunocyto-chemistry		
CD105 reactive against horse	Abacus	SEMCA1557 Clone SN6
CD29 mouse anti-human	Norrie	303001 Clone TS2/16
CD44 rat anti-mouse/human	Norrie	103001 Clone IM7
CD45 mouse anti-human	Abacus	SEMCA87A Clone F10-89-4
MHC-II mouse anti-human	Abacus	SEMCA1085GA Clone CVS20
2nd ab goat anti-mouse FITC	Norrie Biotech/Biolegend	405305
2nd AB rat anti-mouse IgG ALEXA488	Abacus	JI415545166
Flow cytometry		
CD90+	Gentaur	YV0298-01 Clone DH24A
CD105-RPE+	AbD Serotec	MCA1557PET Clone SN6
CD45-Alexa488-	AbD Serotec	MCA87A488T Clone F10-89-4
CD79a_Alexa647-	AbD Serotec	MCA2538A647T Clone CVS36 (HM57)
MHC-II-	AbD Serotec	MCA1085PE Clone CVS20
7-AAD-	Sigma- Aldrich NZ	A9400-1MG
Dapi	AbD Serotec	BUF061

Differentiation		
Stempro Adipose kit	Thermo Fisher Scientific	A10070-01
Stempro Chondrogenic kit	Thermo Fisher Scientific	A10071-01
Stempro Osteogenic kit	Thermo Fisher Scientific	A10072-01
Stains		
Alcian Blue	Sigma- Aldrich NZ	A5268
Alizarin Red	Sigma- Aldrich NZ	A5533
Oil Red O	Sigma- Aldrich NZ	O0625
Organo-limonene mount	Sigma- Aldrich NZ	O8015-30ML
Harris Hematoxylin & eosin	Sigma- Aldrich NZ	HHS16
OCT Jung tissue freezing media	Leica Biosystems	14020108926
Bio Activation Assay		
TNF alpha	Sigma- Aldrich NZ	H8916-1006
allogenic synovial fluid		
ELISA plates		
GSI Equine IL-1b	Genorise USA	106041-SF
GSI Equine IL-10 ELISA jf	Genorise USA	106003-SF
GSI Equine IL-6 ELISA Kit jf	Genorise USA	106001-SF
GSI Equine TGFb1 ELISA Kit jf	Genorise USA	106123-SF
GSI Equine TNF-alpha ELISA Kit jf	Genorise USA	106004-SF
Nori Equine PGE2 ELISA Kit	Genorise USA	GR106222-SF

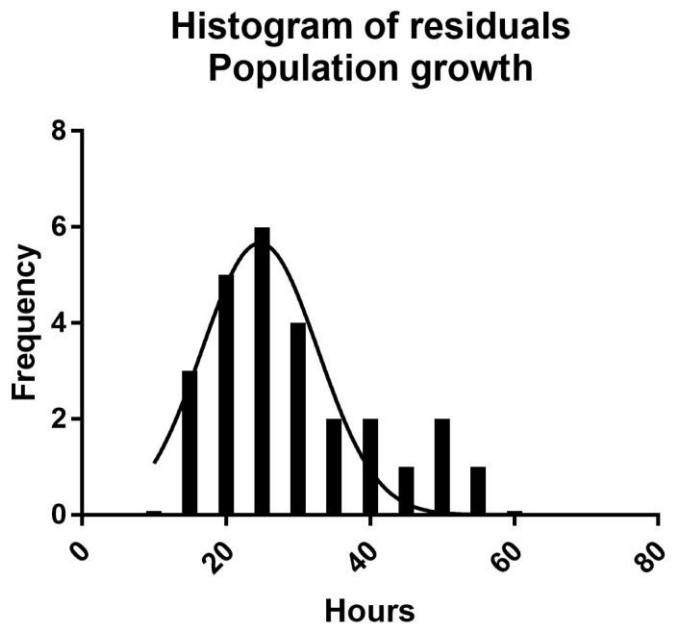
PCR equipment and enzymes

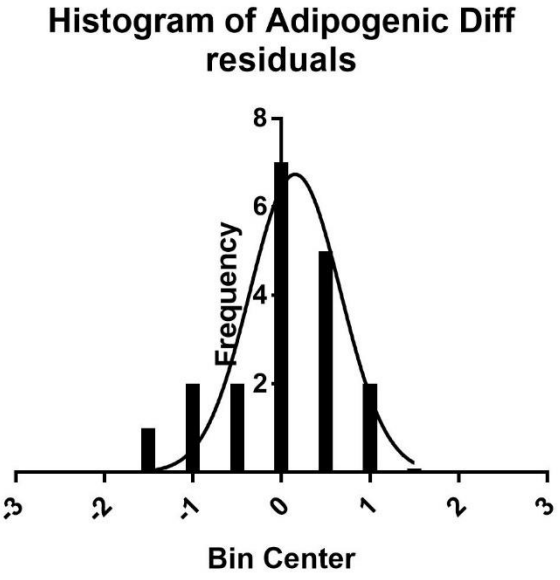
Equipment/enzymes	Supplier	Specifications
Ethidium bromide		10mg/ml
100bp Ladder	GenScript	M102R 500ul
I-Taq 2 PCR	Ngaio Diagnostics	25027
$MgCl_2$	2M and 4M	
Electrophoresis unit	Owl tank and power packs	
Thermal cycler	BioRad	T100
Real Time PCR machine	Corbett Research	Rotor-Gene 6000
UV illuminator	Gibco BRL	UV-TFX-35M

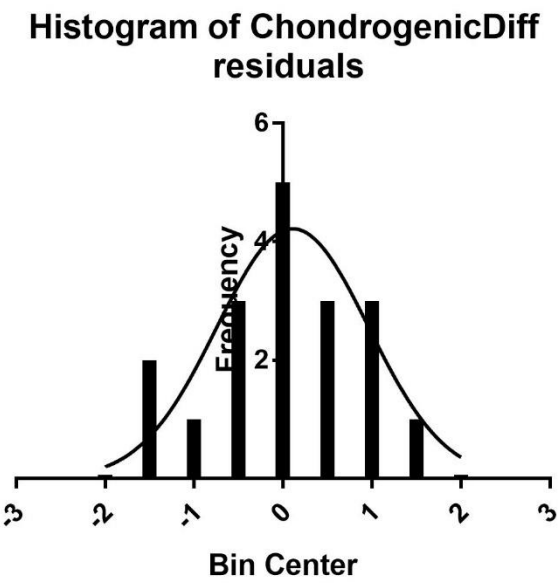
Spectrophotometer	Nanodrop 2000	
PCR eppendorfs		200ul, 500ul
Redsafe, iNtRON Biotechnology	Ngaio Diagnostics	21141
PCR DNA ladder	Genescript	M106R
Proteinase K recombinant PCR grade	Sigma-Aldrich, NZ	EO0491

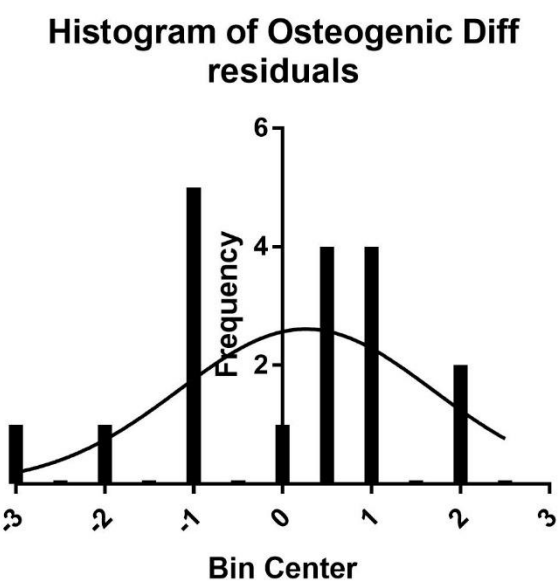
APPENDIX 3: EQUATIONS

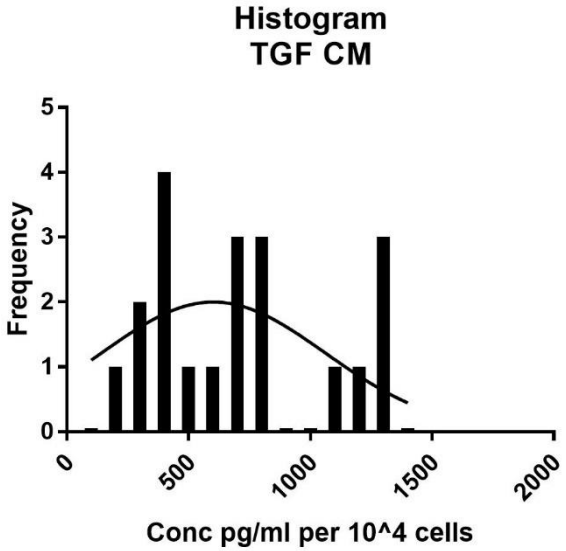
APPENDIX 4: HISTOGRAMS

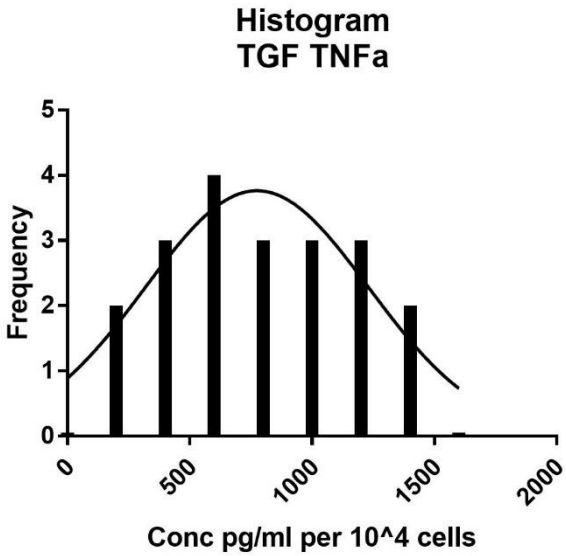


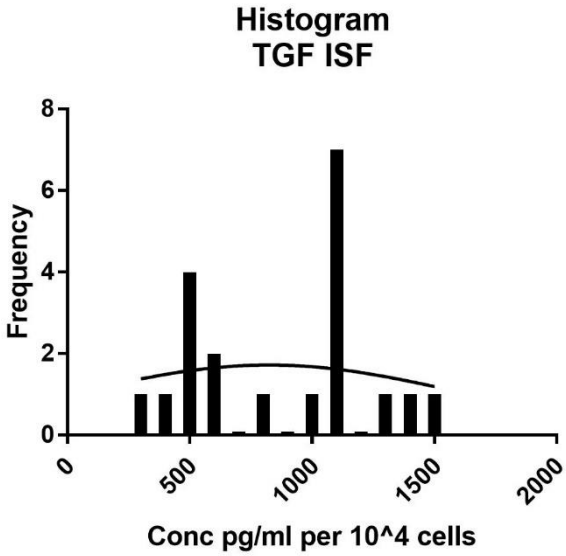

Histogram of Adipogenic Diff residuals
Frequency
8
6
4
2
-3
-2
-1
0
1
2
3
Bin Center

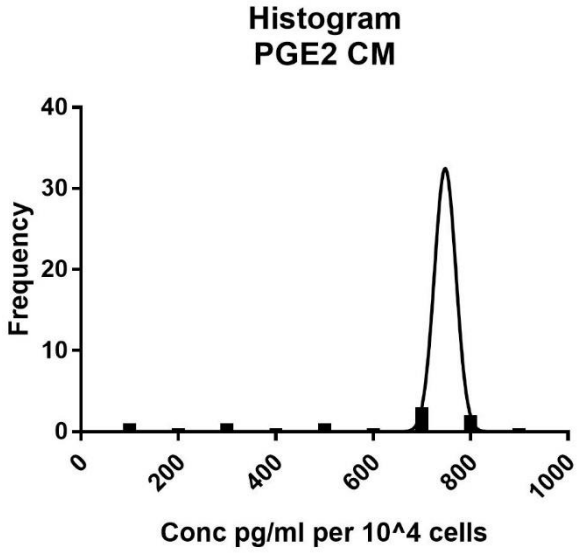

Histogram of ChondrogenicDiff residuals
Frequency
6
4
2
-3
-2
-1
0
1
2
3
Bin Center

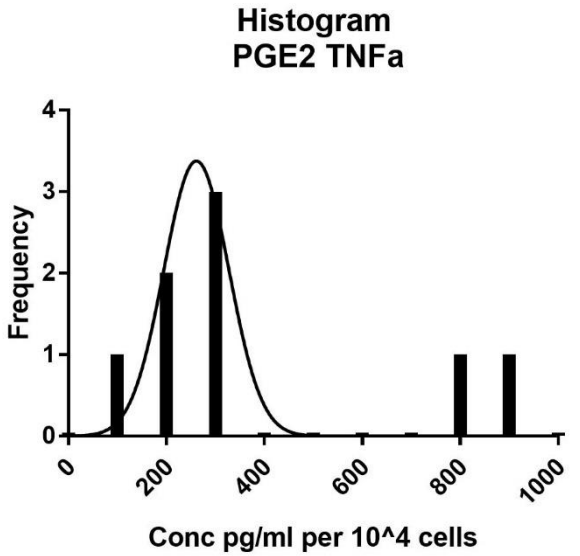

Histogram of Osteogenic Diff residuals
Frequency
6
4
2
-3
-2
-1
0
1
2
3
Bin Center

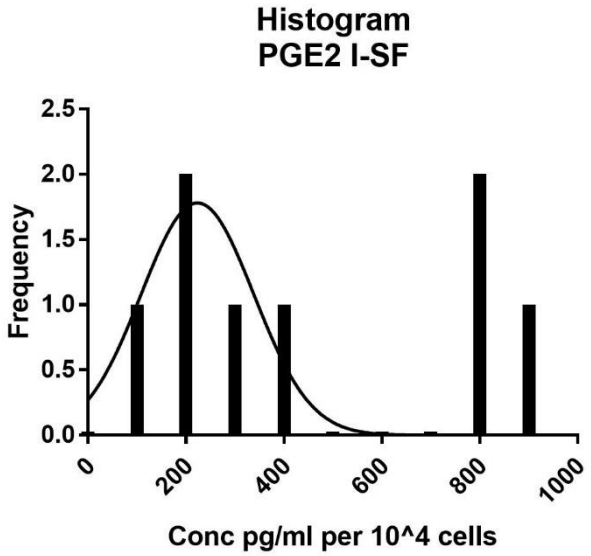

Histogram
TGF CM
Frequency
0
1
2
3
4
5
0
500
1000
1500
2000
Conc pg/ml per 10^4 cells

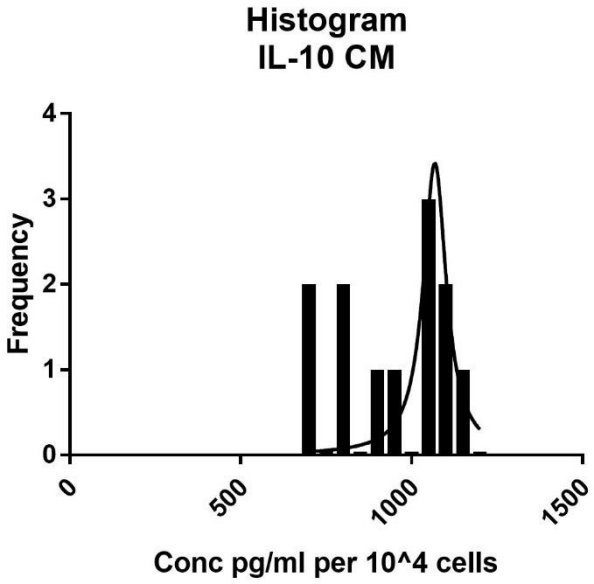

Histogram
TGF TNFa
Frequency
0
1
2
3
4
5
0
500
1000
1500
2000
Conc pg/ml per 10^4 cells

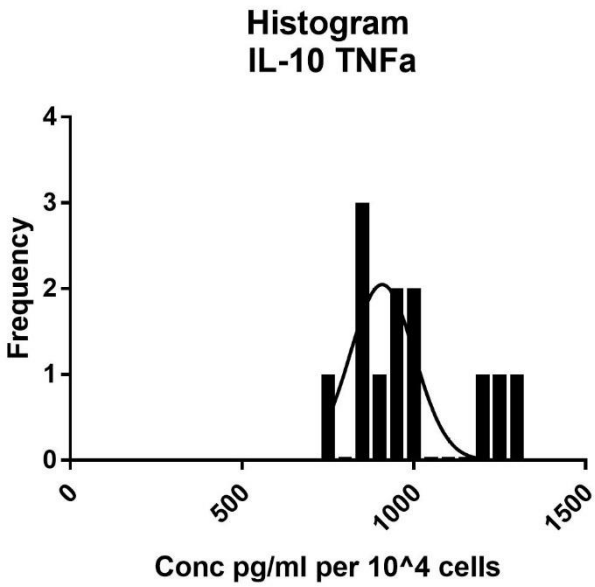

Histogram
TGF ISF
Frequency
0
2
4
6
8
0
500
1000
1500
2000
Conc pg/ml per 10^4 cells

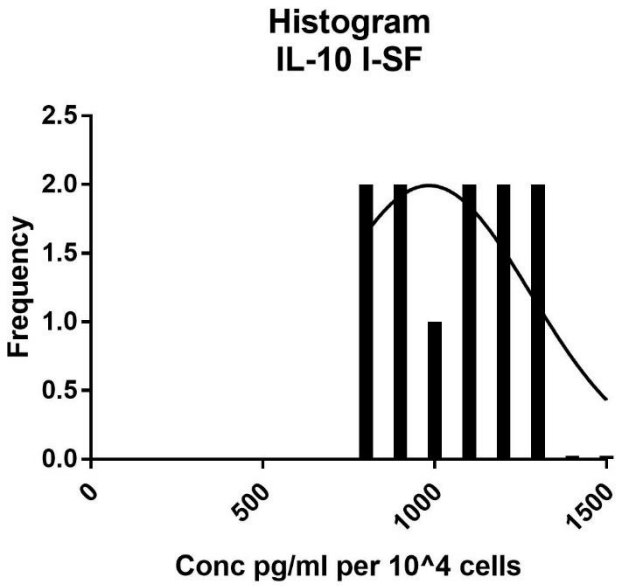

Histogram
PGE2 CM
Frequency
40
30
20
10
0
0
200
400
600
800
1000
Conc pg/ml per 10^4 cells


Histogram
PGE2 TNFa
Frequency
4
3
2
1
0
0
200
400
600
800
1000
Conc pg/ml per 10^4 cells


Histogram
PGE2 I-SF
Frequency
2.5
2.0
1.5
1.0
0.5
0.0
0
200
400
600
800
1000
Conc pg/ml per 10^4 cells


Histogram
IL-10 CM
Frequency
4
3
2
1
0
0
500
1000
1500
Conc pg/ml per 10^4 cells


Histogram
IL-10 TNFa
Frequency
4
3
2
1
0
0
500
1000
1500
Conc pg/ml per 10^4 cells


Histogram
IL-10 I-SF
Frequency
2.5
2.0
1.5
1.0
0.5
0.0
0
500
1000
1500
Conc pg/ml per 10^4 cells