Generation of equine osteochondral constructs using mesenchymal stromal cells

By
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**Abstract**

**Generation of equine osteochondral constructs using mesenchymal stromal cells**

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Focal cartilage injuries are a significant cause of lameness in the horse. Current treatments, such as mosaic arthroplasty, come with significant limitations. Tissue engineered cartilage layered on a bone substrate is a possible alternative to using native osteochondral plugs. The purpose of this thesis is to explore the chondrogenic potential of equine induced pluripotent stem cells (iPSCs) and mesenchymal stromal cells (MSCs) for the purpose of generating osteochondral constructs.

Equine iPSCs were first differentiated into MSC-like cells to generate an intermediate progenitor population. When assessed for trilineage differentiation, these MSC-like cells underwent adipogenesis and osteogenesis, but failed to undergo chondrogenesis. Thus, equine MSCs were used for further studies in cartilage tissue engineering.

Next, cartilage tissues derived from MSCs from two sources were compared to determine which source showed greater chondrogenic potency. 10 unrelated MSC cultures derived from bone marrow (BM-MSCs) and 10 unrelated MSC cultures from umbilical cord blood (CB-MSCs) were differentiated in a membrane culture system. The generated cartilage tissue was assessed at Day 7, 14, and 21. There were large differences in the quality of tissues generated by individual MSC cultures from both sources. Variance component analysis revealed that donor, and not cell source, is the primary contributor to the variance seen in the deposition of matrix components.

CB-MSCs are advantageous for allogeneic use as they can be isolated non-invasively, thus, CB-MSCs were used to generate osteochondral constructs. A novel method was developed
whereby MSCs were directly seeded on top of a calcium polyphosphate (CPP) bone substitute in the presence of a ROCK inhibitor to prevent cell contraction. It was found that a seeding density of 1.5x10^6 cells per CPP generated thick, hyaline-like tissue after 3 weeks of culture. There was no significant difference in tissue quality when the culture length was extended to 6 weeks, or when varying the TGFβ3 exposure in the 6 week cultures.

These results are the first to examine the chondrogenic capacity of equine iPSCs and the first to outline a one-step direct differentiation method of producing MSC-derived cartilage on a bone substrate. Further optimization of construct generation is needed prior to testing the constructs in vivo.
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“Alone we can do so little; together we can do so much.” – Helen Keller
Declaration of Work Performed

I declare that all work in this thesis was performed by Sarah Lepage with the following exceptions: cell sorting was done by Annie Bang at the Samuel Lunenfeld Research Institute at the University of Toronto. Some of the biochemical assays in Chapter 3 were performed by David de Lazzari. Statistics for Chapter 3 were done by Keith Russell in consultation with Gabrielle Monteith. CPP substrates were made by Eugene Hu in the lab of Bob Pilliar at the University of Toronto.
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List of Abbreviations

ACI – Autologous chondrocyte implantation
ALK - Anaplastic lymphoma kinase
ANOVA – Analysis of variance
AT – Adipose tissue
AT-MSC – Adipose tissue mesenchymal stromal cells
BM – Bone marrow
BM-MSC – Bone marrow mesenchymal stromal cells
BMP – Bone morphogenic protein
CAIS – Cartilage autograft implantation system
CB – Cord blood
CD – Cluster of differentiation
CI – Confidence interval
CJRR – Canadian Joint Replacement Registry
CPP – Calcium polyphosphate
DAB - 3,3’-Diaminobenzidine
DMEM-HG – Dulbecco’s modified eagle medium high glucose
DMEM-LG - Dulbecco’s modified eagle medium low glucose
DMMB - Dimethylmethylene blue assay
ECM – Extra-cellular matrix
EDTA - Ethylenediaminetetraacetic acid
FBS – Fetal bovine serum
FGF – Fibroblast growth factor
FITC - Fluorescein isothiocyanate
GAG - Glycosaminoglycan
GFP – Green fluorescent protein
GvHD – Graft versus host disease
HA – Hyaluronic acid
HLA-DR - Human leukocyte antigen D related

IBMX - 3-Isobutyl-1-methylxanthine

IGF – Insulin-like growth factor

IgG – Immunoglobulin G

IgM - Immunoglobulin M

Ihh – Indian hedgehog

IL - Interleukin

iPSC – Induced pluripotent stem cell

iPSC-MSC – Induced pluripotent stem cell-mesenchymal stromal cell

ITS – Insulin transferrin selenium

LIF – Leukemia inhibitory factor

LPL – Lipoprotein lipase

MAB - Mesoangioblasts

MACI – Matrix autologous chondrocyte implantation

MAPK – Mitogen-activated protein kinase

MHC – Major histocompatibility complex

MMLV – Moloney murine leukemia virus

MRI – Magnetic resonance imaging

MSC – Mesenchymal stromal cell

OA - Osteoarthritis

OATS – Osteochondral autograft transfer system

PB – Piggybac

PB-MSC – Peripheral blood mesenchymal stromal cell

PBS – Phosphate buffered saline

PCL - Polycaprolactone

PCR – Polymerase chain reaction

PD – Programmed cell death protein

PDLA – Poly-D lactide
PGA – Polyglycolic acid
PJAC – Particulated juvenile allograft cartilage
PLA - Polylactide
PLGA - Poly(lactide-co-glycolide) acid
PPAR - Peroxisome proliferator-activated receptor
PTHrP - Parathyroid hormone-related protein
qRT-PCR – Quantitative reverse transcription polymerase chain reaction
ROCK – Rho-associated protein kinase
Runx – Runt-related transcription factor
SD – Standard deviation
Shh – Sonic hedgehog
SNAI3 - Snail family zinc finger 3
Sox - SRY-related HMG-box
T3 - Triiodothyronine
TCP – Tricalcium phosphate
TGFβ – Transforming growth factor beta
TNF – Tumor necrosis factor
USDA – United States Department of Agriculture
UT-MSC – Umbilical cord tissue mesenchymal stromal cells
WBC – White blood cells
Preface

This thesis is written in chapter format where each chapter is a published, submitted, or soon-to-be submitted manuscript. Some degree of repetition is inherent with this format, but efforts have been made to optimize readability by omitting or reducing the abstract, introduction, conclusion, acknowledgements and reference sections of each chapter. To ensure complete future experimental reproducibility, all materials and methods sections are unaltered from the original manuscripts.
Introduction

Joint injuries are the major cause of lameness and subsequent lost training days in the equine athlete. As a result, horse owners and trainers undergo significant financial and emotional strain in the form of veterinary costs, loss of use, and loss of market value. The horse industry makes up $19 billion of the Canadian economy (Evans, 2010), and thus swift treatment of these joint injuries is important to reduce the financial and welfare impact of the associated lameness. Injuries to joint cartilage are particularly difficult to treat, as cartilage lacks endogenous healing capabilities. Post-traumatic osteoarthritis is the most common cause of cartilage damage, and often arises as a result of acquired or developmental focal cartilage defects.

Current treatments for focal cartilage defects are insufficient to regenerate hyaline cartilage at the injury site, as they promote the formation of fibrocartilage, which cannot withstand forces within the joint long-term. As a result, stem cell-based treatment tactics emerged as an alternative. While straight administration of progenitor cells, namely mesenchymal stromal cells (MSCs), did not yield satisfactory results in promoting cartilage regeneration, their potential for in vitro tissue engineering remains promising. As it is still unclear what source of MSCs is best for tissue engineering, the chondrogenic potential of bone marrow- and umbilical cord-derived MSCs in a membrane culture differentiation system was explored. In addition, the derivation of MSC-like progenitor cells from equine induced pluripotent stem cells was investigated and assessed for their differentiation capacity.

Mosaic arthroplasty is a technique where a cylinder of cartilage and bone are removed from a healthy area of the joint and implanted into the defect site. As this procedure comes with a high risk of donor site morbidity when using autologous grafts, and allogeneic graft availability is non-
existent in the horse and low in human medicine, a suitable alternative would be to use in vitro-generated osteochondral plugs. Building upon the work above, improvement of the generation of biphasic osteochondral constructs using MSCs for the cartilage phase on top of a calcium polyphosphate (CPP) bone substitute was also investigated. As the lack of MSC adherence to the CPP had previously hindered directly seeding these cells on the bone surface, the addition of a small molecule to prevent cell contraction was evaluated. Following this investigation, the effect of different cell densities, culture time and TGFβ3 duration in culture and their effects on the quality of the cartilage layer on top of the bone substrate was also explored.

Though equine induced pluripotent stem cells (iPSCs) could be differentiated into MSC-like cells, the particular iPSC line tested could not be induced to undergo chondrogenesis. However, the limited number of iPSCs examined does not allow general conclusions regarding iPSCs as a chondroprogenitor source. While both bone marrow and umbilical cord blood-derived MSCs could form cartilage tissue in membrane culture, tissue quality varied widely between donors, more so than between the MSC sources. These findings suggest that the common delineation of cell functions as a consequence of their tissue-origin should be challenged. Cells of interest for a specific function can likely be sourced from many tissues. These results also highlight the challenges of autologous-based cell therapy approaches since cells with the desired function may not be isolated from any given patient. Finally, a novel one-step method of generating scaffold-free cartilage on top of a bone substitute was developed to make in vitro osteochondral constructs. Preliminary work done with three MSC cultures has suggested that a higher seeding density and transient TGFβ3 supplementation are sufficient to generate hyaline-like cartilage tissue on a calcium polyphosphate bone substitute.
Chapter 1: Literature review

Synovial joint structure and function

Synovial or articulating joints are the most common joints in the body. Within the joint capsule, the associated long bones are lined with articular hyaline cartilage and are separated by the synovial cavity. This “joint space” is filled with synovial fluid, which is secreted by the synovial membrane lining the capsule. Synovial fluid is an ultrafiltrate that acts as a nutrient source and lubricant to allow for frictionless motion (Blewist et al, 2007). This lubrication is achieved in part due to high levels of hyaluronan and lubricin in synovial fluid, which also aid in reducing cell and protein adhesion on the cartilage surface (Jay & Waller, 2014; Swann et al, 1974). Hyaline cartilage has an integral function in the joint capsule by providing mechanical force dispersal during joint articulation. As mechanical force during normal weight-bearing activity is applied to the tissue, it releases reserved synovial fluid to enhance lubrication on the cartilage surface (Walker et al, 1968). Proper lubrication and force dispersal are critical for the maintenance of healthy joints.

Developing cartilage

In mammals, articular cartilage develops as a consequence of endochondral ossification in the limb field through a series of cell morphology and phenotype changes as well as signaling cascades. Early in development, mesenchymal stem cells undergo condensation in the limb bud, enhancing cell-to-cell contact and growth factor stimulation of chondrogenesis via TGF-β, BMP, FGF, Shh, and Wnt-3a signaling (Day et al, 2005; Long et al, 2001; Minina et al, 2005). At this time, extra-cellular matrix (ECM) synthesis begins, mostly collagens type I and IIa, fibronectin, and tenascin proteins (Aszódi et al, 2001; Koyama et al, 1995; Kronenberg 2007; Kulyk, Upholt and Kosher 1989). Simultaneously, the transcription factors Sox5, Sox6 and Sox9 signal the cells
to differentiate into chondroprogenitors and subsequently chondroblasts (Akiyama et al, 2002; Smits et al, 2001). This follows with a change in ECM composition to be more cartilage-specific, with type IIb collagen and aggrecan becoming predominant (Knudson & Knudson, 2001; Ryan & Sandell, 1990). Further temporal cellular differentiation and maturation occur to produce chondrocytes, the primary cell type of cartilage.

Upon chondrocyte differentiation, two distinct events occur at this stage to the developing cartilage: bone tissue development through chondrocyte hypertrophy and subsequent differentiation (endochondral ossification), or establishment of permanent mature articular cartilage. During endochondral ossification, chondrocytes undergo hypertrophy as a result of Ihh and PTHrP signaling within ossification centres, which are located in the diaphysis (primary ossification centre) and epiphyses (secondary ossification centres) of a developing long bone (St-Jacques, Hammerschmidt, & McMahon, 1999). Collagen type X becomes highly expressed in the pericellular matrix of hypertrophic chondrocytes, and aids in promoting calcification of the surrounding matrix (Linsenmayer & Linsenmayer, 1995; Shen, 2005). Blood vessels then invade the developing tissue, infiltrating it with osteoblasts and driving the expression of osteogenic factors such as Runx2, eventually replacing the transient cartilage with bone (Chen et al, 2014; Clarkin & Olsen, 2010).

Stable articular cartilage arises from the “interzone” in the developing limb, which separates epiphyseal ossification centres and serves as the eventual location of the limb’s joint. As chondrification proceeds towards the ends of the developing long bone, the interzone arises from the remaining condensed mesenchymal cells, forming a tightly packed cellular region between the ossification centres (Figure 1, Pacifici et al, 2006). Cells in this region form three distinct layers: two outer chondrogenic layers that become the lining of the epiphyses of long bones, and a middle
layer that undergoes cavitation (Lamb et al, 2003). It is speculated that the two outer layers mark the transition zone between calcified cartilage and the newly formed subchondral bone, while the middle layer forms the mature articular cartilage (M. M. Ito & Kida, 2000).

**Figure 1.** Endochondral ossification and the development of permanent articular cartilage. Chondroprogenitors proliferate (P), mature (M), and undergo hypertrophy (H) in ossification centres in developing limbs (blue, purple and pink ovals). These “transient” chondrocytes contribute to the formation of bone. A subset of chondroprogenitors at the interzone (I) become “stable” chondrocytes, which line the epiphyses at the ends of developing long bones and form zonal mature articular cartilage. Adapted from Staines et al, 2013.

*Mature articular cartilage*

Once fully developed, stable cartilage is a cell-poor tissue, containing only 10% chondrocytes, with the balance of the tissue composed of matrix, primarily proteoglycans, collagen, and water. Cartilage components vary in their shape and distribution throughout the
tissue. This variation serves to compartmentalize hyaline cartilage into four distinct zones, delineated parallel to the bone surface: the superficial zone, mid-zone or transition zone, deep zone and calcified zone (Figure 2B and C). Chondrocytes have a flattened morphology and are present in a higher density in the superficial zone, and gradually get rounder and larger as they get closer to subchondral bone (Figure 2A).

The collagen network in the tissue is primarily responsible for the mechanical properties of cartilage. Articular cartilage is comprised of mostly type II collagen (with limited amounts of type IX and type XI) that is arranged in arches from the deep and calcified zones that creates an orthogonal pattern (Clark, 1985; Figure 2C). In the superficial zone, collagen is aligned parallel to the joint surface. Moving through the midzone, the fibers are more randomly arranged, but become perpendicular as they cross into the deep zone.

Proteoglycans are glycosylated proteins that consist of a core protein with covalently attached glycosaminoglycan (GAG) chains (Yanagishita, 1993). The most predominant glycoprotein in cartilage is aggrecan, whose protein core is covalently bound to chondroitin sulfate and keratan sulfate GAG chains (Kiani et al, 2002). Proteoglycans are dispersed within the matrix throughout the zones allowing for force dispersal over the subchondral bone through moderate compression and swelling of the tissue (Korhonen et al, 2003; Mow, Holmes, and Lai 1984).

The load-bearing properties of cartilage tissue depend on the distribution and ratio of collagens and proteoglycans in the matrix. The more parallel arrangement of collagen at the superficial zone is better adapted to tensile strength; as this arrangement becomes more perpendicular in the middle and deep zones, compressive strength increases (Sophia Fox, Bedi, & Rodeo, 2009). GAGs tend to absorb large quantities of water, whereas collagen limits the swelling ability of the tissue (Martel-Pelletier, Boileau, & Pelletier, 2008). The ratio of collagen to
proteoglycans modulates the osmotic swelling of the tissue, allowing for proper force distribution and lubrication through force-dependent fluid movement in and out of the cartilage (Broom & Poole, 1983). As such, proteoglycan content is higher in the deep zone than in the superficial zone, and collagen content is higher in the superficial zone than in the deep zone (Sophia Fox et al, 2009).

Figure 2. Location and composition of hyaline cartilage. A) Structure of a synovial joint. B) Chondrocyte organization throughout the zones of hyaline cartilage. C) Orthogonal orientation of collagen fibers within the tissue. (A: adapted and obtained with permission from http://www.mydr.com.au/sports-fitness/joint-synovial)

**Cartilage damage and repair in horses**

In horses, cartilage injuries account for over 50% of all lameness cases (USDA 2000). A reported 110 training days are lost upon lameness in a performance horse, equating to a substantial loss of income for the owner(s) and trainer(s) (USDA 2001). The leisure horse industry is greatly impacted as well, as steep veterinary costs and the required rehabilitation time are a significant limiting factor for current and future horse owners, who are likely to experience associated emotional distress (Quinn, 2005). Lameness in horses is associated with behavioural and
physiological changes, as the pain causing the protective gait/movement modifications directly impacts the stress response (Viñuela-Fernández et al, 2007). Though analgesics are helpful in managing these changes, they have various associated side effects that may also negatively impact the horse’s welfare (Sanchez & Robertson, 2014).

Modern competition places a great deal of stress on the horse’s joints. The forces applied to the joints during heavy jumping, galloping, sharp turns or advanced dressage movements can result in significant physical damage to the articular cartilage. Blunt, acute trauma or repeated microtrauma are the leading factors in the subsequent breakdown of cartilage tissue and the resulting joint inflammation and pain, also known as post-traumatic osteoarthritis (OA) (McIlwraith, Frisbie, & Kawcak, 2012).

Significant lameness can occur as a result of impact injury to the cartilage or subchondral bone (Hinchcliff, Kaneps, and Geor 2004). As self-healing of the tissue may not be successful (depending on the size of the lesion), lameness can persist long-term in the affected animal (Hurtig et al, 1988). Small defects (under 5 mm²) may undergo “intrinsic” or “extrinsic” endogenous healing. Intrinsic repair occurs in partial thickness defects affecting only the cartilage phase, and extrinsic repair occurs in full thickness defects with the subchondral bone being affected as well (Frisbie 2012). The intrinsic repair mechanism involves a phenomenon called “matrix flow”, where normally stable chondrocytes undergo proliferation and secrete some matrix components into the lesion space. Extrinsic healing occurs when progenitor cells from the bone marrow or vascularized subchondral bone can infiltrate the defect and form fibrocartilage (Fuller and Ghadially 1972, Shapiro, Koide, and Glimcher 1993). Full thickness defect repair through extrinsic healing was detected in the carpal and femeropatellar joints of horses with small defects (5 mm²) in weight bearing regions; these lesions healed significantly better than large (15 mm²) injuries in
non-weight bearing regions (Hurtig et al, 1988). However, there was no evidence of hyaline cartilage restoration to an injury site in the equine joint, which is necessary for long-term joint health and function.

It is this lack of self-repair that ultimately leads to post-traumatic OA. OA in horses occurs most frequently in the metacarpophalangeal (fetlock), carpal (knee), and tibiotalarsal and tarsometatarsal (hock) joints, and less frequently in the stifle and spine (Kidd, Fuller, & Barr, 2010). With repeated minor trauma to the joint, cartilage tissue homeostasis shifts to a degenerative profile of matrix components. Healthy cartilage maintains its architecture and biochemical composition through a balanced secretion of catabolic and anabolic factors by the residing chondrocytes. Cartilage erosion occurs when this balance is disrupted by abnormal joint loading or direct trauma (Goldring & Marcu, 2009). Chondrocytes are highly sensitive to changes in their chemical and mechanical environment; when trauma occurs, release of matrix-degrading enzymes and pro-inflammatory molecules by the cells directly contributes to tissue breakdown (Glyn-Jones et al, 2015). Cytokines such as IL-1 and TNF-α can be released by the chondrocytes themselves or the synovium and upregulate the expression of matrix metalloproteinases that degrade proteoglycans and collagen (Bondeson et al, 2006; Tetlow, Adlam, & Woolley, 2001). This degradation leads to lowered tensile strength and thinning of the cartilage layer, transmitting greater force to the underlying subchondral bone (Maldonado & Nam, 2013). Changes can be observed in the subchondral bone as well, with the development of osteophytes and bone marrow edema (Jacobson et al, 2008). These joint tissue changes can cause fibrosis and inflammation, which result in the pain and stiffness associated with OA.
**Horses as a model for human cartilage damage and treatment**

Research into pathologies and treatment of human cartilage disease conditions often utilizes animal models as a transition phase between *in vitro* studies and human clinical practice, as human tissue is often difficult to procure due to ethical restraints. Small animal models such as mice and rats are most commonly used as they are inexpensive and can be generated as transgenic or gene knockout animals, which allow for the exploration of direct or indirect genetic causes of cartilage-associated pathologies (Helminen *et al.*, 2002). However, while they are suitable models for studying OA pathology and development, their small joint size and relatively thin articular cartilage means it is difficult to study clinically relevant-sized defects. While larger animals such as rabbits, goats, pigs, and dogs have also been studied extensively as a model for cartilage repair (reviewed in Chu, Szczodry, and Bruno 2010), horses have been deemed the superior animal model for a number of reasons. First, they serve as the only model to spontaneously develop chondral injuries and/or post-traumatic OA from athletic pursuits to a similar extent as humans (Lacourt *et al.*, 2012) and traumatic joint injuries are well documented in the horse (McIlwraith *et al.*, 2012). Second, cartilage thickness in horses is similar to that in humans; 1.5-2 mm in horses in the stifle versus 2.2-2.5 mm in the human knee (Frisbie, Cross, & McIlwraith, 2006). In addition, arthroscopic techniques to both create and evaluate lesions can be performed and optimized in the horse joint prior to improving human protocols (McIlwraith, Wright, and Nixon 2014). Thus, cartilage repair protocols optimized in the horse followed by well-established movement rehabilitation practices for the treatment of equine joints may have an added benefit of translating to human medicine as well.
Current treatments

At present, there is no lasting cure for focal cartilage defects. Therefore, prevention of injury through preservation of joint health should be heavily considered throughout a horse’s working life and beyond. Appropriate exercise, weight management, balanced nutrition, good footing and responsible breeding are all important factors in reducing injury risk and managing any present symptoms. Should cartilage damage occur, a number of treatment options are available to alleviate pain and/or stimulate cartilage repair, at least in the short term.

Surgical treatments

Debridement and microfracture are commonly used together to clear out necrotic and fibrous tissue and encourage healing. After the joint is cleared of damaged and calcified cartilage, small holes are then drilled or bored into the subchondral bone, promoting repair through creation of a blood clot and infiltration of mesenchymal stromal cells from bone marrow. In humans, this technique is indicated for small cartilage lesions (<2 cm) that have little to no involvement of subchondral bone (Knutsen, 2007). In horses, microfracture has been shown to promote greater tissue filling with repair tissue that contains more type II collagen than non-microfracture-treated tissue (Frisbie et al, 1999), though with lower GAG accumulation than native cartilage (Frisbie et al, 2003). The repair tissue is thus more fibrous than hyaline in composition and does not fully restore joint function (Frisbie et al, 2006). While more effective at reducing pain than no treatment, microfracture was less effective than treatment with defect implants in some studies. Custers and colleagues (2009) demonstrated that microfracture yielded inferior repair tissue with lower proteoglycan content and more degeneration than in defects treated with ceramic implants in goats (Custers et al, 2009). Human studies have provided mixed outcomes. Using activity level as a
measure of improvement, Krych and colleagues (2012) retrospectively evaluated patients treated with mosaic arthroplasty or microfracture, and found that mosaic arthroplasty-treated patients maintained a superior level of athletic activity (Krych et al, 2012). In addition, reported pain and disease progression occurred less frequently in mosaic arthroplasty-treated patients versus microfracture in a 10-year comparison study (Gudas et al, 2012). However, when assessed through patient-reported outcomes, muscle strength, and radiological outcomes, no significant differences were found between microfracture and mosaic arthroplasty (Ulstein et al, 2014).

Mosaic arthroplasty involves the surgical removal of an osteochondral “plug” from a less weight bearing area of the joint and implanting it into the defect site. Cartilage and bone cylinders of varying diameters are cored out of the donor site, then press-fitted into the defect (Hangody et al, 1998). This one-operation procedure is effective in replacing damaged or missing cartilage with healthy hyaline cartilage already anchored to subchondral bone. Provided the plug has been fitted correctly (level with the joint surface), the donor cartilage will eventually integrate with the surrounding cartilage, and the surrounding bone will infiltrate and integrate with the donor bone (Hangody et al, 2008).

Mosaic arthroplasty was pioneered in horses in 2004 as a method to treat subchondral bone cysts. Out of 11 horses treated, 10 had successful graft incorporation and 7 returned to a similar or higher level of performance (Bodo et al, 2004). Limitations to this treatment include the high risk of donor site morbidity, which increases with the number and/or size of plugs harvested (Laprade & Botker, 2004). As well, since these plugs are harvested from non-weight bearing areas of the joint, cartilage matrix composition and mechanical properties in these sites is different and may not hold up to loads applied to the repair site (Esquisatto, Pimentel, & Gomes, 1997). Autografts from cadaver joints circumvent these limitations, but bring their own in the form of
immunogenicity complications and sourcing/storing allografts (Boscainos, Gross, & Kellett, 2008).

Autologous or allogeneic chondral fragments have been recently introduced as an alternative to the above techniques for use in cartilage defect repair. Cartilage autograft implantation system (CAIS) involves harvesting chondral fragments from a low weight-bearing area of the joint then mincing the cartilage into small pieces (1-2 mm). The pieces are then suspended in a biodegradable scaffold, then injected into the defect site (Cole et al, 2011). This technique was first explored in vivo in a rabbit model. Autologous chondral fragments were suspended in a fibrin glue scaffold and injected into induced osteochondral defects. Compared to the untreated group, chondrocyte proliferation was observed along with deposition of hyaline-like cartilage matrix within the defect (Albrecht, Roessner, & Zimmermann, 1983). In horses, CAIS was compared to autologous chondrocyte implantation (reviewed below) to treat clinical-sized defects (15 mm in diameter). Both techniques resulted in superior tissue regeneration compared to empty defects when evaluated arthroscopically, histologically, and immunohistochemically, with CAIS having the highest overall score for all parameters tested (Frisbie et al, 2009). In humans, CAIS outperformed microfracture in a randomized controlled trial in knee function, reduced pain and stiffness, sports and recreational activities and knee-related quality of life (Cole et al, 2011).

Particulated juvenile allograft cartilage (PJAC) is similar to the CAIS technique but uses allogeneic fragments from young donors (usually younger than 2 years) (Adams et al, 2011). Clinical results for this treatment modality in humans are promising, with good patient-reported scores as well as histological scores (Buckwalter et al, 2014; Coetzee et al, 2013; Farr et al, 2014). Only one research paper has described the results of this technique in an animal model. Following the creation of large defects in the femoral trochlea, adult rabbits were treated with adult cartilage
fragments, juvenile cartilage fragments, or a combination of both. Both juvenile alone and juvenile + adult treatment groups performed better than untreated defects, with juvenile fragments contributing to a higher histological score (Bonasia et al, 2011).

Arthritis-ravaged joints causing severe pain may require total joint replacement. Hip and knee replacements are one of the most common procedures performed by orthopedic surgeons, totalling more than 100,000 surgeries in Canada per year (CJRR, 2015). Joint replacements are most effective at alleviating pain, while adequately restoring joint function (Carr et al, 2012). However, revision surgery is necessary in ~2.5% of cases after 3 years, typically as a result of a weakening bond between the implant and the patient’s bone (CJRR, 2015). Failure rates are estimated to occur at approximately 1% per year (Losina et al, 2004). Thus, total joint replacements are contraindicated for young patients, who will require multiple replacements over their lifetime. At present, total joint replacements are not performed in horses with severe arthritis.

Cell-based treatments

Though microfracture and autograft/allograft transplantation have shown some success in restoring short-term joint function, they fail to restore the full integrity of the articular cartilage at the defect site. This limited tissue regeneration has prompted investigation into new healing modalities using cell-based approaches to stimulate and contribute to cartilage repair.

Autologous chondrocyte implantation

Autologous chondrocyte implantation (ACI) has been increasingly used to treat large focal defects for over the past 15 years (Brittberg et al, 1994; Erggelet, Sietinger, & Lahm, 2003; Peterson et al, 2000). ACI is indicated for larger lesions (normally greater than 2 cm² but less than 10 cm²) in patients with deep or full thickness defects of the femoral condyles, trochlea, and patella.
This technique is a two-stage process, involving the removal of a small amount of healthy cartilage, culture-derived expansion of chondroprogenitor cells, and the subsequently re-implantation into the defect. Following arthroscopic assessment of the lesion, cartilage is harvested from a donor site, typically a less load-bearing region of the joint, then processed through enzymatic digestion, releasing the primary chondrocytes. The chondrocytes are then cultured for a few to several weeks to attain the appropriate number of cells (Minas & Peterson, 1999). These cells are subsequently re-implanted into the injury site, on their own or within a collagen scaffold (matrix-associated ACI) (Behrens et al, 2006; Brittberg et al, 1994). Traditionally, ACI was performed by injecting the chondrocytes underneath a periosteal flap sutured to the surrounding healthy cartilage to minimize cell loss (Brittberg et al, 1994). This commonly resulted in graft hypertrophy, where the repair tissue grew thicker than the adjacent native cartilage (Kreuz et al, 2007; Niemeyer et al, 2008). While graft hypertrophy is still observed in matrix-associated ACI (which does not utilize a periosteal flap), it is observed less frequently, and may not be associated with a negative clinical outcome (Ebert et al, 2015; Pietschmann et al, 2012).

ACI has also proven successful in the equine model. ACI promotes better defect infilling and more hyaline-like repair tissue than untreated defects in the horse (Frisbie et al, 2008; Litzke et al, 2004). In addition, ACI leads to improved matrix organization and significantly higher proteoglycan content in partial thickness defects 8 weeks post-implantation (Nixon et al, 2011). Matrix-associated ACI (MACI), with chondrocytes seeded within a collagen sponge, also showed improvements over untreated and collagen sponge alone; treated defects contained tissue with increased stiffness and but with an inferior shear modulus (measuring tensile strength) (Griffin et al, 2015).
While ACI is considered to be a superior strategy to microfracture in the treatment of larger lesions (Oussedik, Tsitskaris, & Parker, 2015), mosaic arthroplasty is reported to outperform ACI (Dozin et al., 2005; Horas et al., 2003). In cases where subchondral bone loss is <6-8 mm², bone grafting may also be required (Bedi et al., 2010). In addition, chondrocyte isolation and expansion is not as straightforward as with other cell types. As only a limited number of cells can be isolated from the donor cartilage tissue, they must be propagated in culture to obtain the desired number of cells to fill the defect. Chondrocytes tend to dedifferentiate in monolayer expansion culture, and their resulting phenotype after passaging no longer contributes to hyaline cartilage formation; the cells upregulate type I collagen and downregulate matrix molecules such as type II collagen and aggrecan (Cheng et al., 2011; Darling & Athanasiou, 2005). Thus, progenitor cells with better expandability are also under investigation to treat cartilage defects.

Mesenchymal stromal cell therapy

Mesenchymal stromal cell (MSC) therapy is the most recently developed regenerative therapy conducted for cartilage lesions, with large animal studies and clinical trials showing varying degrees of success (reviewed in Bornes, Adesida, and Jomha 2014). MSCs are multipotent immunomodulatory cells derived from the stroma of mesenchymal-derived tissue that are capable of proliferating and differentiating into adipocytes, osteocytes and chondrocytes. As they can be easily isolated from a number of tissue sources, MSCs have emerged as especially promising therapeutic candidates to treat a number of diseases.

Numerous groups have examined the potential of MSCs in the treatment of osteoarthritis and cartilage regeneration in animal models. Caminal and colleagues (2014) reported that in sheep, the injection of MSCs resulted in some improvement of tissue filling, colour and cell distribution
in the repair tissue of induced cartilage defects (Caminal et al, 2014). Similarly, in horses, modest improvement was seen in defect repair with respect to clinical and histological parameters after MSC injection (Frisbie et al, 2009).

The use of scaffolds to encapsulate the MSCs prior to implantation into the defect has also shown some success. Collagen scaffolds are commonly used as they can be prepared as gels or sponges of various stiffness, providing a strong and stable matrix for encapsulating MSCs (Glowacki & Mizuno, 2008). While hyaluronic acid (HA) has reduced strength when compared to collagen scaffolds, it is another popular option as it is a naturally occurring GAG in cartilage (Hardingham & Muir, 1972). Platelet-rich fibrin glue is also used, providing growth factors to aid in MSC differentiation and preventing fibroblast infiltration of the graft (Anitua et al, 2006). Liu and colleagues (2006) demonstrated that MSCs seeded within a synthetic HA extra-cellular matrix formed superior repair tissue and enhanced integration than MSCs seeded alone in rabbits (Liu, Shu, & Prestwich, 2006).

In humans, preliminary studies suggest MSC treatment is both safe and effective. Bone marrow stromal cells isolated from a patient with a full thickness defect in the femoral condyle, expanded for 4 weeks, then embedded in a collagen gel and re-implanted into the defect. The patient returned to his previous activity level one year after surgery (Kuroda et al, 2007). Treatment was also effective in the patella, with clinical improvement in all patients following MSC transplantation (Wakitani et al, 2004; Wakitani et al, 2007).

When compared to ACI, MSC therapy appears to be equally as effective in regards to eliminating symptoms and restoring function. Patients with matched lesion size and age were treated with ACI/MACI or MSC therapy, and no statistically significant differences were found in the clinical outcomes of either treated group (Gobbi et al, 2014; Nejadnik et al, 2010). While more...
research and clinical trials need to be performed to interpret the potency of MSC therapy for osteoarthritis and/or cartilage repair, the current literature indicates it as a safe and effective alternative to ACI.

**Biology and sources of MSCs**

**Biology of MSCs**

In 2006, the International Society for Cellular Therapy outlined specific criteria for the definition of human MSCs: the cells must be plastic-adherent, express a specified panel of surface markers, and be capable of differentiating into osteoblasts, adipocytes and chondrocytes *in vitro* (Dominici *et al*, 2006). Isolation of MSCs is usually performed by the selection of spindle-shaped cells that attach to tissue culture plastic (Friedenstein, Chailakhjan, & Lalykina, 1970). Repeated passaging of the adherent cells depletes any trypsin-insensitive hematopoietic cells in culture, leaving a mostly homogenous population of putative MSCs (Hsiao *et al*, 2010; Sun *et al*, 2003).

MSCs are fibroblastic in morphology, and express a panel of MSC-specific surface markers. In humans, this panel is as follows: CD73$^{\text{high}}$, CD90$^{\text{high}}$, CD105$^{\text{high}}$, CD45$^{\text{low}}$, CD34$^{\text{low}}$, CD14$^{\text{low}}$ or CD11b$^{\text{low}}$, CD79a$^{\text{low}}$ or CD19$^{\text{low}}$, and HLA-DR$^{\text{low}}$ (Dominici *et al*, 2006). In horses, the surface marker panel for MSCs has not been clearly established. Expression of CD29, CD44, and CD90 is mostly consistent, while CD73 and CD105 expression varies across reports and MSC sources (De Schauwer *et al*, 2012; Paebst *et al*, 2014; Ranera *et al*, 2011; Tessier *et al*, 2015).

An important characteristic of MSCs (and any stem/progenitor cell) is their capacity for self-renewal. Self-renewal refers to cell division while maintaining an undifferentiated state. In MSCs, various factors such as leukemia inhibitory factor (LIF), fibroblast growth factor (FGF), and mammalian wingless/integrated (Wnt) actively suppress differentiation and promote self-
renewal (Boland et al, 2004; Jiang et al, 2002; Lee et al, 2015; Nasef et al, 2008). LIF is a potent inhibitor of differentiation in embryonic stem cells, and has been implicated in the self-renewal capacity and immunosuppression of MSCs, although details of its mechanism of action remain unknown (Jiang et al, 2002; Nasef et al, 2008). FGF dually stimulates the proliferation of MSCs and inhibits their differentiation over several passages (Lee et al, 2015). FGF induces the expression of Twist2 and Spry4, involved in the inhibition of osteogenesis and the MAPK pathway (crucial for Sox9 activation), respectively, thus possibly interfering with subsequent MSC differentiation (Bhaskar et al, 2014; Lai, Krishnappa, & Phinney, 2011). Similarly, Wnt3a treatment in MSCs increases their proliferation rate and inhibits osteogenic differentiation (Boland et al, 2004).

MSC differentiation potential has been most extensively studied with regards to adipogenesis, osteogenesis, and chondrogenesis. However, differentiation into other cell types such as muscle, tendon/ligament, and even cells of ectoderm and endoderm lineage have also been reported (Jadalannagari et al, 2015; Schwartz et al, 2002; Wakitani, Saito, & Caplan, 1995).
In addition to their differentiation abilities, MSCs are also capable of modulating several components of the immune system. They have been shown to suppress the immune response through attenuation of T-cell proliferation (Le Blanc et al., 2003), implying that allogeneic MSCs could be used to treat inflammatory conditions such as graft versus host disease (GvHD) and autoimmune diseases in addition to arthritis. In addition, they have been shown to interact with other members of the immune system, such as granulocytes, B cells, and natural killer cells (Brandau et al., 2010; Corcione et al., 2006; Spaggiari et al., 2008). This interaction and mechanism of immunosuppression is not completely understood, but may involve both cell-to-cell contact (perhaps through the activation of the PD-1 molecule [Augello et al., 2005]) and the secretion of anti-inflammatory cytokines such as IL-10, IL-6, TGF-β, and various chemokines [Kyurkchiev, 2014]). These features make MSCs attractive candidates for both tissue engineering and cellular therapy.
Sources of MSCs

Over recent years, the derivation of equine MSCs from a multitude of tissue sources has been reported, although bone marrow is by far the most extensively studied (Smith et al, 2003). Alternative sources of MSCs include adipose tissue, umbilical cord tissue, umbilical cord blood, muscle tissue, periosteum, and peripheral blood (Koch et al, 2007; Koerner et al, 2006; Radtke et al, 2013; Vidal et al, 2008).

All sources have advantages and disadvantages when it comes to isolation, proliferation, and tissue regeneration or differentiation capacity. Bone marrow MSC (BM-MSC) isolation is invasive; bone marrow aspirate is typically taken from the sternum, requiring sedation (Smith et al, 2003). MSCs derived from adipose tissue (AT-MSCs) are isolated from fat pads beside the tail head (Toupadakis et al, 2010), and require an additional tissue digestion step to release the cells. Peripheral blood MSC (PB-MSC) isolation is comparatively less invasive. However, the number of MSCs circulating in peripheral blood is typically low (Chong et al, 2012). BM-MSC, AT-MSC and PB-MSC isolation are performed on adult horses of all ages; however, proliferation and differentiation potential of the MSCs decrease with increasing donor age (Choudhery et al, 2014; Stolzing & Scutt, 2006). Umbilical cord tissue and cord blood derived MSCs (UT-MSC and CB-MSC, respectively) are isolated from neonatal tissue, and the resulting cell populations typically demonstrate higher proliferation rates and a greater capacity for self-renewal compared to MSCs isolated from adults (Baksh, Yao, & Tuan, 2007; Zhang et al, 2011). Multilineage differentiation and surface marker expression has been reported in equine MSCs from all sources (Koch et al, 2007; Koerner et al, 2006; Radtke et al, 2013; Tessier et al, 2015; Vidal et al, 2008).

Donor variation
Variability in both proliferation and differentiation between donors represent significant issues in the use of MSCs therapeutically. Large variations in cell yield and alkaline phosphatase activity were first were observed decades ago in human MSCs (Castro-Malaspina et al, 1980; Jaiswal et al, 1997; Majors et al, 1997). More recently, interspecific donor variation in proliferation and osteogenic potential has also been reported (Phinney et al, 1999; Siddappa et al, 2007). Donor variation has also been observed in equine MSCs from middle-aged horses (Carter-Arnold et al, 2014). Disease status of the donors may also affect MSC function (Escacena et al, 2015).

**Biology of iPSCs**

While MSCs are extremely valuable and well-studied in the field of orthopedic regenerative medicine, these cells are limited in their self-renewal capacity and their ability to differentiate into multiple tissues. Embryonic stem cells are pluripotent and thus are extremely useful to study all tissue types. However, there are no reports on true equine embryonic stem cell lines being derived from the blastocyst, as derivation of these cells has proved to be challenging (Li et al, 2006; Saito et al, 2002). Thus, researchers turned to the strategy of generating equine induced pluripotent stem cells for a pluripotent cell type for use in developmental studies and cell-based therapies in the horse.

*Biology of induced pluripotent stem cells*

Induced pluripotent stem cells (iPSCs) were originally created by Shinya Yamanaka’s group in 2006. By enforcing the expression of only four transcription factors, Oct4, Sox2, Klf4, and c-Myc, Yamanaka’s research team was able to reprogram mouse and human fibroblasts into
cells having embryonic stem cell characteristics, including self-renewal, expression of pluripotency genes, and the ability to differentiate down all three embryonic lineages (mesoderm, ectoderm and endoderm) (Takahashi & Yamanaka, 2006; Takahashi et al, 2007). This pivotal discovery essentially opened a new field of stem cell research, spurring the development of iPSC labs and consortiums of research teams all over the world. iPSC research generally falls into two categories: differentiation of iPSCs for disease modeling or cell replacement therapy, or improving reprogramming techniques to increase efficiency and efficacy.

iPSCs can theoretically be induced to differentiate into any cell type derived from the embryo proper. Therefore, disease modeling can be achieved by generating iPSCs from fibroblasts or disease-specific cells from an affected patient, or by genetically altering healthy iPSCs. There are a number of articles reporting on the application of iPSCs for human disease modeling, which include familial dysautonomia, long QT syndrome, and Parkinson’s disease (Hargus et al, 2010; Lahti et al, 2012; G. Lee & Studer, 2011). Additionally, several reviews have been published in the last year on the use of iPSCs for treatment of ischemic stroke, pediatric heart disease, neurological diseases, pancreatic β-cells, and spinal cord injuries (Ito et al, 2012; Lepski, 2012; Lukovic et al, 2012; Zhu et al, 2012).
Equine induced pluripotent stem cells

In 2011, Nagy and colleagues reported the first generation of an equine iPSC cell line (Nagy et al, 2011). These cells were derived from equine fetal fibroblasts using the piggyBac (PB) transposase system. The PB transposase system involves transfecting cells with a construct containing reprogramming factors (those previously identified by Yamanaka; c-Myc, Klf4, Oct4, and Sox2) flanked by inverted terminal repeats. The transient expression of the PB transposase is sufficient for the excision and integration of the construct anywhere within the genome (Woltjen et al, 2009). Addition of a doxycycline-inducible promoter upstream of these factors provides an
additional layer of control over the reprogramming event. Of particular importance, equine iPSCs display pluripotency characteristics, including the formation of embryoid bodies and the ability to form teratomas in vivo containing mesoderm-, endoderm-, and ectoderm-derived tissues.

Since Nagy et al.’s (2011) study, several other groups have generated equine iPSC lines. Two papers published in 2012 described the generation of equine iPSCs from non-fetal fibroblasts (Breton et al., 2012; Khodadadi et al., 2012). Breton et al. reprogrammed newborn fibroblasts using a Moloney murine leukemia virus (MMLV)-based integration method of the four Yamanaka factors. The resulting iPSCs expressed pluripotency markers, formed embryoid bodies in vitro, and teratomas in vivo. They also reported the reprogramming of fibroblasts from a 2-year old horse, demonstrating an upregulation of pluripotency-associated genes and surface proteins, as well as embryoid body formation (Breton et al., 2012). Adult fibroblasts (age and origin were not reported) were also used by Khodadadi and colleagues (2012) for retroviral-based iPSC generation without the use of c-Myc, which is a proto-oncogene (Nilsson & Cleveland, 2003). They also demonstrated an upregulation of some pluripotency markers as well as embryoid body and teratoma formation (Khodadadi et al., 2012).

The equine iPSCs described above were reported to require both bFGF and LIF supplementation to maintain pluripotency. Whitworth and colleagues (2014) were successful at generating iPSCs from one adult horse using lentiviral-based reprogramming that were not dependent on bFGF for maintenance of pluripotency. While LIF-only cultures proliferated at a slower rate, evidence (in the form of XIST expression as well as decreased H3K27 methylation) indicates that the iPSC colonies were in a more naïve state and thus more closely resembled embryonic stem cells (Whitworth et al., 2014).
With equine fibroblast to iPSCs reprogramming established, researchers then investigated the reprogramming of other equine cell types and their directed differentiation for further use in disease modeling and cell therapy. Sharma and colleagues (2014) reprogrammed equine primary keratinocytes into iPSCs via retroviral induction with the Yamanaka factors, and were successful in culturing the cells under feeder-free conditions. Following reprogramming, they differentiated the cells into functional neurons, as evidenced by their ability to generate action potentials (Sharma et al, 2014). Lee and colleagues (2016) used equine adipose-derived stem cells for lentiviral-based iPSC generation, then injected the reprogrammed cells into Rag/mdx mice following muscle injury. Mice who received injected iPSCs had improved muscle tissue healing through the generation of myofibers by the iPSCs (Lee et al, 2016).

Using the equine iPSCs generated by Nagy and colleagues (2011), our group generated MSC-like cells using a non-embryoid body-based differentiation protocol. These MSC cells were plastic adherent, expressed MSC markers, and were capable of adipogenic and osteogenic differentiation (Lepage et al, 2016). Other work by Aguiar and colleagues (2016) demonstrated that equine iPSCs could be differentiated to keratinocytes that had increased migratory capacity compared to primary keratinocytes (Aguiar et al, 2016).

Finally, Quattrocelli and colleagues (2016) investigated the myogenic and chondrogenic differentiation capacity of equine iPSCs derived from myogenic mesoangioblasts (MAB) and MSCs from peripheral blood. They demonstrated that both cell populations generated iPSCs that could form teratomas. However, while MAB-derived iPSCs differentiated more readily into myocytes and myotubes, MSC-derived iPSCs were reported to have more chondrogenic potential (Quattrocelli et al, 2016). However, chondrogenic pellets generated from both MAB-derived iPSCs and MSC-derived iPSCs stained weakly for proteoglycans, and did not have any cell
lacunae, consistent with our failed attempts at differentiating equine iPSCs into chondrocytes (Lepage et al, 2016).

**Cartilage tissue engineering**

As the long term outcome following surgical repair of cartilage lesions remains unsatisfactory due to the formation of fibrocartilage, *in vitro* cartilage engineering have been considered for treatment as a means to generate hyaline cartilage for defect restoration. Tissue engineering is complex with each aspect of the “triad of tissue engineering”, cells, scaffold/matrix material, and signaling factors, requiring careful optimization to generate implant quality tissue (Risbud & Sittinger, 2002). In cartilage tissue engineering a fourth parameter, mechanical stimulation, must also be taken into consideration.

*Choice of cells*

**Chondrocytes**

As the exclusive cell type of cartilage, chondrocytes are an obvious cell source for generating *in vitro* tissue. Chondrocytes are capable of producing hyaline cartilage when cultured in 3D pellet or membrane culture, which share many characteristics with native hyaline cartilage in terms of proteoglycan content, deposition of type II collagen, and lack of mineralization/hypertrophy (Zhang et al, 2004). However, as chondrocytes tend to dedifferentiate into fibroblast-like cells during routine cell expansion (Cancedda et al, 2003), they may not be the optimal choice for scaled-up, routine tissue engineering. Efforts are under way and have shown promise in the redifferentiation of chondrocytes for the purposes of tissue engineering (Ahmed et al, 2010; Meretoja et al, 2014).
MSCs

MSCs can readily differentiate into cartilage tissue and are thus a suitable cell source for generating cartilage. As with chondrocytes, MSCs can be differentiated into cartilage tissue in 3D pellet culture using defined media. Because they are more easily expandable and exhibit restricted self-renewal, MSCs have an advantage over chondrocytes in that they can be propagated to large numbers over several passages and retain their chondrogenic potency. However, optimal isolation conditions as well as culture conditions for MSC differentiation into implant-grade hyaline cartilage have yet to be determined.

iPSCs

While MSCs are more easily expandable, iPSCs are capable of unlimited self-renewal and pluripotent differentiation, and are thus an attractive alternative source for tissue engineering. In addition, as iPSCs are phenotypically similar to embryonic stem cells, they can provide a platform for looking at chondrogenesis early in development. However, achieving uniform differentiation from iPSCs populations is difficult (Yoshida & Yamanaka, 2010) and thus currently hampers their use for routine cartilage tissue generation.

Types of scaffolds

Scaffolds, typically made of polymer-based materials, are used in tissue engineering to encourage cell attachment and subsequent tissue differentiation in a 3D structure. Scaffolds provide a temporary ECM for cell proliferation, migration, signaling, and also mechanical properties such as rigidity and elasticity. Scaffolds should be made of a material that will degrade at a matched rate to the deposition of nascent ECM by the proliferating cells seeded within the scaffold. Natural scaffolds include fibrin, alginate/agarose, collagen and hyaluronan. All have been used extensively to serve as both a delivery vehicle and stand-alone ECM substitute for cartilage
tissue engineering. Fibrin glue has been used in horses for the treatment of focal defects using both chondrocytes and MSCs, however, morphology and mechanical strength of the repair tissue was inferior to that of native cartilage (Hendrickson et al, 1994; Wilke, Nydam, & Nixon, 2007). Both collagen and hyaluronan scaffolds have also been investigated for equine chondrocyte proliferation and tissue regeneration (Nurnberger et al, 2013; Sams & Nixon, 1995).

Synthetic scaffolds such as polyglycolide (PGA), polylactides (PLA, PDLA), and polycaprolactone (PCL) tend to have higher mechanical strength and a more controllable rate of degradation. However, synthetic polymers are not as biocompatible as natural materials, and are less able to support cell to cell signaling (Borzacchiello et al, 2011).

**Scaffold-free**

Developing a scaffold that mimics the stiffness and biocompatibility of native cartilage ECM is very complex, especially when the difficulty in matching the rate of degradation to the rate of nascent ECM deposition is taken into consideration. Therefore, scaffold-free strategies for engineering cartilage have been explored, which eliminate the requirement for exogenous framework. In scaffold-free systems, only cells contribute to ECM deposition, mirroring fetal cartilage development. Cell sheets have been used recently to engineer neocartilage for defect repair. MSCs or chondrocytes are expanded in monolayer to high confluency until they have grown sufficient ECM, and then the sheet is dissociated from the plastic as a whole (Sato et al, 2014; Zhou et al, 2015). Multiple sheets can be grown and harvested, then subsequently layered, rolled, and/or folded in a process known as tissue fusion or integration (Pérez-Pomares & Foty, 2006). Sheets derived from expanded juvenile allogeneic chondrocytes are currently in Phase III clinical trials (McCormick et al, 2013).
High density 3D cultures can also be used for scaffold-free cartilage engineering. The classic method of cartilage engineering is to employ a centrifugation step to generate a pellet. Once aggregated into pellets, the cells then deposit ECM (Johnstone et al., 1998). The centrifugation step mimics the condensation event early in fetal cartilage development, allowing for increased cell-to-cell contact and signaling down the chondrogenic pathway (Tacchetti et al., 1992). This condensation step can also be performed by seeding chondrocytes (or MSCs) at a high density in a three-sided mold. Membrane culture, shown to be superior over pellet culture for MSC chondrogenesis (Co, Vickaryous, & Koch 2014; Lee et al., 2011), allows for MSC attachment and subsequent differentiation into cartilage disks in 6 mm² cell culture inserts. Cells can also be seeded directly onto bone substrates to generate osteochondral constructs in a process similar to membrane culture (Guo et al., 2004; Kandel et al., 2006; Lee et al., 2011).

**Signaling factors**

Chondrogenesis from MSCs or chondrocyte precursors is regulated by several factors at various stages of cartilage development. In tissue engineering, recapitulating chondrogenic signaling pathways is essential for the generation of mature tissue. One particularly prominent growth factor involved in chondrogenesis is FGF2. Exogenous application of FGF2 has been shown to increase MSC proliferation, essentially “priming” them for chondrogenesis (Handorf & Li, 2011; Solchaga et al., 2005; Stewart et al., 2007). While the addition of FGF2 to cultures for expanding MSCs is beneficial to promote chondrogenesis, maintained supplementation throughout induction has been shown to decrease chondrogenic potential (Weiss et al., 2010).

Classic chondrogenic induction media contains dexamethasone, ascorbic acid, and TGFβ (Johnstone et al., 1998). Dexamethasone increases the accumulation of GAGs and type II collagen by BM-MSCs (Derfoul et al., 2006; Shintani and Hunziker 2011; Stewart et al., 2008). Ascorbic
acid is a necessary cofactor for collagen synthesis (Schwarz, Kleinman, & Owens, 1987). Different TGFβ isoforms have been used successfully to induce chondrogenesis from equine MSCs, with most investigators using either TGFβ1 or TGFβ3 (Co et al, 2014; Kisiday et al, 2008; Vidal et al, 2008; Worster et al, 2000). In particular, TGFβ3 has been shown to induce more GAG accumulation and type II collagen deposition than TGFβ1 in human MSCs (Barry et al, 2001). The length of time in which differentiating MSCs require TGFβ3 supplementation to continue chondrogenesis is not well understood. Gupta and colleagues (2015) showed that continuous TGFβ3 exposure in human MSCs cultured in carboxymethylcellulose hydrogels (20x10^6 cells/ml) for 8 weeks yielded tissue with higher levels of proteoglycans, higher equilibrium modulus, but with greater hypertrophy (Gupta & Nicoll, 2015). Huang and colleagues (2009) demonstrated that MSCs seeded in agarose at 20x10^6 cells/ml and 60x10^6 cells/ml for 7 weeks did not show a difference in mechanical or biochemical properties in either cell density group with continuous or transient (3 weeks) TGFβ3 exposure. However, they did note increased cell hypertrophy in the 60x10^6 cells/ml group with transient TGFβ3 exposure, and when repeated with pellet cultures, the transient group had higher GAG accumulation (Huang et al, 2009).

**Mechanical stimulation**

In addition to the correct combination of cells, scaffolds, and signaling factors, hyaline cartilage requires some degree of mechanical loading to achieve the level of shear and compressive strength necessary to withstand the forces applied to a joint. The collagen network in native cartilage is organized in such a way that it provides a gradient of tensile strength to compressive strength from the superficial aspect of the tissue to the subchondral bone. Mechanical loading during tissue engineering has been shown to influence the collagen alignment of generated neocartilage (Khoshgoftar, van Donkelaar, & Ito, 2011). Compressive mechanical forces have
been shown to enhance chondrogenesis through increased expression of Sox9, type II collagen, and aggrecan (Takahashi et al, 1998). In addition, greater collagen synthesis and a greater compressive modulus are achieved with intermittent compressive loading (Waldman et al, 2004).

All four of these parameters must be considered in some capacity when designing protocols for cartilage tissue engineering. As more research is done on these factors in isolation or in combination, we will be able to pinpoint the optimal combination and sequence of these elements to reproducibly generate hyaline-like cartilage.

**Osteochondral construct engineering**

As mentioned above, mosaic arthroplasty is a successful method for repairing focal cartilage defects. However, with limitations such as donor site morbidity, plug size, and/or sourcing appropriate allografts, an alternative must be sought after. Tissue engineering of biphasic bone and cartilage constructs is now being investigated for *in vivo* use to treat focal defects.

*Biphasic osteochondral constructs*

**Cartilage phase**

The cartilage layer of the engineered osteochondral construct may be generated using a scaffold or in a scaffold-free manner, as reviewed above. The tissue may be developed separately from the bone phase then layered onto the substrate, or induced to form tissue directly onto the bone substitute.

**Bone phase**

For the purposes of *in vivo* work, the bone phase is typically a cell-free scaffold, implanted with the notion that endogenous osteoblasts will infiltrate and deposit ECM that will eventually
replace the substrate as it disintegrates. Several materials have been studied to serve as a bone substitute for osteochondral construct generation. Scaffolds for the bone phase should have a highly porous structure mimicking cancellous bone and have the appropriate mechanical strength to withstand heavy loads. In addition, the material must be able to fuse with a softer tissue such as cartilage, providing a surface suitable for uniform cell attachment (Bal et al, 2010). It must also be biodegradable, preferably at a rate that matches native tissue remodeling, and not elicit an immune reaction.

Calcium phosphates such as hydroxyapatite, tricalcium phosphate (TCP), and calcium polyphosphate (CPP) are widely used in bone tissue engineering. These ceramics have excellent osteoconductive and osteoinductive properties, promoting cell ingrowth and proliferation leading to tissue regeneration and integration (Hutmacher, 2000). Hydroxyapatite is most widely used in bone regeneration because its composition is closest to that of native bone (Yoshikawa & Myoui, 2005), however, it has reduced osteoinductive properties due to its reduced solubility (Ogata et al, 2005). TCP comes in two crystal structures, α and β, and is considered to be both osteoconductive and osteoinductive, and is less stable in aqueous environments than hydroxyapatite (Ducheyne, Radin, & King, 1993). CPP is highly osteoconductive, as implantation of the material into cancellous bone resulted in up to 32% bone ingrowth into the pores of the CPP (Pilliar et al, 2001). It has been shown to support MSC proliferation to a greater extent than hydroxyapatite/TCP or morselized cancellous bone, and to induce osteoblastic differentiation as well (Comeau et al, 2011).

Generating biphasic constructs

Several groups have successfully generated osteochondral constructs in vitro and/or in vivo. Guo and colleagues (2004) implanted tissue engineered plugs into induced defects in sheep, and
found that the repair tissue completely resurfaced the defect after 6 months. The constructs were made by seeding chondrocytes on a β-TCP scaffold, which showed some degradation in vivo after 6 months (Guo et al, 2004). A similar study was performed in 2006, where sheep chondrocytes were instead seeded onto a CPP bone substitute and fitted into induced defects. The tissue engineered cartilage stained positively for toluidine blue, had similar proteoglycan and collagen content to native cartilage, and an equilibrium elastic modulus that increased over time up to 9 months (Kandel et al, 2006).

Follow up work was published by the same group in 2011, where in vitro constructs were generated using MSC-derived chondrocytes seeded on CPP. The BM-MSCs were pre-differentiated in pellet or membrane culture for 2 to 3 weeks, then dissociated from their newly formed matrix to be seeded on top of the CPP scaffold. The resulting cartilage tissue had intense type II collagen staining and proteoglycan content similar to native cartilage, but a lower compressive modulus (Lee et al, 2011).

Porous hydroxyapatite scaffolds have also been used for osteochondral tissue engineering. Kitahara and colleagues (2008) isolated chondrocytes from rabbits, cultured them in alginate beads, then released the cells to seed the top of a hydroxyapatite block. The construct was cultured in vitro for 4 weeks, then implanted into nude mice. After 4 and 8 weeks of culture, the tissue stained positively for toluidine blue, however, the proteoglycan content decreased from 4 weeks to 8 weeks (Kitahara et al, 2008).

**Triphasic or multiphasic constructs**

In biphasic scaffolds, the two distinct mechanical properties of the cartilage and bone layers present a significant risk: the two phases may separate when subjected to shear forces after
implantation into an articulating joint. Adequate anchoring through a transitory phase from cartilage to bone is thus necessary to prevent tissue delamination. Native osteochondral junctions are characterized by a tidemark, which marks the transition from calcified cartilage to subchondral bone. Some groups have tackled the challenge of recreating this tidemark through the addition of mineralizing constituents or layering separately generated cartilage phases. Marquass and colleagues (2010) generated triphasic constructs consisting of an MSC-seeded collagen I hydrogel, an intermediate activated plasma layer, and a β-TCP bone substrate. The plasma layer improved fixation from the cartilage layer to the β-TCP. Twelve months post implantation, the repair tissue showed no significant differences in compressive strength compared to autograft transplants, and was well integrated with the surrounding cartilage (Marquass et al, 2010). Using different materials, Jiang and colleagues (2010) also designed multiphasic constructs. The cartilage layer was constructed using chondrocyte-seeded agarose, and the bone layer was made of PLGA-bioactive glass microspheres. An interface was created using chondrocytes seeded onto a hybrid agarose-microsphere scaffold. Mineralization was detected at the bone-interface region, with no delamination observed between the cartilage and the bone phase (Jiang et al, 2010).

Using a scaffold-free approach and MSCs, Lee and colleagues (2015) generated in vitro constructs with a calcified interface. Using a similar protocol as outlined above, BM-MSCs were pre-differentiated in membrane culture, then seeded onto CPP in the presence of β-glycerophosphate and triiodothyronine (T3). After 4 days, the T3 was withdrawn, and an additional layer of pre-differentiated MSCs not exposed to T3 was seeded on the initial cell layer. After 3 weeks, the resulting tissue showed mineralization specifically at the bone-cartilage interface, and showed higher peak load and energy to failure over control constructs without T3 (Lee et al, 2015).
Challenges with osteochondral tissue engineering

Though significant progress has been made in the realm of in vitro cartilage generation, questions remain concerning methods to reproducibly generate stable, mechanically competent, and functionally relevant hyaline-like cartilage. Generally speaking, most publications report that the compressive and/or shear modulus of tissue engineered cartilage is inferior to that of native cartilage. While the proteoglycan content and/or collagen content of the generated tissue may match native levels, it is the complex organization and orientation of these matrix components that gives hyaline cartilage its unique blend of flexibility and stiffness. In addition, homeostasis must
be achieved between catabolic and anabolic factors expressed in the tissue, and cell differentiation must be halted before progression through cell hypertrophy and subsequent osteogenesis.

Cell source, passage number/number of days in culture, age of donor, and individual donor variation introduce major challenges to consistent high quality cartilage formation. Of all the factors involved in tissue engineering, this is the step that requires the most optimization. Without a reliable source of cells with known chondrogenic potency, the subsequent steps of scaffold/scaffold-free tissue generation, choice of bone material, or choice of growth factor cocktails cannot be held as dogma.
Rationale, Hypothesis, and Objectives

Rationale

Cartilage injuries are a major cause of lameness in horses and, if left untreated, can progress to post-traumatic osteoarthritis. Current treatment options do not contribute to the long-term health and function of the joint, thus cell-based tissue regeneration modalities using MSCs are being explored as an alternative. While significant advances have been made towards generating implant-grade cartilage in vitro, there is a need to identify an optimal source of chondroprogenitor cells for the development of biphasic osteochondral constructs.

Determining the best source and type of stem cell is paramount to the success of tissue engineering. MSCs remain the most studied precursor cell in the context of cartilage generation. However, emerging evidence indicates that iPSCs surpass MSCs in their capacity for almost indefinite proliferation and self-renewal. While previous studies indicate that they do no readily differentiate into chondrocytes, it remains unclear whether they can be transitioned through an MSC phase prior to inducing chondrogenesis. As iPSC differentiation studies are still novel in the equine field, MSCs are still more clinically relevant for the generation of cartilage with the future intent of implantation. MSCs can be derived from various sources, though it is still not clear which or if a particular source is better at generating high quality neocartilage. In addition, donor variation is a significant concern in determining differentiation potency of individual cell cultures. Determining whether MSC donor or source has a greater effect on chondrogenic potential will help contribute to the establishment of criteria for the selection of cells for tissue engineering.

Mosaic arthroplasty, though quite successful in the treatment of subchondral bone cysts and focal cartilage defects, carries a significant risk of donor site morbidity. In vitro generated
biphasic osteochondral constructs alleviate these problems, however current methods for generating *in vitro* plugs with MSCs are cumbersome. Streamlining this process through novel seeding and culture conditions will go a long way in reproducibly generating allogeneic plugs for mosaic arthroplasty.

Taken together, the following studies will narrow the knowledge gap on stem cell source and culture conditions necessary for the robust generation of cartilage and biphasic osteochondral constructs for the treatment of focal cartilage defects.

**Hypothesis**

Equine MSCs from various sources can be used to generate hyaline-like cartilage on top of a bone substrate to create osteochondral constructs

**Objectives**

1. Determine conditions that facilitates chondrogenic differentiation of equine iPSCs.
2. Compare the chondrogenic potential of bone marrow- and umbilical cord blood-derived MSCs in membrane culture.
3. Generation of osteochondral constructs by direct seeding of MSCs on top of a bone substitute.
Chapter 2: Generation, characterization and multi-lineage potency of mesenchymal-like progenitors derived from equine iPSCs.


Introduction

In horses, mesenchymal stromal cells (MSCs) are used primarily in regenerative medicine studies to treat orthopedic injuries. However, these cells are limited in their expandability and differentiation capacity. Recently, the first equine induced pluripotent stem cell (iPSC) lines were reported (Nagy et al, 2011). Induction of iPSCs into MSC-like cells is an attractive option to using MSCs derived from other sources, as a much larger population of patient-specific cells could be generated in hopes of treating large orthopedic defects. However, the differentiation capacity of iPSCs and their potential for use in tissue engineering has yet to be explored. This study outlines the differentiation of equine iPSCs into MSC-like progenitor cells under specific culture conditions.

Materials and Methods

iPSC culture and maintenance

Equine H3-B iPSCs were grown in DMEM-HG (Invitrogen 11960–044) supplemented with 2 mM GlutaMax™ (Invitrogen # 35050), 0.1 mM non-essential amino acids (Invitrogen # 11140), 0.1 mM beta-mercaptoethanol (Sigma M7522), 1 mM sodium pyruvate (Invitrogen #11360-070), 50 U/ml penicillin/streptomycin (Invitrogen #15070), 15% fetal bovine serum (FBS) (HyClone), 1000U/ml leukemia inhibitory factor (LIF; Millipore #ESGRO), 10 ng/ml bFGF (Peprotech #100-18B), and 1.5 μg/ml doxycycline (Sigma #D9891). The cells were plated on
mitomycin-inactivated mouse embryonic feeder cells and cultured in a humidified incubator at 37°C and 5% CO₂. They were passaged every 2-3 days by enzymatic digestion with TrypLE Select (Invitrogen #12563), at a 1:4 ratio.

_Derivation of a MSC-like population from equine iPSCs_

A serial plating strategy was used to differentiate the iPSC culture H3-B (Nagy et al, 2011) into MSC-like cells (Fig. 1). To separate the iPSCs from their mouse embryonic fibroblast feeder layer, the cells were enzymatically digested as described above, centrifuged, and resuspended in sorting medium containing PBS, 25mM Hepes, and 0.2% FBS. Fluorescent activated cell sorting of GFP positive iPSCs was performed using the MoFlo Astrios Cell Sorter (Beckman Coulter). GFP-positive cells were seeded onto gelatin-coated plates at 1 x 10⁴ cells/cm² in MSC induction media consisting of DMEM-HG, 10% FBS, 0.1 mM non-essential amino acids, 50 U/ml penicillin/streptomycin, 10 μM anaplastic lymphoma kinase (ALK) inhibitor SB431542 (StemGent #04-0010) and 5 ng/ml bFGF. Cells were expanded to 80% confluency, and then passaged onto a second gelatin-coated plate for one passage. Thereafter, the cells were passaged onto uncoated tissue culture plates and propagated for 3 to 11 passages. The cells gradually obtained a fibroblast-like, spindle-shaped morphology. ALK inhibitor was removed from the MSC induction media after 10 days. Cells were routinely passaged every 3-4 days, at a 1:3 split ratio, until reaching the 8th passage. This process was reproduced three times with H3-B iPSCs (n=3). The resulting iPSC-MSCs were evaluated at early passage (passage 3-4) and late passage (passage 6-8) by gene expression, flow cytometry and functional differentiation.
**qRT-PCR analyses**

Total RNA was harvested from iPSC-MSCs using the Qiagen RNeasy Kit (Qiagen), and 1 μg of total RNA was reverse transcribed using the Quantitect Reverse Transcription Kit (Qiagen). The reaction was performed at 42°C for 15 min. The cDNA was subsequently analyzed by qPCR using LuminoCt Sybr Green qPCR Ready Mix (Sigma-Aldrich) using the CFX384 TouchTM Real-Time PCR detection system (Bio-Rad). For all samples, a negative control was included that did not contain cDNA. All reactions were completed in triplicate. Values are expressed as 2^ΔΔCt, with ΔΔCt defined as the difference in crossing threshold (Ct) values between experimental and control samples as described using β-actin and Hprt1 as internal standards. Fold difference was compared to the H3-B iPSC parent culture. Oligonucleotide primers are listed in Table 1. Oct4, Sox2, and Klf4 primer pairs do not amplify mouse transcripts from the inserted transgenes (Nagy et al, 2011).

**Flow cytometry**

iPSC-MSCs and CB-MSCs (1 cell culture at passage 3) were expanded and subsequently harvested using AccuMAX (Stem Cell Technologies) and washed with flow buffer (PBS, 5 mM EDTA, 1% horse serum, and 0.1% sodium azide). The following primary antibodies were used to stain the cell suspensions (150,000 – 200,000 cells): mouse anti-human CD73 (Abcam), mouse anti-human CD90 (VMRD), mouse anti-human CD105 (ABD Serotec), mouse anti-horse CD44 (ABD Serotec), FITC mouse anti-human CD29 (Beckman Coulter), mouse anti-human CD45 (VMRD), mouse anti-horse CD4 (ABD Serotec), mouse anti-horse CD8 (ABD Serotec), mouse anti-horse CD11a/18 (ABD Serotec), mouse anti-horse MHC I (ABD Serotec), and mouse anti-horse MHC II (ABD Serotec). Rat anti-mouse IgM-FITC and goat anti-mouse IgG1-FITC were
used as secondary antibodies. Specificity of all antibodies has been validated in our lab using positive controls for the target species of the antibody (horse or human), and with 3 negative controls: isotype negative control, secondary antibody and unstained control (Tessier et al, 2015). 10,000 events were acquired for each experiment on BD FACScan (BD Biosciences) using CellQuest (Becton Dickinson) software, and analysis was conducted using FlowJo (Tree Star).

**Osteogenesis assay**

Osteogenic differentiation was induced as previously described (Koch et al, 2007). In brief, passage 4 iPSC-MSCs were seeded in 6 well plates at a density of approximately 3,000 cells/cm², and grown until 90-100% confluency. To induce osteogenesis, cells were cultured for 10 days in osteogenic media containing DMEM-LG, 10% FBS, 1% L-glutamine, 1% antibiotic-antimycotic (ABAM), 100 nM dexamethasone, 10 mM β-glycerophosphate, and 0.05 mM ascorbic acid-2-phosphate. An equal number of control cells were maintained in MSC expansion medium. Media was changed every 3 days. Osteogenesis was evaluated using Alizarin Red S staining for calcium deposition and von Kossa staining for phosphate accumulation.

**Adipogenesis assay**

Induction of adipogenesis was performed as previously described (Koch et al, 2007). Passage 4 iPSC-MSCs were seeded at a density of approximately 2.1 x 10⁴ cells/ml in 6 well plates. Adipogenesis was carried out in adipogenic induction media, containing DMEM-LG, 15% rabbit serum, 500 μM IBMX, 1% L-glutamine, 1% ABAM, 1 μM dexamethasone, 10 μg/ml insulin, and 200 μM indomethacin. Control cells were maintained in expansion media. Evaluation of positive adipogenesis was done by Oil Red O staining to observe lipid droplets.
Chondrogenesis assay

Passage 6 and passage 11 iPSC-MSCs were induced to undergo chondrogenesis in pellet culture as previously described (Co et al, 2014). Approximately 2.5 x 10^5 iPSC-MSCs were resuspended in 200 µl of chondrogenic induction media, containing high glucose DMEM (DMEM-HG), 200 mM Glutamax, 100 mM sodium pyruvate, 1% ABAM, 0.1 mM dexamethasone, 100 mg/ml ascorbic acid-2 phosphate, 40 mg/ml proline, 1x ITS premix (Life Technologies, #515 00-056), and 10 ng/ml TGFβ3 (R&D Systems, #243-B3). The resuspended cells were plated in V-bottom polypropylene 96 well plates (Phenix #MPG-651201) and centrifuged to pellet the cells at the bottom of the wells. BMP-4 (Roche #E12512) was added alone or in conjunction with TGFβ3 in various final concentrations (10 ng/ml, 50 ng/ml, and 100 ng/ml) to the chondrogenic media in a subset of pellets. BMP-4 was removed from the media after 1 week of pellet culture as previously described (Choudhery et al, 2014). Pellets were incubated at 37°C in 5% CO₂ and 21% O₂ for 2 to 4 weeks. Media was changed every 2-3 days.

Statistical analysis

Graphpad Prism 6 Software was used to perform statistical analysis on qPCR data. One-way ANOVA followed by Tukey’s post hoc test was used to determine expression differences between groups. p-values <0.05 were considered to be statistically significant.
Results

Derivation of iPSC-MSCs from equine iPSCs

After 8 days on gelatin in MSC induction media, the iPSCs lost their colony forming phenotype and acquired a mixed cuboidal and spindle-like morphology (Fig. 2a, b). Upon subsequent passaging onto uncoated petri dishes, the spindle-like morphology predominated (Fig. 2c) and eventually the majority of the differentiated population maintained this MSC-like phenotype (Fig. 2d).

Gene expression of iPSC-MSCs

Gene expression of equine pluripotency markers (Oct4, Sox2, Nanog, and Klf4) in three separately derived iPSC-MSC cultures was compared to their parent H3-B iPSC culture. In both early passage (passage 3-4) and late passage (passage 6-8) cells, iPSC-MSCs showed consistent downregulation of expression in each gene relative to H3-B iPSC (Fig. 3a). Additionally, iPSC-MSCs displayed significant upregulation of MSC-associated genes Sox5, Sox6, Sox9 and CD90, as well as an upregulation of mesodermal lineage genes BMP7 and SNAI3 relative to H3-B iPSC (Fig. 3b). As well, Sox5, Sox6, Sox9, CD44 and CD90 transcription levels approached that of CB-MSCs.

Flow cytometry analyses and derivation efficiency of iPSC-MSCs

Upon flow cytometry analysis, iPSC-MSCs displayed a similar immunophenotype as CB-MSCs, namely positive expression for CD29, CD44 and CD90, and low or absent expression of CD4, CD8, CD105, CD45, CD73, CD11a/18, MHC I and MHC II (Fig. 4a, b). Of cells that remained plastic-adherent throughout the MSC induction process, 74.4% of iPSC-MSCs expressed
CD90, 97.5% expressed CD29 and 97.7% expressed CD44 (Fig. 4). As these values are nearly identical to the percent positive cells in CB-MSCs (CD90: 76.6%; CD29: 97.0%; and CD44: 90.9%; Fig. 5), MSC derivation efficiency of adherent iPSCs using our serial plating method is close to 100%.

Characterization of functional differentiation of iPSC-MSCs

After 10 days in osteogenic induction media, von Kossa staining and Alizarin Red staining revealed that the iPSC-MSCs began to initiate mineralization after induction, indicative of early osteogenesis (Fig. 6). Furthermore, after 14 days in adipogenic induction media, morphology changes and lipid droplets were observed in the iPSC-MSCs following Oil Red O staining (Fig. 6). The results obtained were consistent with what was observed in CB-MSCs induced for osteogenesis and adipogenesis for the same time period (Figure 8). Analysis of adipogenic-associated genes LPL and PPARγ showed no significant difference in gene expression in induced iPSC-MSCs (for LPL: p=0.2156; for PPARγ: p=0.0633) and CB-MSCs (for LPL: p=0.3504; for PPARγ: p=0.1081) compared to non-induced cells (Figure 9). Chondrogenesis was not successful despite several attempts to induce cartilage formation in 3D pellet culture for 2 and 4 weeks using TGFβ3 at 21% oxygen. The pellets were either too small for analysis and/or contained mostly necrotic tissue (Figure 7a, b). In contrast, CB-MSCs induced for 4 weeks showed proteoglycan accumulation and lacunae formation typical of hyaline-like cartilage (Figure 7c). iPSC-MSCs also did not undergo chondrogenesis when treated with BMP-4 alone or with TGFβ3 in 21% or 5% oxygen conditions (data not shown).
Discussion

The aim in this study was to determine whether equine iPSCs could be successfully differentiated into MSC-like cells that exhibit properties similar to MSCs from other sources, such as umbilical cord blood. We hypothesized that iPSCs-MSCs generated from equine iPSCs could be a suitable cell source for chondrogenesis. We report that the iPSC culture H3-B (Nagy et al, 2011) can be reproducibly differentiated into a progenitor cell type that meets many criteria of MSCs: adherence to and proliferation on plastic, expression of certain surface markers, and multi-lineage differentiation potential, though they were unable to undergo chondrogenesis. Our iPSC-MSCs display a significant reduction in gene expression of the pluripotency genes Oct4, Klf4, Sox2, and Nanog while upregulating MSC-associated and mesodermal lineage genes Sox5, Sox6, Sox9, CD44, CD90, BMP7 and SNAI3.

Several different techniques have been employed to derive MSCs from iPSCs. These include culture of adherent outgrowths from embryoid bodies (Koyama et al, 2012; Li, Bronson, and Niyibizi 2010; Wei et al, 2012), culture of iPSCs on various surfaces such as fibrillar collagen (Jin et al, 2013; Liu et al, 2012; Villa-Diaz et al, 2013) or through cell selection (Lian et al, 2010). Serial plating or direct plating is a comparatively simple method to direct the differentiation of iPSCs to MSCs (Guzzo et al, 2013; Hynes et al, 2013; Zou et al, 2013). Our method involved simultaneously seeding equine iPSCs onto gelatin-coated plates in MSC induction media, and this was shown to be sufficient in directing their differentiation into the flattened, fibroblastic morphology typical of MSCs. A homogenous population of cells resulted after only 2-3 passages on uncoated petri dishes in MSC induction media; this technique selected for cells that were plastic-adherent, as any floating cells were lost when changing media. MSC induction media was found to be required for the differentiation of plastic-adherent cells through the serial passaging.
method; simply removing doxycycline from the iPSC culture media was not sufficient in inducing the cells to differentiate into MSC-like cells (data not shown).

Gene expression analysis confirmed that pluripotency-associated genes Oct4, Sox2, Nanog and Klf4 were downregulated in iPSC-MSCs upon differentiation. This suggests a loss of pluripotency, which was correlated with an upregulation of MSC-associated genes Sox5, Sox6, Sox9, CD29 and CD90, and an upregulation of mesodermal lineage markers BMP7 and SNAI3. Sox5 and Sox6 are transcription factors involved in the osteochondroprogenitor lineage program and act upstream of Sox9 (Ikeda et al, 2004). The Sox9 transcription factor is the key regulator in chondrogenesis, initiating the expression of cartilage-specific extra-cellular matrix genes such as Col2a1 (Ikeda et al, 2005). Sox9 is required for the commitment of chondroprogenitors, mesenchymal condensation and proper chondrocyte proliferation, differentiation and maturation (Akiyama 2008). Our data revealed that though Sox5 and Sox6 were upregulated in iPSC-MSCs in both early and late passages, Sox9 expression was only upregulated in late passage cells only. This variable expression of the Sox trio of transcription factors has been observed before in iPSC-MSCs, though chondrogenesis was observed in these cell cultures (Guzzo et al, 2013).

BMP7 is a member of the TGFβ superfamily, and is an early mesodermal lineage marker (Dale et al, 1997; Hahn et al, 1992). Late passage iPSC-MSCs show a distinct upregulation of BMP7 over H3-B iPSCs, though no expression was detected in early passage iPSC-MSCs. SNAI3 is a zinc finger protein and a member of the Snail protein family, and is also an early mesodermal marker. Snail proteins function primarily as transcriptional repressors, and are capable of inducting epithelial-mesenchymal transition (Yang & Weinberg, 2008). SNAI3 was upregulated in both early and late passage iPSC-MSCs.
Expression of the MSC markers CD90, CD29, and CD44, as determined by flow cytometry, are characteristic of a homogenous population of differentiated MSC-like cells successfully derived from iPSCs. This indicates that the efficiency of iPSC differentiation into MSC-like cells is close to 100%. This efficiency assessment method was chosen as it provides the most accurate evaluation of the percentage of cells with an MSC phenotype. However, it must be recognized that this is not a true measure of derivation efficiency from undifferentiated iPSCs to MSCs. This is because of the nature of the serial plating method, where some iPSCs were lost during media replacement due to lack of adherent properties. Efficiency of MSC generation is difficult to establish and reports on iPSC-MSCs or embryonic cell conversion often do not include efficiency data (Guzzo et al, 2013; Lian et al, 2014; Takayama et al, 2012; Yen et al, 2011). However, the flow cytometry results show that the cells that remained attached throughout iPSC-MSC conversion are homogenous and phenotypically similar to CB-MSCs.

Our iPSC-MSCs successfully underwent adipogenesis and osteogenesis after chemical induction as determined by morphology and staining assays. Analysis of the expression of adipogenesis-associated genes LPL and PPARγ did not yield significant results in induced iPSC-MSCs or CB-MSCs. We believe this to be a power issue rather than an indication of minimal adipogenic differentiation, as clear morphological changes and lipid-vacuole formation is seen in induced iPSC-MSCs and CB-MSCs.

Intriguingly, iPSC-MSC response to chemical chondrogenic induction in 3D pellet culture was absent. This may be associated with the low expression of Sox9 in early passage cells; though Sox9 expression increases in late passage cells, the increased passage number may correlate with decreased chondrogenic potential as is seen in MSCs (Vacanti et al, 2005). Other studies have demonstrated the benefit of overexpressing Sox9 to enhance chondrogenesis (Akiyama, 2008;
Bosnakovski et al, 2005; Kimura et al, 2010). It may be that the use of only one equine iPSC line reflects donor-to-donor variation in the chondrogenic potency of MSCs from adult tissues, a known phenomenon (Choudhery et al, 2014; Huang et al, 2013). iPSC-MSCs generated from other equine iPSC lines may possess chondrogenic potency under the described conditions. Several unsuccessful strategies to induce differentiation of iPSCs to chondrocytes were attempted as well; these included embryoid body formation, pellet culture of iPSCs, membrane culture of iPSCs alone, and membrane culture of iPSCs co-cultured with bovine chondrocytes (data not shown). These findings underscore the observation that iPSC differentiation protocols developed for one species (e.g., mouse, human) are not necessarily universal across mammals. This report is a first step towards development of equine specific protocols for iPSC fate conversion towards MSCs.

In conclusion, the present study provides an important look at the capacity of horse iPSCs to differentiate into an MSC-like progenitor population. Using a simplified serial plating technique, we demonstrate that MSC-like cells with properties similar to CB-MSCs can be derived from equine iPSCs. Though this technique is reproducible in one equine iPSC line, the general robustness of the protocol should be tested using a larger number of unrelated iPSC lines. Testing several iPSC lines would also help determine whether the lack of chondrogenic potential is a general feature of equine iPSC-MSCs or the iPSC line used in this study.

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Figures

Figure 1. Study design on the derivation and subsequent evaluation of iPSC-MSCs from equine iPSCs.
Figure 2. Derivation of a MSC-like population from equine iPSCs. iPSCs maintained in iPSC media form ES-like colonies (a). iPSCs cultured in MSC media on gelatin acquired a heterogenous, cuboidal-like morphology with time (b). Upon passaging onto uncoated tissue culture plastic, cells adherent to plastic acquired a more homogenous, spindle-like morphology (c, d) similar to MSC (e).

Figure 3. Gene expression analysis of pluripotency and mesenchymal/mesodermal-related genes by qRT-PCR. a) Expression data of pluripotency genes Oct4, Sox2, Klf4, and Nanog. b) Expression data of MSC-associated genes Sox5, Sox6, Sox9, CD44, CD29, CD90, and mesodermal-associated genes BMP7 and SNAI3. H3-B iPSC: parent iPSC culture. iPSC-MSC early: passage 3-4. iPSC-MSC late: passage 6-8. CB-MSC: MSCs derived from equine cord blood. *p < 0.05; **p < 0.01; ***p < 0.0001 when compared to parent iPSC culture.
Figure 4. Flow cytometry analysis of one representative iPSC-MSC culture for expression of common surface antigens. Dot plots generated from flow cytometry analysis show percent positive cells (FITC positive) after gating around the isotype control population (top row: IgM secondary antibody; bottom row: IgG secondary antibody). The unstained control is shown as being gated around the IgG negative control.
Figure 5. Flow cytometry analysis of one representative CB-MSC culture for expression of common surface antigens. Dot plots generated from flow cytometry analysis show percent positive cells (FITC positive) after gating around the isotype control population (top row: IgM secondary antibody; bottom row: IgG secondary antibody). The unstained control is shown as being gated around the IgG negative control.
Figure 6. *In vitro* adipogenesis and osteogenesis assays of iPSC-MSCs (200x magnification). a) iPSC-MSCs cultured in adipogenic induction medium showed marked changes in morphology and lipid droplet accumulation as shown by positive Oil Red O staining. iPSC-MSCs cultured in osteogenic induction medium acquired mineralization by positive von Kossa staining for phosphate accumulation (b) and Alizarin Red staining for calcium accumulation (c). d) Adipogenic control stained negative for Oil Red O after 14 days in MSC expansion medium. Osteogenic controls stained negative for von Kossa and Alizarin Red after 14 days in MSC expansion medium (e, f).
Figure 7. *In vitro* 3D pellet culture induction of chondrogenesis of iPSC-MSCs. Representative iPSC-MSC cultures cultured in chondrogenic induction medium in pellet culture for 2 weeks (a) or 4 weeks (b) did not show proteoglycan accumulation or lacunae formation through toluidine blue staining. c) Representative control CB-MSC culture showing positive staining for toluidine blue after 4 weeks of culture.

Figure 8. *In vitro* adipogenesis and osteogenesis assays of CB-MSCs (200x magnification). a) CB-MSCs cultured in adipogenic induction medium showed marked changes in morphology and lipid droplet accumulation as shown by positive Oil Red O staining. CB-MSCs cultured in osteogenic induction medium acquired mineralization by positive von Kossa staining for phosphate accumulation (b) and Alizarin Red staining for calcium accumulation (c). d) Adipogenic control stained negative for Oil Red O after 14 days in MSC expansion medium. Osteogenic controls stained negative for von Kossa and Alizarin Red after 14 days in MSC expansion medium (e, f).
Figure 9. Gene expression analysis of adipogenic-associated genes LPL and PPARγ by qRT-PCR in induced and non-induced CB-MSCs and iPSC-MSCs. p values represent relative expression of induced cells over non-induced cells (n=3), normalized to β-actin expression.

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Table 1. Primer pairs used for qPCR.
Chapter 3: Towards engineering equine cartilage using cord blood and bone marrow mesenchymal stromal cells: Donor is a greater contributor to chondrogenic potential than tissue source

This chapter is a modified version of a manuscript to be submitted to Osteoarthritis and Cartilage.

Introduction

Joint cartilage defects and possible secondary disease manifestations such as osteoarthritis (OA) represent more than 50% of all lameness cases in pleasure and performance horses (USDA 2000). Mesenchymal stromal cells (MSCs) isolated from various sources have been shown to be effective for use in cartilage tissue engineering and defect repair (Galle et al., 2010). Our lab has reported that MSCs derived from equine umbilical cord blood (CB-MSCs) have higher chondrogenic potency than MSCs from bone marrow (BM-MSCs) (Berg et al., 2009). However, further work in our lab has revealed large donor-donor variability in CB-MSC culture potency causing us to question these previous reports, which represent relative few biological samples and a limited array of outcome parameters. In this study, our aim was to determine which MSC source produced better quality neocartilage. Few significant differences were found in neocartilage tissues derived from CB- and BM-MSCs. However, tissue quality varied widely among individual cell cultures within both groups. Variance component analysis determined that individual MSC donors contributed more to the variation seen in the data than MSC source.
Materials and Methods

Experimental design

10 CB-MSC and 10 BM-MSC cultures from unrelated donors were expanded in monolayer until confluency. At passage 5, MSCs were seeded on membrane inserts and cultured for 7, 14, and 21 days in chondrogenic induction media. Three technical replicates per time point were collected for histology and immunohistology, and three technical replicates were assigned for biochemistry and mRNA expression analysis (Fig. 1).

CB-MSC and BM-MSC expansion

Cryopreserved CB-MSCs and BM-MSCs (n=10 per group) were thawed and expanded in MSC expansion medium. CB expansion medium: DMEM low glucose (DMEM-LG) (Lonza; Walkersville, MD, USA), 30% fetal bovine serum (FBS) (Invitrogen; Burlington, ON, Canada), 2 mM L-glutamine (Sigma-Aldrich; Oakville, ON, Canada), and 100 U penicillin-streptomycin (Invitrogen; Burlington, ON, Canada). BM expansion medium: DMEM-LG, 10% FBS, 1 mM L-glutamine, and 100 U penicillin-streptomycin. Medium was changed every 2 days, and upon reaching 80-90% confluency, the MSCs were trypsinized and re-plated at a density of 5,000 cells/cm² to reach the desired cell number. All cell cultures were induced to differentiate at passage 5.

Chondrogenic differentiation in membrane culture

Membrane culture was performed as previously described (Co et al, 2014; Lee et al, 2011) with some modifications. Briefly, 12 mm-diameter cell membrane inserts (Millipore; Etobicoke,
ON, Canada) were first prepared for culture by coating them with 100 µl of a solution of 10% fibronectin (Millipore) in DMEM-LG. Membranes were allowed to dry for 48 hours, UV sterilized, then placed in an incubator to solubilize for 6-8 hours. CB- and BM-MSCs were trypsinized and counted, then a 400 µl cell suspension containing 2 x 10^6 cells in 10% FBS MSC expansion media was deposited inside the membrane insert in a 12-well plate. 600 µl of 10% expansion media was added to the outside of the membrane insert, and then cells were incubated overnight at 38˚C in 21% O₂. The following day, the expansion medium was exchanged for chondrogenic medium containing DMEM high glucose (DMEM-HG) (Sigma-Aldrich; Oakville, ON, Canada), 1X insulin-transferrin-selenium (ITS) (BD Biosciences; Mississauga, ON, Canada), 10 mg proline (Sigma-Aldrich; Oakville, ON, Canada), 100 nm dexamethasone (Sigma-Aldrich; Oakville, ON, Canada), 100 mM sodium pyruvate (Invitrogen; Burlington, ON, Canada), 200 mM GlutaMAX (Invitrogen; Burlington, ON, Canada), 100 ug/ml ascorbic acid (Sigma-Aldrich; Oakville, ON, Canada), and 10 ng/ml TGFβ3 (R&D Systems; Minneapolis, MN, USA). 10 µM of Y-27632 (ROCK inhibitor; Sigma-Aldrich) was added for the first 72 hours of culture. The membrane inserts were incubated at 38˚C in 21% O₂ for 7 days, 14 days, and 21 days. Media was changed every 2-3 days.

Histology and immunohistology

Membrane inserts and associated tissue was removed from the well, washed in PBS and then fixed in 10% formalin. Tissue was then paraffin embedded and sectioned (5 µM sections). For histology: slides were stained with either hematoxylin and eosin or toluidine blue then visualized under light microscopy. To visualize collagen I and collagen II, immunohistochemistry performed as previously described (Co et al, 2014). Briefly, de-paraffinized and rehydrated sections were subjected to enzymatic antigen retrieval through hyaluronidase incubation for 10
minutes at room temperature followed by proteinase K incubation at 37°C for 20 minutes. Sections were then blocked with 3% FBS, then incubated with antibodies raised against type I collagen (Millipore, clone 5D8-G9, Cat# MAB3391) or type II collagen (Developmental Studies Hybridoma Bank; Iowa City, Iowa, USA, Cat#II-II6B3) overnight. HRP-conjugated secondary (DAKO, Burlington, ON, Canada) was added the following day for 1 hour, then rinsed with PBS. Colour was developed using DAB chromogen. Sections were counter-stained with hematoxylin, then mounted with coverslips and visualized under light microscopy. Negative controls were treated in parallel, but without the addition of a primary antibody.

**Biochemical analysis**

Day 7, 14 and 21 old neocartilage-tissue was digested in 65 µg/ml papain (Sigma-Aldrich) for 48 hours then frozen at -20°C until further analysis. DNA, glycosaminoglycan and hydroxyproline contents were quantified as previously described (Co *et al*, 2014; Lee *et al*, 2011).

**mRNA expression**

Day 7, 14 and 21 neocartilage-tissues were removed from their substrates and placed into RNAlater (ThermoFisher, Burlington, ON, Canada) and stored at -20°C until ready for RNA extraction. RNA was extracted by removing tissue from RNAlater, snap freezing in liquid nitrogen, then pulverizing the frozen tissue using a Retsch® Mixer Mill (RETSCH, Haan, Germany) for 3 minutes at 30 Hz. Following bead milling, RNA isolation was done using the mirVana total RNA isolation kit, then the RNA was cleaned using RNA Clean and Concentrator (Zymo Research, Irvine, California, USA). RNA was DNase-treated and reverse transcribed using the SuperScript II reverse transcription kit (ThermoFisher). Quantitative real-time PCR (RT-PCR) was performed using the CFX96 Touch™ Real-Time PCR detection system (Bio-Rad, Hercules, 60
CA, USA). Chondrogenesis-associated gene expression was normalized to 18S rRNA expression, and fold difference was compared to expression from primary chondrocytes. Primer sequences can be found in Table 1.

Statistical analysis

With the assistance of a statistician, we analyzed results as general linear mixed models using cell source as a fixed effect and day and donor as random effects (McCulloch & Neuhaus, 2005). Residuals were tested for normality and plotted against predicted values and factors to assess ANOVA assumptions. Data was log-transformed where necessary and subsequently back-transformed for readability. Least squares means were determined and significance was assessed based on the differences of least squares means. In the case of gene expression analysis, means were converted to fold changes with the $2^{\Delta\Delta CT}$ method (Livak & Schmittgen, 2001). Variance component analysis was performed by taking the estimate values produced by the general linear mixed model for each effect (cell source, day, and donor) and dividing each effect estimate by the total of all estimates to generate a percentage value. Significance was assigned at p<0.05.

Results

Wet mass, histology, and immunohistochemistry

Both BM-MSCs and CB-MSCs were capable of producing neocartilage resembling hyaline cartilage by day 21 (Fig. 2). Tissue quality varied widely between cell cultures in the BM and CB groups; some cell cultures produced transparent, thin tissue with little matrix and with fibrous morphology (“low” chondrogenic potential), while other cell cultures produced opaque, thick tissue with positive toluidine blue and type II collagen staining (“high” chondrogenic potential). Both BM- and CB-MSC-derived tissues stained positively for type I collagen, with varying
staining intensities. Tissue mass increased significantly with time in both groups, but did not differ significantly between tissues derived from BM- or CB-MSCs collected at the same time point (Fig. 2d).

**Biochemistry**

Proteoglycan content normalized to DNA content was not different between BM- and CB-derived MSC groups at any given time point (day 7: \( p=0.5624 \); day 14: \( p=0.0921 \); day 21: \( p=0.6299 \)). Collagen content normalized to DNA content did not differ between cell sources at any time points (day 7: \( p=0.0761 \); day 14: \( p=0.4568 \); day 21: \( p=0.8833 \)). CB-MSC derived neocartilage showed an increase in proteoglycan content from day 7 to day 14 (\( p=0.0101 \)). There was no observed increase in collagen content in the BM group from day 7 to day 21 (\( p=0.4018 \)) however there was a large increase in the CB group from day 7 to day 21 (\( p=0.0019 \)) (Fig. 3).

**mRNA expression**

*Col10* gene expression at day 7 was significantly greater in CB-MSCs than BM-MSCs (\( p=0.0152 \)), though it was not significantly different at subsequent time points. *Sox9* expression was significantly greater in CB-MSCs at day 7 and day 21 (\( p=0.0118 \) and \( p=0.0130 \), respectively), though not at day 14. *ACAN, Col1a2* and *Col2a1* were not significantly different between MSC sources at any time-point. (Fig. 5).

**Variance component analysis**

Variance component analysis revealed that donor is the greatest contributor to the variance seen in proteoglycan and collagen content (Table 2). Individual donors from either source contribute to 73% of the variance seen in GAG/DNA values, and 85.5% of the variance seen in
the collagen/DNA values from Figure 5. GAG to collagen ratio for each parameter was also assessed, and once again found that donor has the greatest variance (52%).

**Discussion**

Here, membrane culture-generated neocartilage tissue was qualitatively and quantitatively compared from multiple unrelated BM-MSC and CB-MSC cell lines at three time points. Macroscopically, there were large differences in the opaqueness and thickness of the tissue. Thicker tissues corresponded with more hyaline-like traits such as positive toluidine blue staining, presence of cell lacunae, greater indication of type II collagen and minimal type I collagen staining. Of note, all tissues showed some degree of positive staining for type I collagen. As early differentiating MSCs express type I collagen in their matrix (Goldring, Tsuchimochi, & Ijiri, 2006), it may be that 3 weeks of differentiation are not sufficient to see a marked decrease of type I collagen, or that the cells retain their ability to synthesize type I collagen. mRNA expression data did not show a significant change in Colla2 expression over the three week time period, similar to that noted during chondrogenic in vitro differentiation of sheep BM-MSCs in membrane culture (Lee et al, 2011).

GAG and type II collagen accumulation in the extra-cellular matrix (ECM) are key components in the production of tissue-engineered cartilage. The addition of TGFβ3 to the chondrogenic induction media induces the expression of ECM-related genes such as ACAN and Col2a1 (Baugé et al, 2011), and accumulation of GAG and total collagen increases in a time-dependent manner (Lee et al, 2011; Taylor et al, 2012). Among CB-MSCs an increase in sulfated GAGs was observed from Day 7 to Day 14, as well as total collagen accumulation, from Day 7 to Day 21; no significant difference was seen over time in the BM group. This increase in GAG deposition may be associated with the observation that CB-MSCs reportedly have a higher
proliferation rate than BM-MSCs (Kern et al, 2006). It is speculated that their metabolism is different as well; a faster metabolism is correlated with increased proliferation and GAG deposition (Quinn et al, 2002). As well, increased proteoglycan deposition can inhibit collagen deposition, therefore possibly explaining the observed trend of delayed collagen deposition in CB-MSCs (Bastiaansen-Jenniskens et al, 2008). Early inhibition of collagen deposition has been shown to improve the maturation of collagen crosslinks, enhancing integration (Mcgowan & Sah, 2005).

Upon TGFβ3-mediated induction of chondrogenesis, Sox9 is upregulated in MSCs, which acts as a transcription factor for cartilage-associated genes ACAN and Col2a1 (Han & Lefebvre, 2008; Ng et al, 1997). In developing cartilage from chick embryos, Kosher et al. (1986) demonstrated that Col2a1 mRNA transcripts greatly increase within the first few days in culture (Kosher, Kulyk, & Gay, 1986). Consistent with our data, they did not see a corresponding decline in Colla2 transcripts in the same time period; high expression of Colla2 was detected in 7-day limb cartilage. Our data did not demonstrate any differences in gene expression over time. This is broadly similar to sheep BM-MSCs expanded in membrane culture, which also show no obvious increase or decrease in gene expression over time (Lee et al, 2011). Although temporal changes in chondrogenesis-associated gene expression have been reported among different MSC cultures, there are no consistent trends of gene expression changes over time (Berg et al, 2009; Reich et al, 2012).

Inter-donor variation remains a central obstacle when working with cell populations. Variation in proliferation and differentiation capacity between donors limits efforts to standardize tissue engineering protocols, and hence is a particular concern for autologous-based MSC strategies. While phenotypic and immunological evaluation shows that unrelated equine CB-MSC
cultures consistently express select surface markers and suppress lymphocyte activity (Tessier et al., 2015), multilineage potency can vary widely. For example, Siddappa et al. (2007) evaluated 19 different human BM-MSC cultures for osteogenic potential, and found significant variation in early ALP expression between donors, which correlated with differences in in vivo bone formation. Digirolamo et al. (1999) demonstrated that adipogenic potential was correlated with the colony-forming ability of individual human BM-MSC cultures, which again varied widely. The data suggests that the same is true for chondrogenic potency in equine MSCs. Despite having a large number of individual cell cultures in the BM and CB groups (originally intended to increase power for statistical analysis), the error bars at each evaluation point indicates very large inter-donor variability between cell cultures in both groups. This variability thus abrogated almost all significant differences found during statistical analyses of the quantifiable parameters of tissue quality evaluation. In fact, variance component analysis of these data determined that donor is the greatest contributor to the variance seen, greater than both day and cell source.

In conclusion, a thorough analysis of the chondrogenic potential of equine BM- and CB-MSCs at various time points revealed considerable variation among donors, underscoring the potency of the donor as a key factor when considering a cell population for tissue engineering. The origin of the cell source also deserves careful consideration, especially in the context of collection risks, age of donor, as well as immunological phenotype, to name a few. BM-MSCs and CB-MSCs do differ in two of these respects; CB-MSC isolation is non-invasive and donor age always remains the same. At present, the primary factors that enhance chondrogenic potency among MSC populations remains unclear. A more in-depth look at MSC growth characteristics, gene expression, and additional surface marker expression may expose associations to predict or enhance chondrogenic potency.
Acknowledgements

I wish to acknowledge Gabrielle Monteith for her help with statistics and the variance component analysis, as well as Carmon Co for her intellectual contributions and guidance in histology and biochemical analyses.

Figures

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**TB**

**Col-I**

**Col-II**

Scale: 100 µm
Figure 2. Characterization of neocartilage derived from core blood (CB) and bone marrow (BM) mesenchymal stromal cell populations (MSC) at multiple time points. At each of the 7 day (A), 14 day (B) and 21 day (C) time points, we stained for Toluidine blue (TB) and immunostained for type I (Col-I) and type II (Col-II) collagen. One representative high chondrogenic (high) and one low chondrogenic (low) tissue is shown for each source at time point. Insets for immunostained panel represent omission (negative) controls. Top left insets in C) show macroscopic images of representative CB-MSC-derived tissues from the “high” and “low” groups. Some tissues were processed with the underlying membrane insert still attached (asterisks).
Figure 3. Average tissue mass from BM- and CB-MSC-derived neocartilage at days 7, 14, and 21. Letters denote significant differences (p<0.05) between time points.
Figure 4. GAG content and total collagen content normalized to DNA content for all samples at Day 7, 14, and 21. Data is presented as mean ± 95% CI. No significant differences were observed between groups at any given time point. n=10.
Figure 5. mRNA expression of chondrogenesis-associated genes for all samples at Day 7, 14, and 21. Data is presented as log2 fold changes ± 95% CI. Expression was normalized to the 18s rRNA housekeeping gene, and is relative to primary chondrocytes. *p<0.05, n=10.

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Table 2. Variance component analysis. Estimation of the contribution to variance by the fixed effect (tissue source) and random effects (day and donor).
Chapter 4: One-step generation of biphasic osteochondral constructs using equine cord blood-derived MSCs

Introduction

Full thickness chondral lesions are a substantial cause of lameness in equine athletes. Osteochondral autograft transfers have lead to some successful treatments, however this technique is associated with donor site morbidities and difficulties with autograft harvest. Therefore, a novel one-step osteochondral generation protocol was developed through the direct seeding of undifferentiated MSCs on top of a calcium polyphosphate (CPP) bone substrate. Direct seeding of undifferentiated MSCs was achieved through the inhibition of ROCK, a Rho-associated kinase that plays a role in cell contraction (McBeath et al, 2004). Reducing cell contraction allowed for a uniform tissue layer to form on the CPP. In addition, it was determined that by optimizing MSC seeding density as well as adjusting TGFβ3 exposure time, tissue could be produced that has many of the histological and biochemical hallmarks of hyaline cartilage. This abbreviated chapter describes a series of pilot studies designed to investigate the utility of strategies that logically emerged from common issues described in the literature review, namely tissue contraction on substrates, choosing the appropriate cell seeding density, and length of TGFβ3 exposure.

Materials and Methods

Experimental Designs

Cell contraction study (Figure 1): The aim was to determine whether the addition of a fibronectin coating and/or the addition of the ROCK inhibitor Y-27632 (ROCKi) reduced MSC contraction on the CPP. Previous membrane culture experiments revealed that the addition of ROCKi reduced MSC contraction rates to fibronectin-coated membrane substrates to 0% (Appendix 1). Thus, it
was determined whether ROCKi reduced MSC contraction on CPP as well. Five CB-MSC cultures were expanded in monolayer until 80% confluency. At passage 5, 2×10^6 CB-MSCs were seeded on CPPs that were either coated with fibronectin or uncoated, with or without the addition of ROCKi. Contraction was scored on Day 2 (48 hours post-seeding), where the presence of contraction (sphere forming on CPP) was determined to be 100% contraction, and uniform cell distribution was determined to be 0% contraction.

**Cell density study (Figure 2):** The aim was to determine an optimal cell seeding concentration to produce thick neocartilage on top of the CPP. Three CB-MSC cultures were expanded in monolayer until 80% confluency. At passage 5, CB-MSCs were seeded at different cell concentrations (0.5×10^6, 0.67×10^6, 1×10^6, 1.5×10^6, and 2×10^6 cells) on CPPs in MSC expansion medium containing ROCKi, which was then replaced the following day with chondrogenic medium containing ROCKi. MSC continued to be cultured in the ROCKi chondrogenic medium for 2 days, then removed. After 3 weeks, tissues were evaluated for histology, immunohistology, and biochemical content (see Chapter 3).

**TGFβ3 exposure study (Figure 3):** The aim was to determine whether a longer culture time and/or varying the duration of TGFβ3 exposure altered the quality of neocartilage generated. Three CB-MSC cultures were expanded in monolayer until 80% confluency. At passage 5, 1.5×10^6 CB-MSCs were seeded on CPPs in medium containing ROCKi. Tissues were generated under 3 culture conditions: 3 weeks total culture time with continuous TGFβ3 exposure (3 week continuous group), 6 weeks total culture time with 3 weeks of TGFβ3 exposure followed by an additional 3 weeks of culture without TGFB3 (transient TGFβ3 group), and 6 weeks total culture time with continuous TGFB3 exposure (continuous TGFβ3 group). Tissues were evaluated for histology, immunohistology, biochemical content and mRNA expression (see Chapter 3).
Isolation and expansion of MSCs

Cryopreserved cord blood MSCs (CB-MSCs, n=5 for cell adherence study, n=3 for cell density and TGFβ3 exposure studies), isolated as previously described (Koch, Thomsen, & Betts, 2009), were thawed and plated onto polystyrene flasks. Expansion culture medium consisted of DMEM-LG (Lonza), 30% FBS (Sigma), 10,000 U/ml penicillin-streptomycin (Sigma), and 5,000 U/ml L-glutamine (Sigma). Cells were expanded until they reached 80% confluency, then were trypsinized (0.04% trypsin-EDTA) and induced to differentiate at passage 5.

Direct seeding and differentiation of MSCs on CPP

CPP cylinders (4 mm wide by 2 mm high) were generated by gravity sintering calcium polyphosphate powders as previously described (Waldman et al., 2002). The CPPs were placed in Tygon tubing and autoclaved, then soaked in 1X tris buffered saline for 24-72 hours. For the cell adherence study, 20 CPPs were coated with 10% fibronectin (Millipore) in DMEM-LG as described in Chapter 3. Various concentrations of cells (0.5x10⁶, 0.67x10⁶, 1x10⁶, 1.5x10⁶, and 2x10⁶ cells) were seeded on top of CPPs in 40 µl of MSC expansion media. The following day, the medium was exchanged to chondrogenic induction medium composed of DMEM high glucose (DMEM-HG) (Sigma-Aldrich), 1X ITS+ premix (BD Biosciences), 10 mg proline (Sigma-Aldrich), 100 nm dexamethasone (Sigma-Aldrich), 100 mM sodium pyruvate (Invitrogen; Burlington, ON, Canada), 200 mM GlutaMAX (Invitrogen), 100 µg/ml ascorbic acid (Sigma-Aldrich), and 10 ng/ml TGFβ3 (R&D Systems). For all three experiments, 10 µM of Y-27632 (ROCK inhibitor; Sigma-Aldrich) was added to select wells for the first 72 hours of culture (including initial MSC expansion medium and chondrogenic induction medium). Media was completely exchanged every other day for 3 to 6 weeks. In the continuous exposure groups, TGFβ3
was maintained in the chondrogenic induction medium for the duration of tissue generation, either 3 weeks or 6 weeks. In the transient exposure group, TGFβ3 was maintained in the chondrogenic induction medium for 3 weeks, followed by 3 weeks of culturing in chondrogenic induction medium in the absence of TGFβ3.

Native cartilage and primary chondrocytes as positive biological controls

Biochemical and mRNA expression analysis of CB-MSC-derived neocartilage in the TGFβ3 exposure study was compared to that of native equine articular cartilage and primary equine chondrocytes. Full thickness native cartilage was isolated from the distal aspect of the cannon bone from adult female horses of various breeds (n=3). The animals ranged in age from 15 to 19 years, and were euthanized for reasons unrelated to this study. For biochemical analysis, cartilage was snap frozen in liquid nitrogen and stored at -80°C. Tissues were digested in 80 µg/ml papain (Sigma-Aldrich) for 48 hours and then frozen at -20°C until further analysis. For mRNA expression, primary chondrocytes were isolated from fresh cartilage by collagenase digestion. Tissue was minced with a scalpel and digested overnight at 37°C in 1.5 mg/ml collagenase type I (Sigma-Aldrich). The samples were then strained through a 70 um filter and the RNA was immediately isolated using the mirVana total RNA isolation kit (ThermoFisher).

Histology and Immunohistology

After 3 and 6 weeks, newly generated neocartilage adhered to the CPP and required the use of a scalpel to isolate from the construct. This neocartilage was processed as previously described (Chapter 3). For histological analysis, slides were stained with hematoxylin and eosin, toluidine blue or double stained with toluidine blue and von Kossa, then visualized using light microscopy as previously described (Co et al, 2014; Lee et al, 2015). Immunohistochemistry for type I and type II collagen was performed.
type II collagens was performed and visualized as previously described (Co et al, 2014, Chapter 3). Negative controls were treated in parallel, but without the addition of a primary antibody.

**Biochemical analysis**

Tissues were digested in 40 µg/ml papain (Sigma-Aldrich) for 48 hours and then frozen at -20°C until further analysis. DNA, glycosaminoglycan and hydroxyproline contents were quantified as previously described (Co et al, 2014; Lee et al, 2011).

**mRNA expression**

Neocartilage tissues were removed from CPP (using a scalpel), then snap frozen and stored at -20°C until ready for RNA extraction. The frozen tissue was pulverized using a Retsch® Mixer Mill (RETSCH, Haan, Germany) for 3 minutes at 30 Hz. Following bead milling, RNA isolation was done using the mirVana total RNA isolation kit (ThermoFisher), then the RNA was cleaned using the RNA Clean and Concentrator (Zymo Research, Irvine, California, USA).

RNA was DNase treated and reverse transcribed using the qScript cDNA Synthesis Kit (Quanta BioSciences, Beverly, MA). Quantitative PCR was performed on the CFX96 Touch™ Real-Time PCR detection system (Bio-Rad) with gene-specific primers (listed in Table 1). Chondrogenic gene expression was normalized to 18S RNA and relative to primary chondrocytes.

**Statistical analysis**

For all analyses, ordinary one-way ANOVA with Tukey’s multiple comparisons was performed. GraphPad Prism 6 Software was used to perform statistical analyses. One-way ANOVA with Tukey’s multiple comparisons was performed to determine the differences in biochemical content
and mRNA expression between all treatment groups and positive controls. Significance was assigned at p<0.05.

Results

MSC contraction on CPP

In the absence of the ROCK inhibitor Y-27632 (ROCKi), all cell cultures contracted and effectively detached completely or near completely from the CPP (Figure 2a, top), beginning as early as 24 hours following exposure to the chondrogenic induction medium (Day 2). In the presence of ROCKi, the contraction rate dropped to approximately 20%, and the MSCs formed a uniform cell layer on the CPP (Figure 2a, bottom, and Figure 2b). The addition of a fibronectin (fib) coating to the CPP did not reduce contraction without the addition of ROCKi, nor did it appear to further reduce contraction when treated in combination with ROCKi (Figure 2b).

Cell seeding density study: weight, histology and immunohistology

After 3 weeks of culture, tissues from the 5 seeding densities (0.5x10^6, 0.67x10^6, 1x10^6, 1.5x10^6, and 2x10^6 cells) were evaluated using histochemistry, immunohistochemistry, and biochemistry. Overall, tissue mass increased with cell density (Figure 4). Tissues generated from cells seeded at a higher initial density (e.g., 1.5x10^6 and 2x10^6) generated thicker, whiter, more opaque tissue after 3 weeks than tissues generated from lower seeding densities (e.g., 0.5x10^6, 0.67x10^6, 1x10^6). The tissues generated from cells seeded at a higher initial seeding density showed presence of cell lacunae and proteoglycan deposition in the matrix of H&E and toluidine blue stained sections (Figure 5). Regardless of their original seeding densities, all generated tissues demonstrated positive staining for type I and type II collagen (Figure 5).
**Cell seeding density study: Biochemistry**

Tissues from each group were further evaluated for total collagen, sulfated GAG and DNA content. As expected, total GAG and collagen increased with increasing cell density, but did not differ significantly between the 1.5x10^6 and the 2x10^6 groups (Figure 6a, b) and GAG levels did not differ significantly among the various seeding densities when normalized to DNA content (Figure 6c). However, collagen levels normalized to DNA content were significantly higher in the 1x10^6, 1.5x10^6, and 2x10^6 groups compared to the 0.5x10^6 and 0.67x10^6 treatment groups (Figure 6d). Based on these findings, a cell seeding density of 1.5x10^6 was employed for the TGFβ3 exposure study.

**TGFβ3 exposure study: wet-weight, histochemistry and immunohistochemistry**

In vitro generated neocartilage demonstrated a similar appearance (comparatively thick, glistering and opaque; Figure 8) and mass (Figure 9) at both the 3 weeks and 6 weeks of culture time points. However, at 6 weeks of culture time both transient and continuous TGFβ3 exposure produced neocartilage that was relatively thicker than the 3 week tissues, with larger-sized cell lacunae. Six week tissues also appeared to have an increased cell density near the tissue-CPP interface. Toluidine blue staining revealed proteoglycan accumulation in all tissue cultures at both 3 and 6 weeks (Figure 10). However, none of the cultures revealed evidence for mineralization following von Kossa staining (Figure 10). Tissues stained positively for collagens type I and II. Type I and II immunoreactivity did not appear to differ between the three groups (Figure 9). A cell-free matrix layer formed at the superficial aspect of the tissues in both of the 6 week groups; this is also observed in the 3 week cultures, though the layer appears thinner.
TGFβ3 exposure study: biochemistry and mRNA expression

No significant differences were detected between groups for total sulfated GAG, total collagen, total DNA, GAG/DNA content and collagen/DNA content (Figure 11). However, total collagen, GAG/DNA and collagen/DNA did differ significantly when compared to values obtained for native articular cartilage (p<0.05, p<0.05, and p<0.0001, respectively). Total DNA only differed significantly from 6 week continuous to native cartilage (p<0.05). mRNA expression was also evaluated for the 3 week, 6 week transient and 6 week continuous TGFβ3 groups (Figure 12). Col1a2 expression was significantly higher in the 3 week and 6 week continuous TGFβ3 group compared to primary chondrocytes (p<0.05). Col2a1 expression was also significantly higher in the 6 week continuous group compared to MSCs and primary chondrocytes (p<0.05). Sox9 showed no significant differences between groups and undifferentiated MSCs, but was significantly higher in chondrocytes (p<0.005), while Runx2 (a marker for osteogenesis) was significantly higher in both of the 6 week groups (p<0.05 for 3 week versus MSC and chondrocytes, p<0.005 for 6 week groups versus MSC and chondrocytes).

Discussion

To date, scaffold-free cartilage has been generated on bone substitutes, such as CPP, by seeding primary chondrocytes or pre-chondrocytes differentiated from MSCs on the substrate (Kandel et al, 2006; Lee et al, 2011). However, attempts at seeding MSCs directly on CPP have been largely unsuccessful as a result of cell contraction on the substrate (Lee et al, 2011). Here, it is reported that the addition of a ROCK inhibitor virtually eliminated this problem, allowing for reliable, uniform MSC seeding on the CPP.
The Y-27632 ROCK inhibitor (ROCKi) acts by preventing phosphorylation of the myosin light chain (Riento & Ridley, 2003), thereby preventing cell contraction. Cell contraction, and the associated contractile phenotype, is a known antagonist of in vitro chondrogenesis in 3D culture (Rottmar et al, 2014; Yoo et al, 1998). A total exposure time of 72 hours to ROCKi was sufficient to prevent cell contraction on CPP in 80% of cultures. At present it is unknown if a longer exposure to ROCKi would significantly improve or diminish tissue quality on CPP. Inhibition of ROCK in chondrocytes has been shown to increase Sox9 gene expression (Woods, Wang, and Beier 2005). However, this may only occur in monolayer culture, as increased expression of Sox9 target genes was not found in chondrocyte pellet culture (Woods and Beier 2006). No obvious differences in terms of histology or immunohistochemistry were seen in membrane culture tissues when ROCKi was supplemented for 2 weeks versus 48 hours (see Appendix 1). As a result, it is expected that brief exposure to ROCKi did not significantly diminish the chondrogenic induction in our CPP cultures.

In the seeding density experiment, total sulfated GAG content was similar among the three lower seeding densities (0.5, 0.67, and 1x10⁶ cells seeded), and significantly increased at the 1.5x10⁶ and 2x10⁶ seeding densities. However, GAG/DNA content was not significantly different across all seeding densities, consistent with previous studies on human MSCs that observed a consistent GAG/DNA content with seeding densities varying from 3-48 x 10⁶ cells/ml (Ponticiello et al, 2001). In contrast, other groups have reported that total GAG/DNA content was significantly lower at higher seeding densities (Huang et al, 2009; Kavalkovich et al, 2002). It is worth noting that the higher seeding densities in these previous studies (50-60x10⁶ cells/ml, construct diameter of 4-6 mm) corresponded with the highest seeding densities tested in this study. It therefore
remains possible that with further increases in cell number seeded on CPP, a decline in GAG/DNA deposition may be observed.

Total collagen was also significantly increased in the higher seeding densities, though the 2x10^6 group did not significantly differ from the 1x10^6 group. This pattern was most similar to collagen/DNA, where 1, 1.5, and 2x10^6 seeding densities showing the highest values, with 1.5x10^6 displaying the highest level of collagen/DNA. In contrast, other studies using agarose gels did not observe differences in total collagen among different seeding densities (Erickson et al, 2012; Huang et al, 2009). It is speculated that the observed plateau of collagen deposition is consistent with the notion that there is a threshold at which matrix deposition is reached within a given number of differentiating cells in a specified space and/or volume (Huang et al, 2009). At higher seeding densities, perhaps 2x10^6 cells and above in our CPP cultures, formation and/or secretion of matrix components may in fact be inhibited. Based on our histological, immunohistological, and biochemical analysis, 1.5x10^6 cells per CPP is the preferred seeding density, and thus further optimizations were carried out using this seeding number to improve tissue quality.

In the TGFβ3 exposure experiment, differences in matrix components were not observed among any of the groups. In contrast, Lee and colleagues (2011) reported that pre-differentiated MSCs grown on CPP for 4 weeks were significantly lower in GAG/DNA and collagen/DNA content than cultured tissues grown for 8 weeks (Lee et al, 2011). This may indicate that after 3 weeks of culture, the differentiated MSCs may have reached their threshold for the synthesis and/or secretion of matrix components, or it may indicate that longer culture time may be necessary to for a difference to be observed. The previously reported work pre-differentiated MSCs for 3 weeks in membrane culture followed by 4 and 8 weeks of CPP culture, therefore totalling a 7 week and 11 week culture period (Lee et al, 2011).
Varying the length of TGFβ3 exposure in the 6 week tissues did not appear to affect GAG and collagen levels. Previous studies investigating transient vs. continuous exposure of TGFβ3 in chondrocytes revealed a marked increase in GAG synthesis and the compressive modulus was observed upon removing TGFβ3 after 2 weeks (Byers et al, 2008; Ng et al, 2011). Both studies seeded chondrocytes within agarose hydrogels and cultured the cells for 4 weeks (Ng et al, 2011) or 8 weeks (Byers et al, 2008). MSC chondrogenesis, on the other hand, seems to respond differently to varying TGFβ3 exposure times in a context-specific manner. In agarose gels, MSCs cultured for 7 weeks showed no significant differences in GAG or collagen content in transient (3 week) or continuous TGFβ3 supplementation, though the equilibrium compressive modulus was higher in the transient group. However, when the same MSCs were induced in pellet culture, GAG was significantly increased while collagen levels were not (Huang et al, 2009). Gupta and Nicoll (2015) demonstrated that transient TGFβ3 (2 weeks) resulted in a greater accumulation of collagens I and II after 8 weeks of MSC culture in hydrogel, but that the continuous group had significantly greater GAG (Gupta & Nicoll, 2015). In addition, they observed that the compressive modulus and peak stress were higher in the continuous group. Thus, transient TGFβ3 supplementation appears to be sufficient for collagen and GAG deposition, though further studies are necessary to determine which exposure time (if any) confers significantly greater mechanical properties.

In the TGFβ3 exposure time experiment, collagen content in the generated neocartilage reached only about 25% of native cartilage values. Lack of collagen accumulation is a recognized pitfall of tissue engineered cartilage and other studies have similarly reported comparatively low collagen values (Eyrich et al, 2007; Hu & Athanasiou, 2006a; Miot et al, 2006). Lack of collagen is usually correlated with reduced mechanical properties, compared to native cartilage (Vunjak-
Novakovic et al., 2002). Varying growth factor supplementation, such as IGF-1 (Jenniskens et al., 2006), has been shown to improve collagen synthesis. In addition, the application of mechanical stimulation also drastically improves collagen deposition, distribution, and subsequently tensile strength of the resulting neocartilage (Elder & Athanasiou, 2008; Hu & Athanasiou, 2006b; Mauck et al., 2007; O’Conor, Case, & Guilak, 2013). Neocartilage tissue generated on CPP showed higher GAG content than collagen, although these values were still lower than typically observed in native cartilage. Early and perhaps excessive deposition of GAG may interfere with collagen secretion, and is also speculated to result in inferior tissue mechanical properties (Responsye, Natoli, & Athanasiou, 2007).

In addition to producing matrix components, MSCs are expected to display a chondrogenic phenotype after induction that persists for extended in vitro culture and eventual tissue implantation. It has been shown here that the expression of Col1a2, which is associated with the dedifferentiation of chondrocytes and loss of the chondrocytic phenotype, is significantly higher in undifferentiated MSCs and generated neocartilage from all three groups compared to primary chondrocytes. A significant upregulation of Col2a1 compared to MSCs and chondrocytes was observed, but no significant differences were seen between treatment groups. This is in contrast with the pattern observed in human MSCs undergoing chondrogenesis, where TGFβ withdrawal resulted in decreased Col1a2 but increased Col2a1 expression (Buxton et al., 2011). Increased expression of Col1a2 has been reported in high density micromass cultures during in vitro differentiation (Tallheden et al., 2004) and may indicate the eventual formation of less desirable fibrocartilage if expression persists upon tissue maturation in vitro or in vivo (Bedi, Feeley, & Williams, 2010). ACAN expression is another marker of hyaline cartilage, does not differ
significantly between treatment groups either; this is consistent with what other groups reported (Buxton et al, 2011; Huang et al, 2009).

*Sox9*, widely recognized as the master regulator of chondrogenesis, showed no difference in expression among treatment groups or undifferentiated MSCs. However, all groups had significantly lower expression of *Sox9* than native primary chondrocytes, consistent with the decreased expression of this transcription factor observed in equine CB-MSC pellet culture by Day 21 relative to primary chondrocytes (Buechli, Lamarre, & Koch, 2013). Interestingly, the *Sox9* transcription factor has been shown to repress Runx2 (Zeng et al, 2002), which may explain the increased levels of mRNA expression seen in *Runx2* over primary chondrocytes.

*Col10* expression was not detected in undifferentiated MSCs or the three treatment groups. *Col10* expression is a marker of hypertrophic chondrocytes, and type X collagen is deposited prior to tissue calcification (Cancedda et al, 2003). *Col10* has been shown to be upregulated with continuous TGFβ3 application in MSC chondrogenesis (Buxton et al, 2011; Gupta and Nicoll 2015). MSC differentiation typically continues on with eventual hypertrophy and further mineralization of the tissue engineered cartilage, as it mimics endochondral ossification (Mueller & Tuan, 2008). Interestingly, the upregulation of *Runx2*, a marker of osteogenesis, has been shown to enhance expression of *Fgf18*, a regulator of chondrocyte maturation and an inhibitor of hypertrophy (Hinoi et al, 2006). It is possible that the increased expression of *Runx2* in the 6 week transient and 6 week continuous groups affects the *Col10* expression in the developing neocartilage, however further work to examine the expression of hypertrophy inhibitors such as *Fgf18* is required. Gene expression of undifferentiated MSCs and generated neocartilage was relative to primary chondrocytes, which were derived from older animals with no signs of osteoarthritis (see Materials and Methods). However, it is unknown whether primary chondrocytes
from younger animals may be more similar to the generated neocartilage with respect to gene expression.

Neocartilage thickness appeared to increase slightly by 6 weeks of culture. The layer of cell-free matrix observed at superficial aspect of the tissue, which varies in thickness depending on the cell culture and time in culture, does not occur in native cartilage and is thus a by-product of our tissue engineering method. It is speculated that this may be a result of matrix deposition in the absence of mechanical stimulation and/or media perfusion in prolonged (6 week) culture. As the cell-free layer stains positively for toluidine blue and type I and II collagen, there appears to be an accumulation of proteoglycans and collagen on the superficial aspect of the tissue. Mechanical stimulation has been shown to improve the distribution of proteoglycans (Khoshgoftar et al, 2014), perhaps serving to more homogenously distribute proteoglycans and collagen and improve the tissue’s mechanical properties. In addition, Freyria and colleagues (2004) observed an increase in cell proliferation and GAG deposition, as well as a more homogenous distribution of cell and matrix components, with media perfusion versus static culture. More homogenous matrix dispersal was associated with a noted decrease in the superficial matrix layer formed in their static culture tissues after 3 months (Freyria et al, 2004). Experimenting with mechanical stimulation and/or perfusion may cause this superficial cell-free layer to diminish or disappear, perhaps resulting in more homogenous tissue throughout.

No mineralization was detected with von Kossa staining in the absence of supplemented organic phosphate at the cartilage-CPP interface or elsewhere throughout the tissue. This is consistent with what has been seen with pre-differentiated sheep BM-MSCs induced to form cartilage on CPP, even with the addition of β-glycerophosphate (Lee et al, 2011). Polyphosphate release from the CPP has been suggested to inhibit tissue mineralization (Allan et al, 2007). As
cartilage calcification at the interface greatly reduces the risk of delamination, induction of mineralization is a logical next step to improving the construct’s shear strength.

The work described in this chapter demonstrates that osteochondral constructs can be generated with equine CB-MSCs by directly seeding the cells on top of a CPP substrate in the presence of the ROCK inhibitor Y-27632 for the first 72 hours of culturing.

Acknowledgements

I sincerely thank Justin Parreno, Vanessa Bianchi and Matthew Vickaryous for their intellectual contributions.
Figures

Figure 1. Experimental design for cell contraction study. CB-MSCs (n=5) were seeded on CPP in four different treatment conditions: no ROCK inhibitor and no fibronectin (-ROCKi -fib), no ROCK inhibitor with fibronectin (-ROCKi +fib), with ROCK inhibitor and no fibronectin (+ROCKi -fib), and with ROCK inhibitor and with fibronectin (+ROCKi +fib). Adherence was scored on Day 2.
Figure 2. Experimental design for cell density study. CB-MSCs (n=3) were seeded on CPP at 5 different seeding densities (0.5x10^6, 0.67x10^6, 1x10^6, 1.5x10^6, 2x10^6) and cultured for 3 weeks. 3 CPPs were seeded for each condition per cell line, and resulting tissues were cut in half for evaluation (1/2 tissues).
Figure 3. Experimental design for TGFβ3 duration study. CB-MSCs (n=3) were seeded on CPP and cultured for 3 or 6 weeks. The culture conditions were as follows: 3 weeks total culture time with TGFβ3 (3 week continuous), 6 weeks total culture time with 3 weeks exposure to TGFβ3 followed by 3 weeks without TGFβ3 (6 weeks transient), and 6 weeks total culture time with 6 weeks exposure to TGFβ3 (6 weeks continuous).
Figure 4. ROCK inhibition reduces MSC contraction on CPP. a) Top panels: hatched line indicates contracted MSCs without ROCKi on day 2 post-seeding. Bottom panel: Uniform adherence of MSCs to CPP in the presence of ROCKi on day 2 post-seeding. b) 100% of all cell lines and replicates contracted without ROCKi, even with the presence of a fibronectin (fib) coating on the CPP. n=5, b=p<0.0001.
Figure 5. Wet weight of tissues generated from different seeding densities after 3 weeks of culture on CPP. Means sharing the same superscript are not significantly different from each other (Tukey’s, p<0.05). n=3 cell lines, 3 replicates each.

Figure 6. Histology and immunohistochemistry of neocartilage generated from various cell seeding densities. Hematoxylin and eosin (H&E), toluidine blue (tol blue), type II collagen (col-II) and type I collagen (col-I) staining and immunostaining on tissues from three different MSC cultures generated from different seeding densities after 3 weeks of culture on CPP. Insets represent negative controls for immunostaining.
Figure 7. Total GAG, total collagen, total DNA, GAG content and collagen content normalized to DNA in neocartilage generated from various seeding densities after 3 weeks in culture. Means sharing the same superscript are not significantly different from each other (Tukey's, p<0.05). n=3 cell lines, 3 replicates each.
Figure 8. Macroscopic images of 3 week and 6 week tissues on CPP.

Figure 9. Wet weight of CPP tissues. Cont: continuous; trans: transient.
Figure 10. Hematoxylin and eosin (H&E), toluidine blue and von Kossa (Tol blue/von Kossa), type II collagen (Col-II), and type I collagen (Col-I) stain of 3 week continuous, 6 week transient, and 6 week continuous CPP tissues from one representative cell culture. Insets represent negative (omission) controls. See Appendix 2 for images from all three MSC cultures.
Figure 11. Total GAG, total collagen, total DNA, GAG content and collagen content in 3 week continuous, 6 week transient, 6 week continuous CPP tissue and native cartilage. Means sharing the same superscript are not significantly different from each other (Tukey's, p<0.05). n=3 cell lines, 3 replicates each.
Figure 12. Quantitative RT-PCR of chondrogenesis- and osteogenesis-associated gene expression on 3 week continuous, 6 week transient, 6 week continuous CPP tissue and primary equine chondrocytes. mRNA expression is normalized to 18S RNA and relative to undifferentiated MSCs (Day 0). Means sharing the same superscript are not significantly different from each other (Tukey's, p<0.05). n=3, cell lines, 3 replicates each (pooled).
Tables

<table>
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<tr>
<th>Gene</th>
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<td>Sox9</td>
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<td>Co et al, 2014</td>
</tr>
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| Runx2        | AACCCACGGAAGCAGATGCACTATCCA | GGGACATGCCGTAGGTAAGT | Figueroa et al,
| 18S rRNA     | GTAACCCGTTGAAACCCATT | CCATCCAATCGGTAGTAGCG | Chen et al, 2003|

Table 1. Primer pairs used for qPCR.
General discussion and perspectives

Tissue engineering has emerged as a promising alternative to current surgical treatments in the repair of focal cartilage defects. While much progress has been made to improve tissue engineering protocols in the realm of human medicine, the equine veterinary field has not advanced at the same rate. The debilitating impact that cartilage defects have on the equine industry, coupled with the horse representing a strong animal model for studying human cartilage injuries and repair modalities, suggests the need from more work to be done on this species. In this thesis, the chondrogenic potential of two types of progenitor cells was investigated: equine iPSCs and MSCs. Following this, I evaluated whether hyaline-like MSC-derived neocartilage tissue could be generated on top of a bone substrate to create in vitro osteochondral constructs. The research within this thesis has not only provided evidence that progenitor cells vary quite widely in their propensity to undergo chondrogenic differentiation, but also outlines a novel direct cell seeding method of creating osteochondral constructs from equine MSCs.

The investigation of suitable progenitor cells is an essential first step in the process of tissue engineering. Equine iPSCs were first explored for their potential in chondrogenesis. Previously, attempts at differentiating equine iPSCs alone or in co-culture with bovine chondrocytes in membrane culture were unsuccessful (Nagy and Kandel labs, unpublished). Several groups have reported on the requirement of a differentiation step to first generate embryoid bodies, followed by the subsequent isolation and culture of adherent fibroblastic cells, to successfully differentiate iPSCs into chondrocytes (Chen et al, 2012; Guzzo et al, 2013; Wei et al, 2012). As embryoid body formation leads to the generation of a heterogeneous group of cells, a method to derive MSCs from equine iPSCs was sought after to generate a more homogenous population of precursor cells prior to inducing chondrogenesis. Using a serial plating protocol and defined MSC induction media,
iPSCs were differentiated into MSC-like cells with spindle-like morphologies. These MSC-like cells also demonstrated a downregulation of pluripotency genes, an upregulation of MSC-associated genes, and shared the same expression pattern of surface markers as previously reported for equine CB-MSCs. However, while it was found that iPSC-derived MSCs could be induced along osteogenic and adipogenic lineages, they failed to undergo chondrogenesis in pellet culture.

TGFβ3 is a known inducer of chondrogenesis and is the most commonly used TGFβ isoform when differentiating MSCs (Hao, Varshney, & Wang, 2008). Indeed, this growth factor alone is sufficient to induce equine MSCs to undergo chondrogenesis (Berg et al, 2009; Co et al, 2014; Koch et al, 2007). Other growth factors are also known to participate in chondrogenesis. For example, TGFβ1, another TGFβ isoform, has been shown to promote chondrogenic differentiation in equine MSCs (Hgewald et al, 2004; Kopesky et al, 2014; Worster et al, 2000). In addition, members of the BMP family (part of the TGFβ superfamily), such as BMP-2 and BMP-4, have also been shown to be sufficient in differentiating MSCs into chondrocytes (Hatakeyama, Tuan, & Shum, 2004; Liao et al, 2014; Schmitt et al, 2003; Yoon & Lyons, 2004). IGF-1, FGF-2, and GDF-5 (part of the TGFβ superfamily) have also been implicated in the induction of chondrogenesis (Feng et al, 2008; Ge et al, 2006; Kou & Ikegawa, 2004; Park & Na, 2008; Schmidt, Chen, & Lynch, 2006). While our iPSC-MSCs failed to differentiate in chondrogenic induction media containing TGFβ3 and/or BMP-4, further investigation into whether these additional factors can stimulate chondrogenesis in equine iPSCs or iPSC-MSCs would provide more information as to the chondrogenic potential of these cells.

Unexpectedly, the differentiation technique described in Chapter 2 was only successful in one equine iPSC culture. Consistent with previous findings (Pevsner-Fischer, Levin, and Zipori 2011), equine MSCs from different donors have shown highly variable differentiation capacities
(Chapter 3). Thus, it remains unknown whether the lack of chondrogenesis observed in equine iPSC-MSCs is representative of all iPSC donors or if the particular equine iPSC line used has an intrinsic low propensity for cartilage differentiation.

Donor variability represents an enormous obstacle when assessing the practical development of cell based therapies. Compounding the issue of donor-to-donor variation is the fact that MSCs are frequently isolated from different sources. Therefore, expanding upon the scope and depth of an earlier study (Berg et al, 2009), the chondrogenic potential of equine MSCs from two sources: umbilical cord blood (CB-MSCs) and bone marrow (BM-MSCs) was explored. Previous work suggested that CB-MSCs were more chondrogenic than BM-MSCs, although only a limited number of cultures were tested. Therefore, the power of this study was increased to interrogate 10 unrelated CB-MSC cultures and 10 unrelated BM-MSC cultures to determine which source showed a higher potential for chondrogenesis. The chondrogenic assessment was performed using membrane culture, which has been shown to be superior to pellet culture (Co et al, 2014; Lee et al, 2011) and thus would be more representative of our eventual goal of creating implant-grade cartilage. In addition, tissues were analyzed at three time points with multiple evaluation tools, including histology, immunohistology, biochemistry, and gene expression.

Tissue quality varied widely among donors from both MSC sources. Broadly stated, poor quality tissue formed limited matrix and stained weakly for toluidine blue and type II collagen. In contrast, good quality tissue was comparatively thicker, stiffer, and more opaque, and stained intensely for toluidine blue and type II collagen. The sampled tissues were then quantified at all three time points (Day 7, 14, and 21) for sulfated GAG and collagen content. Unexpectedly, there were no significant differences found between MSC sources at any of the time points assessed. In addition, while mRNA expression of ACAN and Sox9 was higher in BM-MSCs at Day 7, there
were no significant differences between sources for any of the remaining genes. All of these data, combined with variance component analysis of the biochemical data, points to donor being a larger contributor to variance than MSC source.

With the results presented here, it cannot be stated conclusively whether CB-MSCs or BM-MSCs are better candidates for cartilage tissue engineering using the membrane culture system. Rather, this work strongly indicates that chondrogenic potency comes from individual cell cultures, rather than a particular source of MSCs. In this case, the question that follows is: what makes any given MSC population have good chondrogenic potential? It has been shown in BM-MSCs that the age of the donor has an effect on the chondrogenic potential of the donor’s cells (Kretlow et al, 2008). However, CB-MSCs are all of neonatal origin (and thus donors are the same age), though it has not been addressed whether the age of the mare or her number of births correlates with the potency of the derived MSCs. Some studies have demonstrated that variations in isolation practices and/or cell propagation and passaging might have an effect on subsequent cell proliferation (Iftimia-Mander et al, 2013; Surdo and Bauer 2012; Wagner & Ho 2007). Alternatively, the passage number of the MSCs may also affect the variability seen. Generally speaking, increased passage number leads to a decreased differentiation potential (Kretlow et al, 2008; Tan et al, 2015; Zaim et al, 2012). However since CB-MSCs have been shown to senesce later than BM-MSCs, they may prove to be more chondrogenic at later passages (Jin et al, 2013). Lastly, differences seen in chondrogenic potential may be related to basal expression differences in undifferentiated MSCs in genes or surface markers associated with chondrogenesis (Campbell & Pei, 2012). The CD56 surface marker, for example, has been correlated with increased pellet size in CD56+ BM-MSCs (Battula et al, 2009).
Previously, osteochondral-like plugs have been successfully generated using sheep BM-MSCs and equine CB-MSCs (Co 2013; Lee et al, 2011). However, generation of scaffold-free uniformly attached neocartilage tissue on top of a bone substrate required the pre-differentiation of MSCs in membrane culture for 2-3 weeks (Lee et al, 2011). As the overarching goal of this thesis is to optimize the generation of these constructs for eventual in vivo implantation, the improvement of the initial cell seeding step was investigated to reduce culture time, reagent use, and the initial number of cells required. The intermediate MSC pre-differentiation step was necessary in earlier works, as direct seeding of the undifferentiated MSCs onto the CPP bone scaffold resulted in contraction upon the initiation of chondrogenic induction (Lee et al, 2011). A contractile phenotype is not conducive to chondrogenesis (Rottmar et al, 2014), and thus a balance must be met to ensure adequate cell communication without initiating cell structural changes.

The phenomenon of cellular contraction has also been observed during membrane culture conditions (Co et al, 2014), though to a lesser extent than that observed on the CPP. In an effort to reduce contraction in membrane culture, the ROCK inhibitor Y-27632 (ROCKi) was used, which reduced contraction rates from ~40% to ~0% (Appendix 1). A brief addition of ROCKi to MSCs seeded directly on CPP (for the first 72 hours of culture) greatly reduced cell contraction on the bone substrate as well. This permitted the sustained culture of a uniformly attached multi-layered cell sheet, where the high cell density within the confines of the CPP and surrounding Tygon tubing presumably promoted increased cell-to-cell contact and chondrogenic signaling. This breakthrough set the foundation for our subsequent work in improving the quality of the cartilage tissue generated on top of the CPP.

For the remainder of the thesis work, the optimization of cell seeding density, duration of culture, and TGFβ3 exposure time was investigated in the generation of osteochondral constructs.
A moderately high seeding density (1.5x10^6 cells per CPP) was found to produce thick, hyaline-like cartilage tissue with high GAG and collagen levels after 3 weeks of culture. Subsequently, no differences between the levels of matrix components were found between cultures grown for 3 weeks, 6 weeks with continuous TGFβ3 exposure, and 6 weeks with transient TGFB3 exposure (first 3 weeks only).

One caveat to this study was that the investigation included only three CB-MSC cultures. As described in Chapter 3, significant donor variation was observed in chondrogenic potential among equine MSC cultures. To maximize the opportunity for success, three cultures were selected that generated the highest quality neocartilage in pellet and/or membrane culture. Two additional cell cultures that had low chondrogenic potency in membrane culture were tested and found that they produced no matrix on the CPP (data not shown). While two of our three CB-MSC cultures performed similarly in CPP culture, one culture (labeled 1108) produced thinner tissue (see Appendix 2) and correspondingly less total GAG and collagen. This may indicate that chondrogenic potential is influenced by the method of cartilage generation, and thus one method of producing neocartilage (e.g., membrane culture) is not necessarily a predictor of good quality tissue when moved to another system (e.g., CPP culture). Thus, it is very difficult to predict the chondrogenic potential of any given equine MSC donor, and future studies investigating other predictive methods will be invaluable in moving forward with osteochondral construct engineering.

Several other factors could also be considered for further optimization. Firstly, the mechanical properties of the newly generated neocartilage were not investigated. The mechanical properties of cartilage are important for maintaining the tissue’s integrity when force is applied. Tissue stiffness (compressive strength) is typically low in tissue engineered cartilage (Kock et al, 2014).
2012), although transient (but not continuous) exposure to TGFβ3 increases the compressive modulus (Gupta & Nicoll 2015; Huang et al, 2009; Ng, Ateshian, and Hung 2009). One strategy to improve tissue stiffness is to “prime” the tissue with mechanical stimulation prior to implantation. As little as one application of cyclic loading has been shown to significantly improve the compressive strength of engineered tissue over static culture (Waldman et al, 2006). This mechanical conditioning has also been shown to improve the distribution of deposited matrix components (O’Conor et al, 2013).

Shear strength is also a challenge when designing tissue engineered cartilage (Vahdati & Wagner, 2013). Strong integration between the cartilage tissue and the underlying subchondral bone or bone substitute is necessary to prevent delamination. Native osteochondral junctions are recognized by a tidemark, which marks the transition from unmineralized cartilage to calcified cartilage. This calcification is believed to reduce delamination at the cartilage/bone interface by force distribution (St-Pierre et al, 2012). As polyphosphate release from the CPP has been suggested to inhibit tissue mineralization (Allan et al, 2007), researchers investigated “coating” the CPP to prevent polyphosphate release. Using an inorganic sol-gel deposition method, hydroxyapatite films were deposited on the CPP, which allowed for the mineralization of deep zone chondrocytes to take place at the interface (St-Pierre et al, 2012). Lee and colleagues (2015) improved the shear strength their MSC-CPP osteochondral construct by first coating the CPP, then seeding a layer of T3 induced pre-differentiated MSCs, followed by another layer of cells without T3. The T3 supplementation induced mineralization at the MSC-CPP junction, with no other mineralization detected throughout the rest of the tissue (Lee et al, 2015). Future studies looking into the generation of a zone of calcified cartilage using equine CB-MSCs should also improve the construct’s shear strength.
Lastly, and most relevant to this issue of clinical repair, constructs will need to be tested in vivo. For our experiments we employed a CPP 4 mm² in diameter and 2 mm² in height; to investigate clinical-sized defects (>6 mm in diameter), the size of the constructs will have to be scaled up. With increased size, preventing tissue shearing will be even more important, and the mechanical properties of the generated cartilage must be sufficient to withstand forces in the articulating joint. Previous work done with chondrocytes seeded on CPP have shown that these constructs were maintained in induced defects in sheep joints for up to nine months’ post-implantation (Kandel et al, 2006). Upon finding the appropriate culture conditions and mechanical conditioning, our CB-MSC-based plugs can be tested in induced defects in the horse and evaluated for short-term safety and long-term efficacy.

**General conclusions**

In conclusion, the work presented in this thesis describes the generation of an MSC-like cell population from equine iPSCs, the contribution of donor variability to chondrogenic potential, and outlines a novel, one-step method for creating osteochondral constructs. Our discovery that donor variability plays a bigger role in the quality of membrane-based cartilage tissue highlights the challenges that still need to be overcome to standardize cell therapy and/or tissue engineering protocols using MSCs. Our one-step seeding procedure of CB-MSCs on a CPP bone substrate is an important step towards the replacement of autologous or cadaver osteochondral plugs with in vitro tissue engineered osteochondral constructs.
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Appendix 1: ROCK inhibitor improves membrane culture

Summarized Methods and Results

CB-MSCs were thawed, expanded, and seeded on prepared fibronectin-coated membranes in MSC expansion media as previously described in Chapter 3. 10 lines were assessed for adherence and 3 lines were assessed for the effect of ROCK inhibition on membrane culture chondrogenesis. EM = expansion medium, CM = chondrogenic medium.

To determine contraction rate: In the +EM group, 10 μM of ROCK inhibitor Y-27632 (ROCKi) was added to the expansion medium immediately upon cell seeding (Day 0). After 24 hours, the medium was changed to chondrogenic medium containing TGFβ3 and ROCKi (+EM/+CM group) (Day 1). In select cultures, ROCKi was only added to CM (-EM/+CM). The control group (-EM/-CM) received no ROCKi supplementation in either the expansion medium or chondrogenic medium. MSC contraction on the membrane substrate was observed on Day 2. Without ROCKi supplementation (-EM/-CM group), contraction rate is approximately 40%. However, with the addition of ROCKi in both the +EM/+CM and -EM/+CM groups, contraction decreased to 100% (Figure 2).

To determine effect of long-term exposure of ROCKi on membrane culture: CB-MSCs were seeded on membranes in expansion medium and switched to chondrogenic medium containing ROCKi after 24 hours. In the -ROCKi group, media was changed to CM without ROCKi 48 hours later. In the +ROCKi group, ROCKi was maintained in the media for the duration of culture. After two weeks, the tissues were evaluated by histology and immunohistochemistry. These preliminary results suggest that there is no difference in tissue quality between treatment groups.

Figures
ROCK inhibitor improves MSC attachment to membranes

Figure 1. Representative image of cell contraction trial on Day 2. A, B: ROCK inhibitor added to both MSC expansion media (+EM) upon seeding (Day 0) and to chondrogenic media (+CM) 24 hours later (Day 1). C, D: ROCK inhibitor added only to chondrogenic media (+CM). E, F: No ROCK inhibitor added (-EM/-CM); contraction can be seen within the hatched lines.

Figure 2. Percentage of cell adherence to membranes after 24 hours in chondrogenic media (CM; Day 2) for the three experimental conditions: No ROCK inhibitor (ROCKi), no ROCK inhibitor in EM (-EM, Day 0), but added to CM (+CM, Day 1 and ROCK inhibitor added to EM (+EM, Day 0) and to CM (+CM, Day 0). n=10, with 2 replicates per cell line. p<0.01, one-way ANOVA with Tukey’s post-hoc test.
**Prolonged ROCK inhibitor exposure has no apparent effect on chondrogenesis**

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Figure 3. Histochemical analysis of neocartilage tissue sections with continuous or transient application of the ROCK inhibitor (ROCKi). Hematoxylin and eosin (H&E) and toluidine blue (tol blue) stains of membrane culture tissues grown for two weeks with continuous ROCKi (+ROCKi) or only transient ROCKi added on days 1 through 3 (-ROCKi).
Figure 4. Immunohistochemical analysis of neocartilage tissue sections with continuous or transient application of the ROCK inhibitor. Type I collagen (Coll) and type II collagen (ColII) immunostaining of membrane culture tissues grown for two weeks with continuous ROCKi (+ROCKi) or only transient ROCKi added on days 1 through 3 (-ROCKi). Insets: negative controls (no primary antibody) for immunostaining.
Appendix 2: Histology and immunohistology of all cell cultures tested in the TGFβ3 exposure study

Figure 1. Hematoxylin and eosin stain of 3 week continuous, 6 week transient and 6 week continuous (x axis) CPP tissues from three MSC cultures (named 1108, 1204, and 1206, y axis).
Figure 2. Toluidine blue and von Kossa staining of 3 week continuous, 6 week transient and 6 week continuous (y axis) CPP tissues from three MSC cultures (named 1108, 1204, and 1206, x axis).
Figure 3. Type II collagen immunostaining of 3 week continuous, 6 week transient and 6 week continuous (x axis) CPP tissues from three MSC cultures (named 1108, 1204, and 1206, y axis). Insets represent omission (negative) controls.
Figure 4. Type I collagen immunostaining of 3 week continuous, 6 week transient and 6 week continuous (x axis) CPP tissues from three MSC cultures (named 1108, 1204, and 1206, y axis). Insets represent omission (negative) controls.