

**Membrane Culture and Reduced Oxygen Tension Enhances Chondrogenesis of  
Equine Cord Blood Mesenchymal Stromal Cells in Vitro**

**By**

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## **ABSTRACT**

### **Membrane Culture and Reduced Oxygen Tension Enhances Chondrogenesis of Equine Cord Blood Mesenchymal Stromal Cells in Vitro**

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Variations of culture conditions impact the quality of neocartilage generated in vitro from mesenchymal stromal cells (MSC). The objective of this study was to evaluate and compare the effect of reduced oxygen and membrane culture during in vitro chondrogenesis of equine cord blood (CB) MSC. CB-MSC (n=5 horses) were expanded at 21% oxygen prior to 3 week differentiation in membrane or pellet culture at 5% and 21% oxygen. Assessment included histological examination (H&E, Toluidine Blue, Immunohistochemistry for type I and II collagen), protein quantification by Hydroxyproline assay and Dimethylmethylene assay, and mRNA analysis for collagen IA1, collagen IIA1, collagen XA1, HIF1 $\alpha$  and Sox9. The combination of reduced oxygen and membrane culture produced neocartilage that most closely resembled hyaline cartilage. Membrane culture resulted in increased wet mass, homogenous matrix morphology and an increase in total collagen content, while 5% oxygen culture resulted in higher GAG and type II collagen content.

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## **DECLARATION OF WORK PERFORMED**

I declare that all work submitted in this thesis was performed by me with the exception of CB-  
MSC isolation which was performed by Crystal Tse and Lauren Tracey, and primer design by Laurence  
Tessier.

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## **LIST OF ABBREVIATIONS**

AC	Autologous chondrocytes
ACI	Autologous chondrocyte implantation
ACPC	Articular cartilage progenitor cells
ALP	Alkaline phosphatase
AT	Adipose tissue
B2M	Beta-2-microglobulin
BM	Bone marrow
CB	Cord blood
CBP	Creb binding protein
CM	Chondrogenic media
CPP	Calcium polyphosphate
DMMB	Dimethylmethylen blue
DMSO	Dimethyl sulfoxide
ECM	Extracellular matrix
ELIZA	Enzyme linked immunosorbent assay
EM	Expansion media
FBS	Fetal bovine serum
FIH	Factor inhibiting hormone
GAG	Glycosaminoglycan
H&E	Hemotoxylin & Eosin
HG-DMEM	High glucose Dulbeccos modified eagles media
HIF	Hypoxia inducible factor
IHC	Immunohistochemistry

iPSC	Induced pluripotent stem cells
LG-DMEM	Low glucose Dulbeccos modified eagles media
MACI	Matrix assisted autologous chondrocyte implantation
MMP-13	Matrix metalloprotease 13
MSC	Mesenchymal stromal cells
NCAM	Neural cell adhesion molecule
OSX	Osterix
PHD	Prolyl hydroxylase
PTFE	Polytetrafluoroethylene
PThrP	Parathyroid related protein
PTOA	Post traumatic osteoarthritis
qPCR	Quantitative real-time polymerase chain reaction
RunX2	Runt related transcription factor 2
SOX9	Sex determining region Y 9
TGF $\beta$	Transforming growth factor beta
VEGF	Vascular endothelial growth factor
VHL	Von hippel landau

# INTRODUCTION

Articular cartilage damage is a major contributor to musculoskeletal or limb injury in performance horses (Riggs, 2006). Articular cartilage damage may result from a single traumatic event or from progressive weakening of the tissue from repetitive high speed and high impact loading experienced by horses during racing or show jumping (McIlwraith and Trotter 1996; Riggs, 2006). If left untreated, the initial injury may progress into chronic osteoarthritis (OA), characterized as progressive and permanent deterioration of cartilage accompanied by joint inflammation (McIlwraith and Trotter 1996).

The equine industry contributes approximately \$19 billion dollars to the Canadian economy annually (Equine Canada, 2010). A major financial burden in the equine industry is the loss of performance days and wastage resulting from lameness due to orthopaedic injuries (Kaneene, Ross et al. 1997; Williams, Harkins et al. 2001; Boden, Anderson et al. 2006; Beisser, McClure et al. 2011). Thus there is high demand for permanent repair options for horses.

Multipotent mesenchymal stromal cells (MSC) are emerging as a potent source of cells to repopulate chondrocytes for cartilage repair. However, while MSC are readily acquired in large numbers and capable of undergoing chondrogenic differentiation, the quality of the generated cartilage is inferior in comparison to hyaline cartilage phenotype (Toh, Liu et al. 2005). Majority of in vitro generated neocartilage is of hybrid fibro-hyaline phenotype and express hypertrophic markers such as type X collagen (Markway, Tan et al. 2010; Buechli, Lamarre et al. 2012).

To address the issue of inferior cartilage, this thesis explores two important factors, mode of culture and oxygen tension, and their contribution to neocartilage phenotype using cord blood (CB)-MSC in vitro. The study compared the effect of 5% and 21% oxygen concentrations during in vitro

chondrogenesis of CB-MSC in two different culture systems, pellets and membranes. Assessment of generated neocartilage was done using histochemistry, immunohistochemistry (IHC) for type I and II collagens, biochemical quantification of total collagen content and glycosaminoglycan content (GAG), and mRNA expression analysis (for collagens 1A1, 2A1 and XA1, HIF1 $\alpha$  and Sox9). My results demonstrate that mode of culture and oxygen concentration are significant contributors to in vitro generated neocartilage. Under the set experimental groups, desirable cartilage phenotype was maximized in membrane culture at 5% oxygen. Under these culture conditions, the newly formed cartilage was matrix-rich with homogenous distribution of extra cellular matrix (ECM) and rich in GAG and type II collagen.

## LITERATURE REVIEW

### **What is cartilage?**

Cartilage is a stiff and flexible connective tissue composed of primarily water, ECM and resident cartilage cells or chondrocytes (Athanasίου, 2009). Unlike most other tissues, cartilage is typically avascular and aneural (Frisbie, 2012). Histologically, there are three main types of cartilage – elastic, fibrocartilage and hyaline (Athanasίου, 2009). Elastic cartilage contains elastin, and is found in the pinna and epiglottis (Krstic, 1985) Fibrocartilage is found in intervertebral disks and menisci. Fibrocartilage matrix is mainly composed of type I collagen bundles and low amounts of proteoglycan, giving this tissue its characteristic stiffness and strength (Athanasίου, 2009). Hyaline cartilage is normally found at the ends of long bones, ribs, larynx, tracheal ring and bronchi. It is dominated by type II collagen and has relatively high levels of hydrophilic proteoglycans, giving it great compressive strength (Frisbie, 2012).

### Articular cartilage

Articular cartilage consists of hyaline cartilage that covers the subchondral bone at the diarthrodial joint. Its function is to provide low friction movement and absorb and dissipate loading during skeletal movement (Poole, Kojima et al. 2001). The load bearing capability of articular cartilage is primarily attributed to articular cartilage's ability to remain hydrated under significant pressure. This is attributed to the osmotic pressure associated with high concentrations of proteoglycan and the unique configuration of collagen fibrils that provide great tensile strength (Todhunter, 1996; McCutchen, 1959).

In histological section, articular cartilage can be subdivided into 4 zones, each demonstrating a specific function and structural organization. From the articular surface to the subchondral bone these include: the superficial zone, transitional zone, radial zone (or mid zone) and deep zone (Figure 1). The superficial zone has high collagen content arranged parallel to the surface which functions to resist both shear and tensile force (Akizuki, Mow et al. 1986). The superficial zone has the lowest proteoglycan content but secretes and is covered by a film of lubricin, which is responsible for lubrication and friction

reduction (Buckwalter and Mankin, 1998). The transitional zone marks the transition from superficial to radial zone, and is characterized by an increase in proteoglycan content, a decrease in collagen content, and an alteration in chondrocyte morphology (cells become more rounded) and orientation (begin to adopt a perpendicular alignment to the articular surface) (Poole, Kojima et al. 2001). The radial zone has the highest proteoglycan content and confers the most compressive strength (Poole, Kojima et al. 2001). Collagen fibres are arranged randomly, giving this zone a characteristically amorphous appearance (Poole, Kojima et al. 2001). Lastly is the deep zone, anchored to the subchondral bone. Deep zone cartilage has lowest cellularity and cells are arranged in a stacked pattern. Collagen fibres are large and lie perpendicularly, anchoring this cartilage to the underlying calcified zone (Poole, Kojima et al. 2001). Chondrocytes in the deep zone uniquely express type X collagen, a potent marker for hypertrophy and a precursor to calcification (Cancedda, Castagnola et al. 2000). The phenotype in this zone is similar to the transitional phase of endochondral ossification, where the cartilage is gradually replaced by bone. The transient phenotype during this period is calcified cartilage, having the morphology of cartilage, coupled with expression of hypertrophic and ossification markers. However, unlike endochondral ossification, the deep zone is permanently in this hybrid state, as vascularisation is resisted (Athanasίου, 2009).

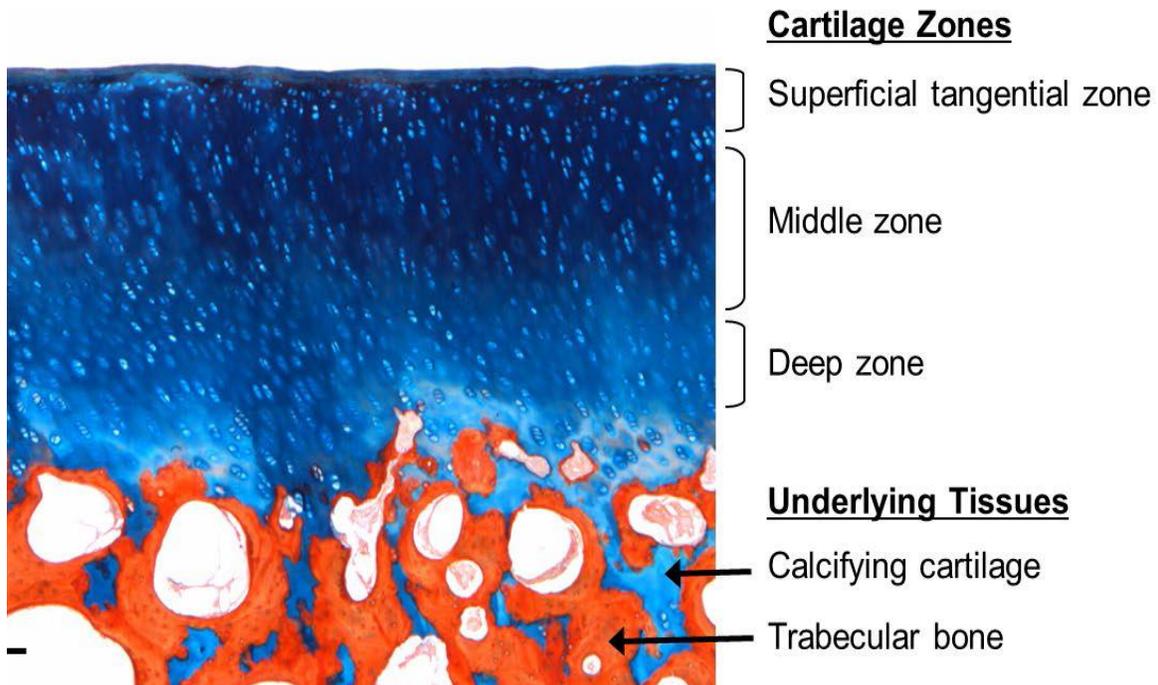


Figure 1. Histological representation of cartilage zones.

*Illustrative representation of the different zones of articular cartilage in an unspecified mammalian model (Johnstone, Alini et al. 2013). Superficial tangential zone is representative of the superficial and transitional zones.*

## **Cartilage Repair**

### Defect classification and endogenous repair

Cartilage defects can be classified as partial thickness, affecting only part or all of the non calcified layers, or full thickness, penetrating into the calcified cartilage layer (Hunziker, 1999). Due to the aneural nature of cartilage, partial thickness defects are often unnoticed until continued erosion leads to lesions into the subchondral bone, or until inflammation and/or swelling occur within the joint space. Defects under 5mm in diameter activate an endogenous repair response which can be separated into intrinsic and extrinsic repair. Intrinsic repair occurs in partial thickness defects and refers to a phenomenon termed ‘matrix flow’ where surrounding chondrocytes secrete matrix in attempt to fill in

smaller lesions (Frisbie, 2012). Full thickness defects that penetrate into the subchondral phase activate the extrinsic repair mechanism, which describes the inflow of progenitor cells and growth factors from a source outside the joint, such as the bone marrow or the vascularised subchondral bone (Frisbie, 2012). The endogenous replacement tissue is composed of scar tissue and fibrous type I collagen, which is mechanically unsuitable to withstand the high impact loading experienced at the joint (Lietman, Miyamoto et al. 2002).

### Non-surgical therapies

Non-surgical options for cartilage repair are generally palliative and aim to prevent or delay progression to osteoarthritis. Such therapies may include weight loss/control, physiotherapy, rest from exercise, rehabilitation training, and pain medication, anti-inflammatory therapies, disease modifying therapies that aim directly at joint lubrication, interleukin receptor antagonists, platelet rich plasma oral, intravenous injections or intra-articular injections. In depth review of equine non-surgical options is provided elsewhere (Frisbie, 2012). These types of therapies are short term remedies and long term, surgical intervention procedures aim for long term sustained repair. Common surgical intervention procedures include marrow stimulation, chondrocyte implantation and grafting, which are discussed in detail below.

### Surgical therapies

Surgical therapies aim to provide long term relief from cartilage defects. Surgical procedures can be categorized into repair, regeneration or replacement strategies. Repair strategies aim to restore tissue functionality and are often associated with scar tissue formation. Regeneration strategies refer to an aim of de novo generation of an identical tissue with identical function, usually by means of cellular replacement or tissue engineering. Replacement strategies are grafting of intact autologous, allogeneic, or synthetic materials. Common procedures and details for each strategy are discussed below.

### Repair: Marrow stimulation or stimulated endogenous cartilage repair

Marrow stimulation is a surgical technique aimed at initiating and mobilizing the endogenous repair response and exposes the site of injury to the underlying bone marrow (Steadman, Rodkey et al. 2001). Various techniques are available including abrasion arthroplasty, spongialization, osteostixis and microfracture (Frisbie, 2012). Currently, abrasion arthroplasty and microfracture are most favoured. Microfracture is a procedure where an awl is used to puncture small holes 2-3mm apart through any overlying cartilage and into the subchondral bone plate (Steadman, Rodkey et al. 2001). Debridement accompanies microfracture in order to remove necrotic or fragmented cartilage. Debridement and microfracture is a first line treatment due to ease and low cost. However, as in endogenous healing, the resulting neocartilage is typically fibrocartilage or hybrid fibro-hyaline cartilage with high accumulation of type I collagen (Breinan, Martin et al. 2000; Steadman, Rodkey et al. 2001). Additionally, short-term improvements are typically not sustained (Shapiro, Koide et al. 1993; Widuchowski, Lukasik et al. 2008; Fortier, Potter et al. 2010). Autologous chondrocyte implantation

### Regenerate: Autologous chondrocyte implantation

Autologous chondrocyte implantation (ACI) is an alternative procedure that strives for cartilage regeneration rather than repair (Brittberg, Lindahl et al. 1994). Unlike repair, the aim of regeneration is to restore tissue form and function without scarring. Autologous chondrocytes (AC) are harvested from an autologous cartilage biopsy from a low weight-bearing region, or from cartilage fragments of the lesion. Cells are expanded in vitro and then injected into the defect site (Chaipinyo, Oakes et al. 2004; Giannini, Buda et al. 2005). A periosteal flap is sutured in place to create a tight seal keeping the chondrocytes in place. This suturing method is technically laborious and does not ensure homogenous cell distribution (Bhosale and Richardson 2008)

Matrix-assisted AC implantation (MACI) is an improvement over traditional ACI, in that it circumvents the need for a periosteal suture. Expanded chondrocytes are incorporated into a matrix and then injected into the defect (Bartlett, Skinner et al. 2005; Zheng, Willers et al. 2007). The polymerized matrix ensures homogenous distribution and helps hold the cells in place. Disadvantages of both ACI and MACI include donor site morbidity and limited autologous chondrocyte sources. Chondrocyte recovery from native cartilage is low, averaging only 22% of total chondrocytes available per gram of tissue, thus requiring large amounts of cartilage tissue (Jakob, Demarteau et al. 2003). In vitro expansion of harvested chondrocytes often results in dedifferentiation, an increased expression of type I collagen and a decreased expression of type II collagen (Darling and Athanasiou 2005). Therefore, this expansion step may be responsible for the formation of fibrocartilage that is commonly observed following ACI/MACI (Bentley, Biant et al. 2003; Roberts, McCall et al. 2003). Many studies continue to use AC as a cell source, working to improve on the redifferentiation and resulting phenotype of the cartilage or on preventing dedifferentiation of chondrocyte phenotype (Ahmed, Gan et al. 2009).

## Replace: Mosaic arthroplasty

Chondral and osteochondral allografts and autografts have been transplanted into joints for the purpose of cartilage repair. Mosaic arthroplasty is a popularized variation of transplant grafting (Hangody and Karpati 1994). Multiple small cylindrical osteochondral cores are harvested from a low weight bearing region and implanted into the site of defect. Fibrocartilage fills in the spaces between the cores (Hangody, Vasarhelyi et al. 2008). This method conserves the integrity of hyaline cartilage at the articular joints, where the dominant collagen is type II collagen. However, there is limited donor source and harvesting the graft may result in donor site morbidity. Furthermore, grafts are commonly taken from low weight bearing areas, which may be biomechanically unsuitable to withstand the high impact loading at articulating joints (Hurtig, Pearce et al. 2001).

## **Progenitor cells for cartilage repair**

### Progenitor cells from cartilage

Articular cartilage progenitor cells (ACPC) may be used in place of AC for tissue engineered grafts or injection therapy. Progenitor cells have recently been isolated from articular cartilage with demonstrated ability for trilineage differentiation and in vitro expansion (Alsalameh, Amin et al. 2004). ACPC chemically induced to chondrogenic fate after (in vitro expansion) using conventional differentiation methods are immunopositive for type I and II collagen, as well as aggrecan (Williams, Khan et al. 2010; McCarthy, Bara et al. 2012). Among the advantages of ACPC are that they are expandable in culture and, upon differentiation, do not exhibit evidence of hypertrophy, including collagenXa1 mRNA expression (McCarthy, Bara et al. 2012). However, ACPC are relatively novel cell types and reliable methods of isolation and identification have not yet been established (Williams, Khan et al. 2010). Furthermore, initial harvest of articular cartilage would be necessary to obtain tissue for ACPC isolation, thereby suffering from similar limitations previously discussed for ACI (e.g., two-step surgery, limited material and possible donor site morbidity).

## Multipotent Mesenchymal Stromal Cells

Mesenchymal Stromal Cells (MSC) are a progenitor population external to the joint space that is able to differentiate into chondrocytes. As an external alternative, the use of MSC overcomes the limitations, such as donor site morbidity and limited sources, commonly associated with techniques that use materials from the joint. Furthermore MSC can be expanded to high cell numbers without losing their phenotype. Postnatal MSC can be isolated from a variety of sources. Common MSC sources used for cartilage regeneration are bone marrow (BM), CB and adipose tissue (AT).

The accepted minimal defining criteria for human MSC are: plastic adherence, a specific panel of surface antigen expression, and the capacity for trilineage differentiation (Dominici, Le Blanc et al. 2006). Although surface antigen profiles vary by species, and no consensus has been reached for horses, evidence from our lab (L.Tessier, unpublished data) and others (De Schauwer, Meyer et al. 2011) suggests that equine CB-MSC express CD29, CD44, CD90, and not MHCI, MHCII, CD4, CD8, CD11a/18, and CD73. Trilineage differentiation refers to the potential to differentiate towards the osteocyte, chondrocyte or adipocyte lineages in vitro. This chondrogenic potency makes MSC an attractive cell source for regeneration of cartilage.

## CB-MSC and chondrogenesis

Koch et al. (2007) was the first to isolate MSC from equine cord blood (eCB) and several independent research groups have since successfully isolated eCB-MSC (DeShauwer, Van de walle et al. 2012; Reed and Johnson, 2007; Schuh, Riedman et al. 2009; Burk, J., I. Ribitsch et al. 2012). CB collection is less invasively collected compared to BM and AT and may offer an off-the-shelf option, alleviating the pressure of time associated with isolation, process and possibly differentiation of MSC once injury has occurred. Additionally, various animal and human studies have suggested that MSC proliferation and chondrogenic potentials decline with age (Zheng, Martin et al. 2007; Stolzing, Jones et al. 2008). CB-MSC represents a 'younger' phenotype and has demonstrated enhanced chondrogenic

efficiency compared to BM-MSC and AT-MSC (Kern, Eichler et al. 2006; Berg, Koch et al. 2009; Wang, Tran et al. 2009; Arufe, De la Fuente et al. 2012, Zhang, Mirai et al. 2011)

### Induced pluripotent stem cells (iPSC)

Pioneered by Yamanaka and colleagues in 2006, iPSC have become a rapidly emerging stem cell alternative for cell therapies or related research (Takahashi and Yamanaka 2006). iPSC are generated by genetic reprogramming of mature somatic cell (such as fibroblasts) such that they express Oct4, Sox2, Klf4 and cMyc. Cells induced to express these factors develop stem cell-like characteristics, such as self-renewal, and pluripotent differentiation. Equine iPSC were generated by the Nagy lab in 2011 (Nagy, Sung et al. 2011) and represent an attractive alternative to embryonic stem cells that can be expanded or obtained in high numbers. However iPSC research is still in its infancy, and chondrogenic differentiation has proven difficult (Guzzo, Gibson et al. 2012). Preliminary data indicates that iPSC-derived cartilage is inferior to MSC-derived cartilage (unpublished data, Sarah Lepage). Advantages and disadvantages of the cell types discussed are summarized in table 1.

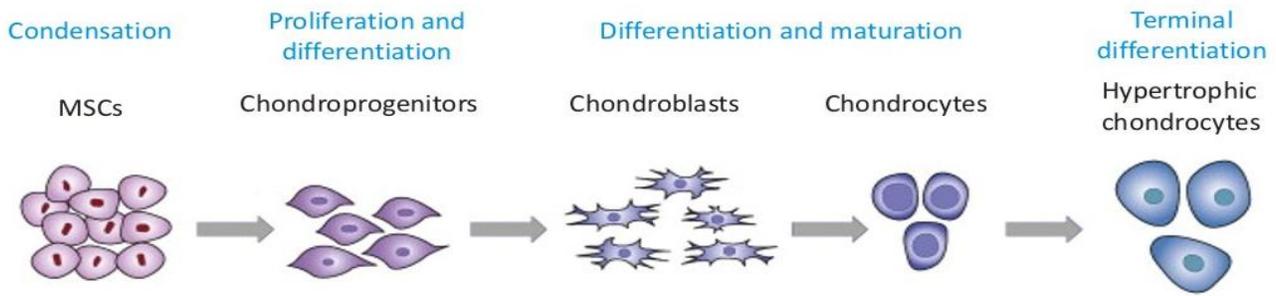
*Table 1. Advantages and disadvantages of various cell sources*

<b>Cell type</b>	<b>Advantages</b>	<b>Disadvantages</b>
AC	Native phenotype Immune compatible	Low isolation efficiency De-differentiation upon expansion
Allogeneic Chondrocytes	Large cell amount available	Limited donor availability Risk of disease transmission
ACPC	Expandable to high numbers Low indication of hypertrophy	Limited donor availability Reliable identification required
MSC	Expandable to high numbers Various sources available Able to differentiate to chondrocytes	Heterogeneous population of cells Stable differentiation still problematic Potential for hypertrophy
iPSC	Large source of patient specific cells	Stable differentiation still problematic Potential for teratoma

*Adapted from Johnstone, Alini et al. 2013*

## **In vivo embryonic chondrogenesis**

The classical model for studying in vivo chondrogenesis is the formation of cartilage anlagen, a transient cartilage model that precedes replacement bone formation. The initial events of cartilaginous anlagen formation occur early in development, beginning with a population of chondroprogenitor cells that migrate into the limb field, undergo a hallmark event termed “condensation”, and then differentiate into chondrocytes (Onyekwelu, Goldring et al. 2009). During the condensation phase, cells become packed tightly together (without increase in proliferation), and initiate a series of important cell-cell signalling mechanisms (Thorogood and Hinchliffe 1975). The importance of cellular condensation for chondrogenesis is underscored by studies that found interruption of chondrogenesis by ablation of cell adhesion molecules, such as neural cell adhesion molecule (NCAM) or N-cadherin during limb development (Delise and Tuan 2002). Following condensation, BMP/TGF $\beta$  signalling then initiates differentiation of the mesenchymal cells into chondroblasts (Yoon and Lyons 2004). Subsequent induction of Sox9, Sox5 and Sox6 marks the onset of chondrogenic differentiation, and the expression of chondrogenic markers such as type II collagen, aggrecan, link protein and others (Yoon and Lyons 2004). Eventually, chondrocytes mature and reach terminal hypertrophic differentiation (Figure 2). Hypertrophy is characterized by a significant increase in cell size, matrix remodelling and expression of hypertrophic genes such as type X collagen, alkaline phosphatase (ALP), matrix metalloproteinase (MMP-13) and runt related transcription factor 2(RunX2).

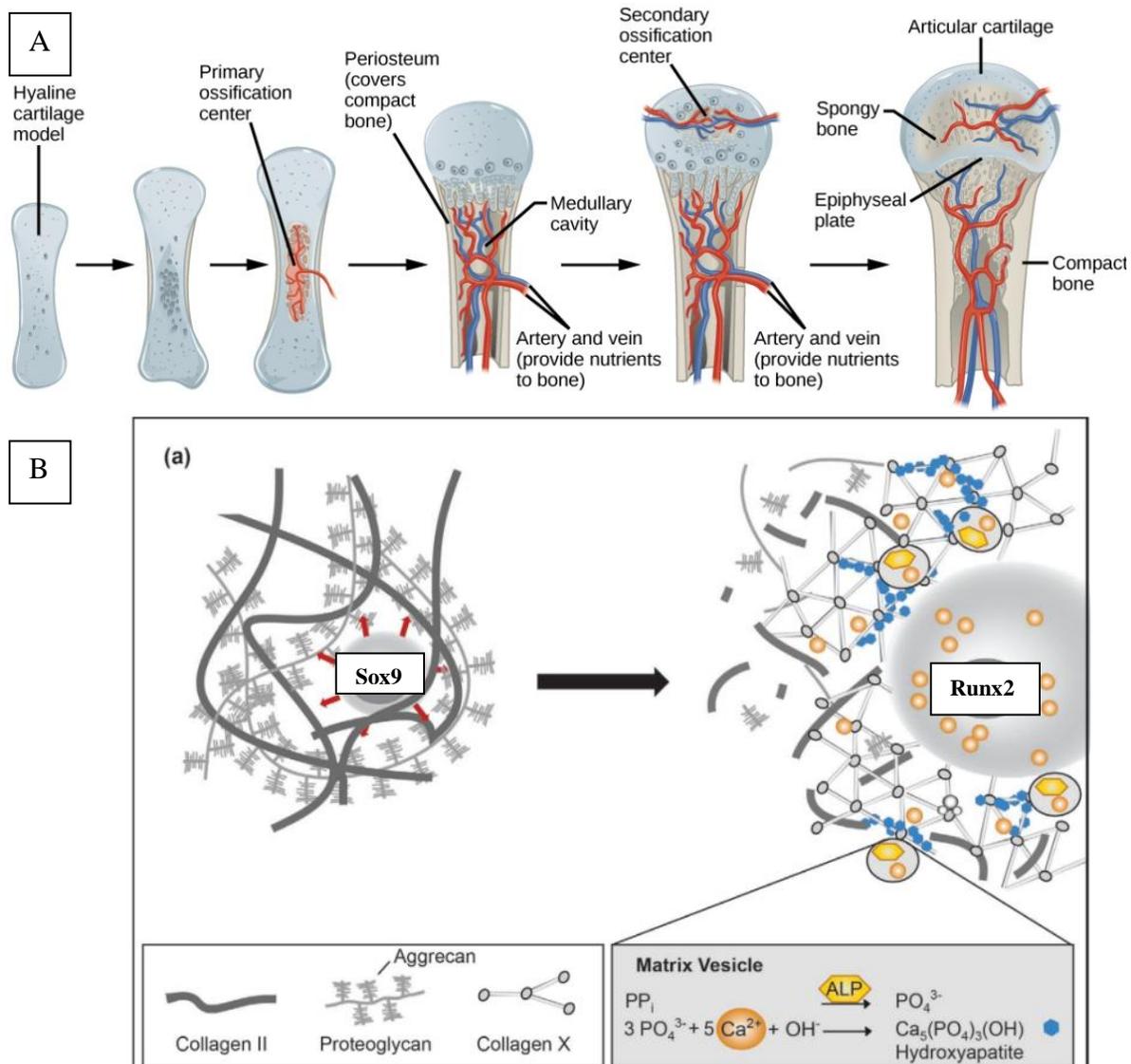


*Figure 2. Chondrogenesis: from MSC to terminal hypertrophy*

*Cartilage progenitors derive from MSC and differentiate along a pathway leading to hypertrophy. Figure retrieved from Robi and Kelc, 2013.*

### Endochondral ossification

Endochondral ossification describes the process of the cartilage anlagen being replaced by bone (Figure 3A). This process follows chondrocyte hypertrophy, where transcription factor RunX2 initiates the process of ossification. RunX2 upregulates ossification factors such as vascular endothelial growth factor (VEGF), MMP-13, and ALP (Studer, Millan et al. 2012). VEGF promotes critical angiogenesis during ossification, where vascularisation aids the replacement or transdifferentiation of the predominant cell type from chondrocytes to osteoblasts and osteoclasts (Zelzer, Glotzer et al. 2001). MMP-13 among others are responsible for matrix remodelling (collagen and aggrecan degradation) and ALP is responsible for matrix mineralization (Figure 3B)(Mackie, Ahmed et al. 2008). For long bones, endochondral ossification is initiated separately in each of the metaphysis (the primary ossification center) and diaphysis (the secondary ossification centers). These centres grow radially and eventually meet and fuse (Kronenberg 2006).

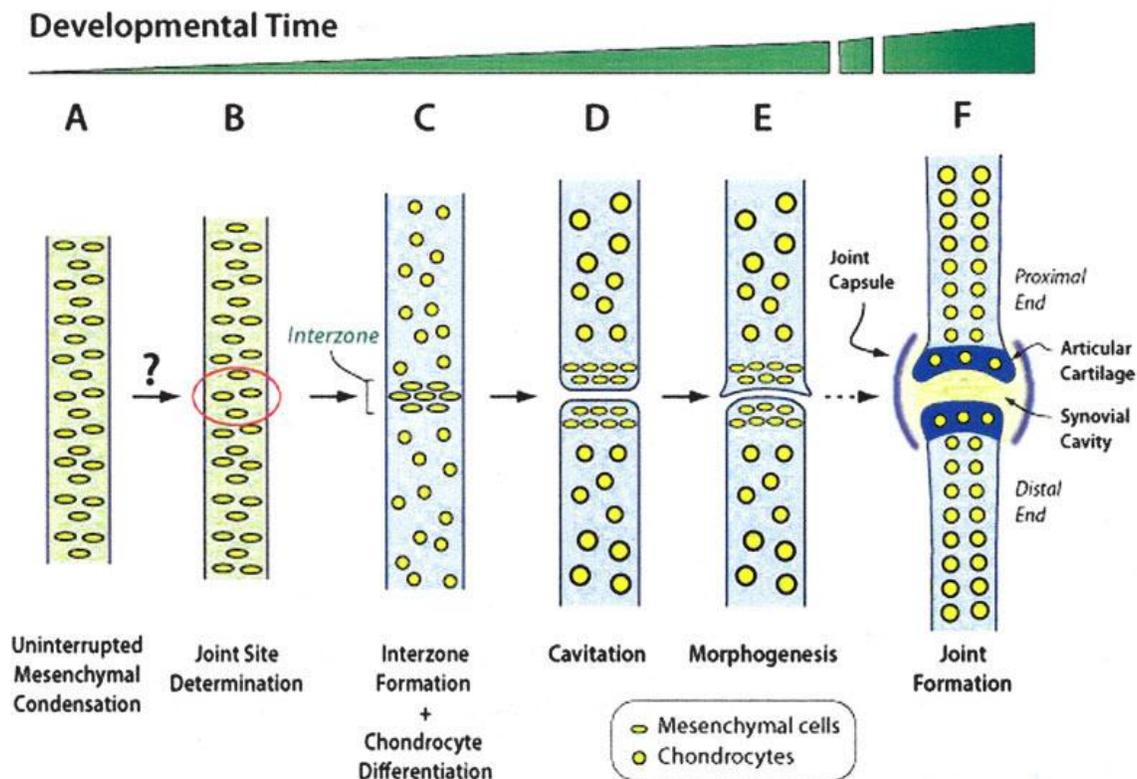


**Figure 3. Endochondral ossification**

Figure 3A depicts the process of endochondral ossification and vascularization, beginning first at the primary ossification center, then secondary ossification center (College 2013). Figure 3B shows the increase in chondrocyte size (left: red arrows) and the calcification framework laid down by collagen X (right). Calcification occurs within the matrix vesicles, which are bound to type X collagen. Within this, ALP hydrolyses  $PP_i$  to  $PO_4^{3-}$ , facilitating hydroxyapatite formation (Studer, Millan et al. 2012).

## Joint formation

In contrast to the transient existence of the cartilage anlagen, permanent cartilage is found lining articular joints. This permanent cartilage is believed to arise from the interzone, which marks the physical interruption of the once continuous mesenchymal condensation (Figure 4)(Ito and Kida 2000). The interzone is characterized by flattened mesenchymal cells with gap junctions, split into three layers. The two outer layers become the epiphysis of long bones, where secondary ossification takes place, while the central layer undergoes cavitation (Figure 4)(Ito and Kida 2000). Little is known about the mechanisms of joint formation, and it is unclear which layer ultimately gives rise to articular cartilage. Furthermore, it is also unclear whether cellular cavitation is a result of cell death or matrix reorganization, resulting in an open, fluid filled space that becomes the synovium (Pacifci, Koyama et al. 2005).



*Figure 4. Joint formation*

*Joint formation by interzone determination and cavitation (Pacifci, Koyama et al. 2005).*

### **In vitro chondrogenesis**

To induce progenitor cells to a chondrogenic cell fate in vitro, a variety of induction stimuli have been explored (Table 2). Important culture stimuli/parameters include chemical growth factor supplementation, mode of culture (scaffold or scaffold free) and environmental factors (e.g., mechanical stimulation, oxygen tension). Detailed review of scaffold assisted culture and mechanical stimulation are discussed in detail elsewhere (Schulz and Bader 2007; Johnstone, Alini et al. 2013).

*Table 2. Various growth factor and culture methods for in vitro chondrogenesis*

<b>CULTURE METHOD</b>	<b>CELL TYPE</b>	<b>INDUCTION FACTOR</b>	<b>REFERENCE</b>
Pellet culture	Human BM-MSc	TGFβ1 or 3	Mueller and Tuan 2008; Mueller, Fischer et al. 2010
		TGFβ3 with BMP 2, 4 or 6	Sekiya, Larson et al. 2005
		TGFβ3 with GDF5	Coleman, Vaughan et al. 2013
	Equine BM- MSC	TGFβ3 with BMP 6	Vidal, Robinson et al. 2008
		TGFβ1 with FGF2	Stewart, Byron et al. 2007
	Murine BM-MSc	TGFβ1 with IGF-1	Longobardi, O'Rear et al. 2006
Alginate bead culture	Human BM-MSc	TGFβ 3 with BMP 2	Shen, Wei et al. 2009
	Porcine primary chondrocytes	TGFβ 3	Bernstein, Dong et al. 2009
Polyethylene glycol diacrylate (PEGDA)	Human BM-MSc	TGFβ 3 without Dexamethasone	Erickson, Kestle et al. 2012
		TGFβ 1 and FGF	Bahney, Hsu et al. 2011
Hyaluronic acid	Bovine BM-MSc	TGFβ 3	Erickson, Huang et al. 2009

### Transforming growth factor (TGFβ)

The most common supplemental growth factors to stimulate chondrogenic differentiation in vitro are a combination of TGFβ, ascorbic acid and dexamethasone (Johnstone, Hering et al. 1998). Additional factors such as bone morphogenic proteins (BMP) or fibroblastic growth factors (FGF) have also been explored (Table 2). TGFβ subfamily isoforms (TGFβ 1, TGFβ 2, TGFβ 3) have been suggested to exert differential effects for chondrogenesis (Chimal-Monroy and Diaz de Leon 1997). TGFβ 2 and 3 (but not TGFβ 1) has been shown to increase type II collagen protein and mRNA levels and proteoglycan synthesis in both human and equine BM-MSc (Worster, Nixon et al. 2000; Barry, Boynton et al. 2001).

### Pellet culture

Pellet culture, also known as micromass culture, is the primary mode of culture for chondrogenic differentiation (Johnstone, Hering et al. 1998). High density pellet culture mimics the 3 dimensional cellular condensation step observed during embryonic chondrogenesis, where intercellular interactions

are crucial. In the pellet culture method, progenitor cells are pelleted by centrifugation in polypropylene plastic culture ware that prevents cell adherence to the substrate and thus promoting cell-cell interactions (Welter, Solchaga et al. 2007). Attempts to differentiate MSC in monolayer have displayed negligible mRNA levels type II collagen or Sox9 (Bosnakovski, Mizuno et al. 2006).

Recently, Penick et al, (2005) established high throughput generation of cartilage pellets by use of 96-well polypropylene plates as opposed to 15-ml conical tubes (Penick, Solchaga et al. 2005). Although pellet differentiation systems permit multiple cell lines to be simultaneously screened for chondrogenic potency (for example, using a 96-well culture plates), the resulting 3D cell aggregates are limited in size due to diffusion limitations: as pellet volume increases, oxygen and nutrient diffusion diminishes. Histological evaluation reveals that pellets often develop areas of necrosis, as well as cortical deposition of type I collagen (Murdoch, Grady et al. 2007; Mueller and Tuan 2008; Markway, Tan et al. 2010; Adesida, Mulet-Sierra et al. 2012). Consequently, there are inherent physical limitations to the overall number of chondrogenic cells that can be generated from each pellet. The starting MSC seeding density for chondrogenic conditioning is limited to  $2.5 \times 10^5$  -  $5.0 \times 10^5$  cells per pellet, which, upon cellular extraction for cytotherapeutic strategies may yield an even lower amount due to cell death or leakage (Penick, Solchaga et al. 2005). As a result, pellet culture is impractical for cellular therapy and tissue engineered constructs, which require high cell numbers, upwards of 2 million cells/cm<sup>2</sup> (Kandel, Gryn timer et al. 2006; Brittberg 2008),

### Membrane culture

Lee and colleagues have recently developed a scaffold-free alternative to pellet culture, termed membrane culture (Lee, Hurtig et al. 2011). MSC are seeded at high density on top of a type IV collagen coated polytetrafluoroethylene (PTFE) membrane for chondrogenic induction and culture. Membrane culture systems yield a homogenous hyaline-like neocartilage, with lower levels of type I collagen compared to pellet culture (Lee, Hurtig et al. 2011). Tissue homogeneity is likely the result of the porous

membrane, permitting a more homogenous penetration of nutrient and gas. The reduction in surface tension in membrane culture likely contributed to the reduction of type I collagen expression. A major benefit of the membrane culture system is the possibility for scale-up. Lee et al. 2011 report a starting seeding density of  $2 \times 10^6$  per 12mm membrane insert, compared to a  $2.5 \times 10^5$  -  $5.0 \times 10^5$  initial seeding density in pellet culture. Furthermore, larger culture inserts are commercially available, although their feasibility for generating cartilage has yet been explored.

### Neocartilage hypertrophy

A persistent problem of neocartilage formed from a conventional pellet-style culture with TGF $\beta$  induction is the expression of type X collagen mRNA, indicating a trend towards hypertrophy (Mueller, Fischer et al. 2010). TGF $\beta$  may be both stimulatory to chondrogenesis and inhibitory to terminal differentiation, or stimulatory to terminal differentiation (Ballock, Heydemann et al. 1993). TGF $\beta$  acts down the Smad2/3 pathway to block terminal differentiation and Smad 1/5/8 pathway to permit hypertrophic differentiation (van der Kraan, Blaney Davidson et al. 2009). It is still unclear what causes this transition from one pathway to another, or whether it is a result of particular TGF $\beta$  isoforms. However, the static, continuous addition of TGF $\beta$  in culture is an inexact substitute of the complex and dynamic spatiotemporal microenvironment observed during in vivo chondrogenesis (Figure 4). A variety of additional factors, temporal and environmental cues may be important in regulating chondrogenesis.

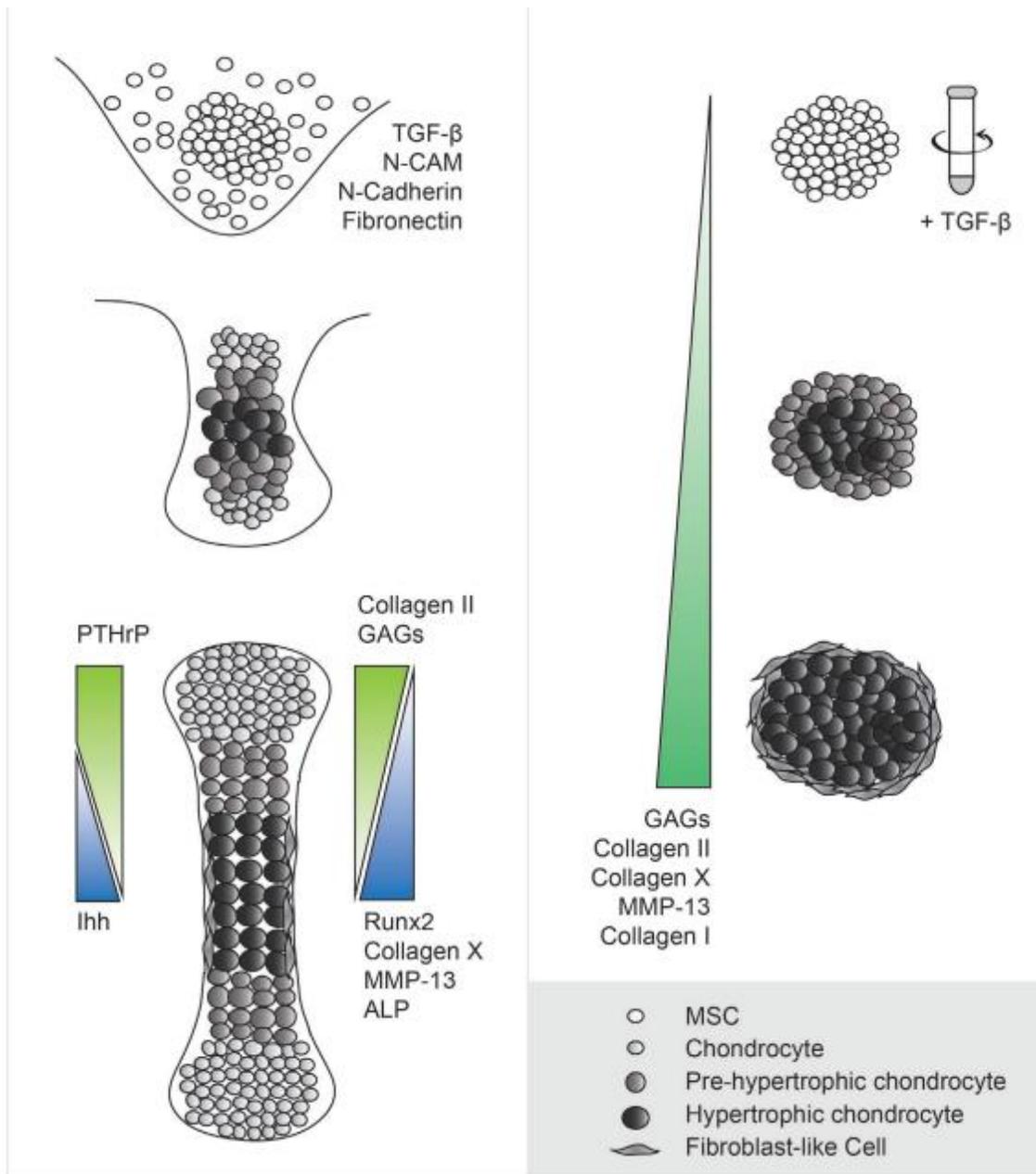


Figure 5. Comparison of in vivo and in vitro chondrogenesis

*During in vivo chondrogenesis (left) a host of growth factors and spatiotemporal cues regulate chondrogenesis, with cartilage at articular ends expressing collagen II and GAG. For in vitro chondrogenesis, MSC are induced using TGF $\beta$  and spun down to form a pellet mass. Without the combination of growth factors and spatiotemporal cues, GAGs, different collagen types including collagen X are concomitantly expressed (Studer, Millan et al. 2012).*

## Oxygen tension

### Low oxygen culture

Compared with atmospheric oxygen levels of ~21%, the oxygen concentration of in vivo cartilage is low, ranging between 1-10% depending on thickness and proximity to subchondral bone or synovial fluid (Zhou, Cui et al. 2004). Reduced oxygen conditions may therefore be considered 'normoxic' for cartilage cell cultures, whereas those at an atmospheric concentration of 21% represent a state of 'hyperoxia'. Various studies have demonstrated that reduced oxygen culture conditions significantly influence in vitro chondrogenesis of BM and AT MSC in various species (Table 3).

*Table 3. Oxygen tension studies using MSC for chondrogenesis in vitro*

Cells	Expansion O <sub>2</sub> levels	Differentiation O <sub>2</sub> levels	O <sub>2</sub> level and phase which enhances chondrogenesis	Reference
Rabbit Chondrocytes	21% or 5%	21% or 5%	5% expansion	Henderson, Ginley et al. 2010
Human BM-MSC	3% or 21%	3% or 21%	3% expansion	Adesida, Mulet-Sierra et al. 2012
	2%	2% or 20%	2% differentiation.	Markway, Tan et al. 2010
Porcine BM-MSC	N/A	21% or 5%	5% differentiation.	Meyer, Buckley et al. 2010
Ovine BM-MSC	5% or 20%	5%	5% expansion.	Zscharnack, Poesel et al. 2009
Rat BM-MSC	21%	21% or 2%	2% differentiation.	Kanichai, Ferguson et al. 2008
Human AT-MSC	N/A	21% or 5%	Better in 5%	Wang, Fermor et al. 2005
	21%	21%, 15%, 10%, 5% or 1%,	Better in 21 or 15%	Pilgaard, Lund et al. 2009
Mouse AT-MSC	N/A	2% and 21%	Poor chondrogenesis in both	Malladi, Xu et al. 2006
	2% and 21%	21%	2% expansion	Xu, Malladi et al. 2007

### MSC niche and oxygen

Each source of MSC represents a distinctive niche. A MSC niche refers to the anatomical location in which the cells have specific cellular, molecular and environmental cues that direct and maintain their characteristics (Mohyeldin, Garzon-Muvdi et al. 2010). The BM niche is estimated to range from 1-7% oxygen (Chow, Wenning et al. 2001; Harrison, Rameshwar et al. 2002), while that of AT is slightly higher at 10-15% oxygen (Bizzarri, Koehler et al. 2006). To date, the oxygen level in the in vivo CB niche remains unknown, although it is presumed not to exceed 8% oxygen in congruence with the female reproductive tract (Fischer and Bavister 1993). Differences in niche microenvironments may contribute to their response to reduced oxygen culture. Chondrogenic differentiation of BM-MSCs is enhanced in reduced oxygen culture (Markway, Tan et al. 2010; Meyer, Buckley et al. 2010; Adesida, Mulet-Sierra et al. 2012), but results for AT-MSCs have been inconsistent (Wang, Fermor et al. 2005; Malladi, Xu et al. 2006). Reduced oxygen for chondrogenic differentiation of CB-MSCs has not been studied. However, reduced oxygen culture for MSC expansion demonstrates evidence of an altered metabolic activity in comparison to BM-MSCs and AT-MSCs (Lavrentieva, Majore et al. 2010).

### Hypoxia Inducible Factor - HIF

Semenza and colleagues identified in 1992 hypoxia inducible factors (HIF) as the principle response factors that regulated erythropoietin levels during reduced oxygen (Semenza and Wang 1992). Since then, HIF has been found to be involved with the regulation of more than 100 genes (Weidemann and Johnson 2008). With respect to cartilage and chondrogenesis, HIF1 has generated the most interest. HIF1 consists of the subunits HIF1 $\alpha$  and HIF1 $\beta$ . Heterodimerization of the two is necessary to mediate translocation to the nucleus and DNA binding to hypoxia responsive elements (HRE) which influences downstream gene expression. Both  $\alpha$  and  $\beta$  subunits are constitutively expressed but it is the HIF1 $\alpha$  subunit that confers oxygen sensitivity. HIF1 $\alpha$  has a half-life of less than 5 minutes at oxygen levels >6% (Jewell, Kvietikova et al. 2001; Dery, Michaud et al. 2005).

## HIF regulation

HIF1 $\alpha$  is regulated by two oxygen dependant mechanisms, one involving prolyl hydroxylase (PHD), and the other Factor Inhibiting Hormone (FIH)(Figure 5). PHD enzymes hydroxylate specific proline residues of HIF1 $\alpha$  in the presence of oxygen (Berra, Benizri et al. 2003). Von Hippel-Lindau (VHL) then binds and promotes ubiquitylation of HIF1 $\alpha$ , which then makes it a target for proteosomal degradation (Ivan, Kondo et al. 2001; Jaakkola, Mole et al. 2001). Similar to PHD, FIH action is also oxygen dependent (Lando, Peet et al. 2002). FIH hydroxylates specific asparagine residues of HIF1 $\alpha$  (Mahon, Hirota et al. 2001; Lando, Peet et al. 2002). This asparagine hydroxylation prevents HIF1 $\alpha$  from binding to cofactors p300/CBP, which are required for full HIF1 activity (Arany, Huang et al. 1996).

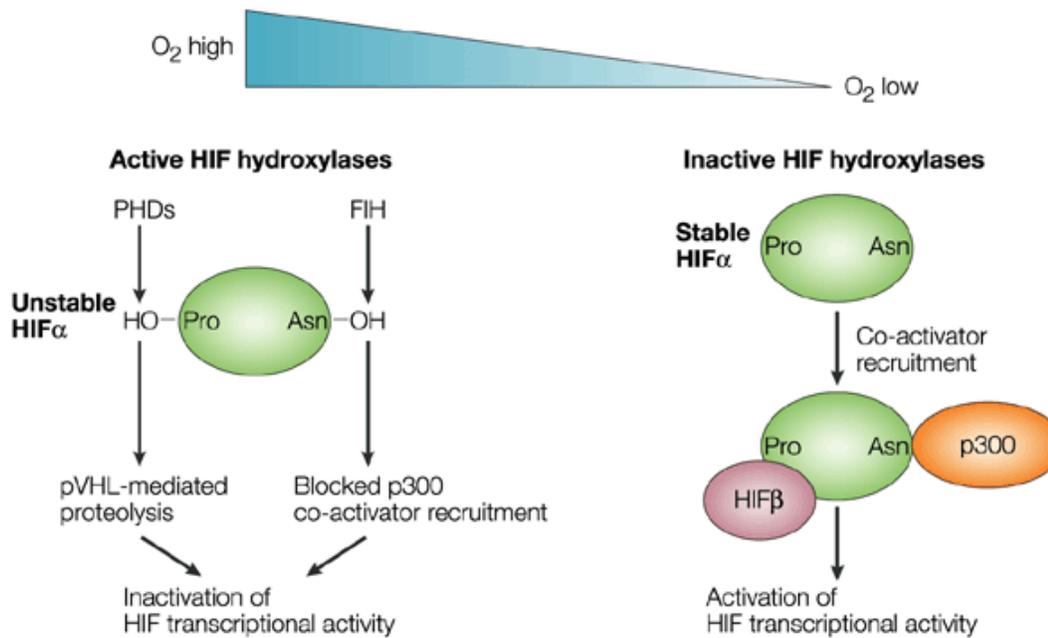


Figure 6. HIF mechanism

*At adequate oxygen levels (left), PHD hydroxylates proline residues direct HIF1 $\alpha$  degradation by ubiquitin ligase von Hippel Lindau (pVHL). Additionally FIH hydroxylates an asparagine residue, which blocks the binding of cofactors p300 and Creb binding protein (CBP). At low levels of oxygen, hydroxylation does not occur, therefore stabilizing HIF1 $\alpha$ . (Schofield and Ratcliffe 2004)*

## HIF and Chondrogenesis

HIF1 $\alpha$  is widely recognized as being directly involved in chondrogenesis. HIF1 $\alpha$  null mouse models have abnormal cartilaginous growth patterns resulting in limb deformation and early cell death (Schipani, Ryan et al. 2001). HIF1 $\alpha$  expression and activity has also been confirmed in the limb bud and mesenchymal condensations (Provot, Zinyk et al. 2007). In vitro, HIF1 $\alpha$  was shown to directly bind to the Sox9 promoter region, where Sox9 and associated cartilage markers type II collagen and aggrecan were positively correlated with HIF1 $\alpha$  nuclear accumulation (Robins, Akeno et al. 2005; Amarilio, Viukov et al. 2007). Induced mutation of hypoxia responsive elements (HRE) in the Sox9 promoter resulted in abolishment of those findings (Robins, Akeno et al. 2005). Several in vitro studies using knockdown or forced expression of HIF1 $\alpha$  have confirmed these findings in both protein and mRNA levels (Pfander, Cramer et al. 2003; Kanichai, Ferguson et al. 2008; Duval, Leclercq et al. 2009).

## **RATIONALE, HYPOTHESIS AND OBJECTIVES**

Current treatment options for cartilage repair palliative and aim to delay progression of osteoarthritis. Cell based regeneration strategies using MSC are currently being studied as an alternative option. Although in vitro differentiation techniques are able to produce a ‘hyaline-like’ phenotype, presence of a significant amount of type I and X collagen remains a problem. Two important culture parameters to alter are mode of culture and oxygen availability.

The spherical shape of pellet culture results in a heterogeneous cartilage phenotype and limits the number of cells that can be conditioned at a time. The recently devised membrane culture method shows promise in overcoming these limitations.

Reduced oxygen has been shown to enhance the generated matrix phenotype of both BM-MSC and AC. Although characterization of proliferative capacity and ‘stemness’ of CB-MSC has been reported, the effect of low oxygen culture on chondrogenic differentiation remains unknown.

Thus, to determine the influence of reduced oxygen tension and membrane culture on differentiation of equine CB-MSC in vitro, simultaneous evaluation on the separate and combined effects of membrane culture and reduced oxygen tension was conducted with the following hypothesis and objective:

### **Hypothesis**

I hypothesize that mode of culture and oxygen level significantly affects the quality of in vitro chondrogenesis of equine CB-MSC.

### **Objective**

To characterize and compare neocartilage generated by pellet and membrane culture in 5% and 21% oxygen.

## MATERIALS AND METHODS

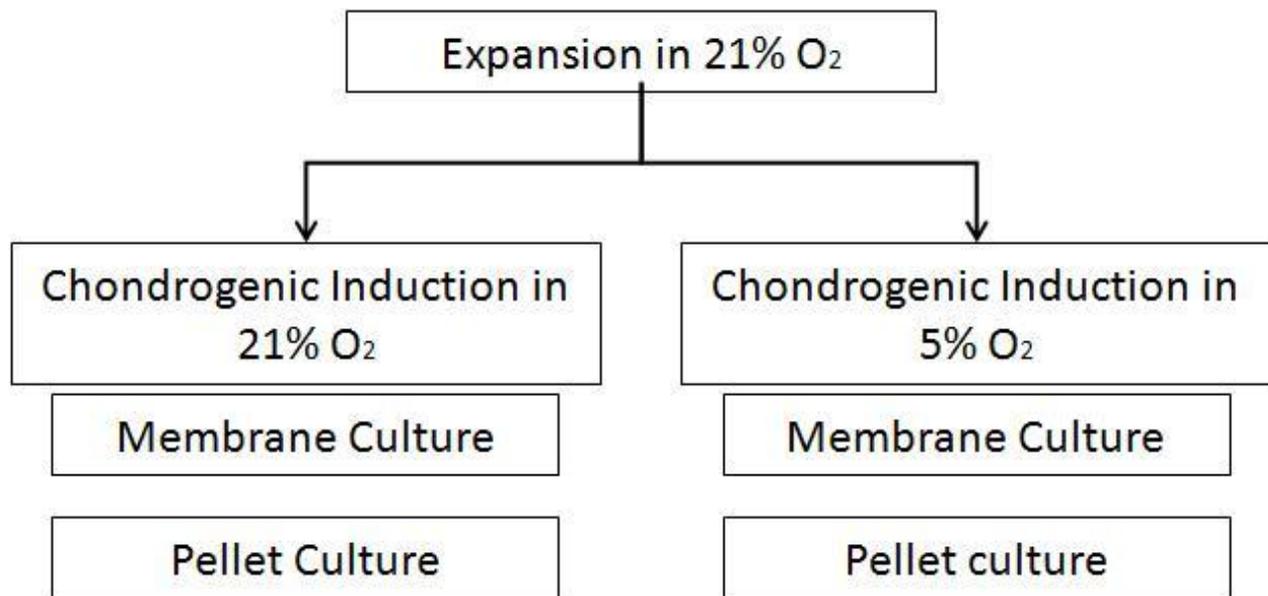
### CB-MSC Isolation and Expansion

The nuclear cells (NC) were isolated from five equine CB samples using PrepaCyte®-WBC (BioE, Inc; St. Paul, MN, USA) according to manufacturer's instructions. As previously described (Koch, Thomsen et al. 2009), the NC fraction was resuspended in isolation media consisting of low-glucose Dulbecco's modified Eagle medium (LG-DMEM: Lonza; Walkersville, MD, USA), 30% Fetal Bovine Serum (FBS) (Invitrogen; Burlington, ON, CAN), 100 IU/mL Penicillin (Invitrogen; Burlington, ON, CAN), 0.1mg/mL Streptomycin (Invitrogen; Burlington, ON, CAN), 2mM L-glutamine (Sigma-Aldrich; Oakville, ON, CAN) and 0.1mM Dexamethasone (Sigma-Aldrich; Oakville, ON, CAN). The cell fraction was then plated on polystyrene tissue culture flasks at a density of  $1 \times 10^6$  cells/cm<sup>2</sup> (Sigma-Aldrich; Oakville, ON, CAN). Colonies were trypsinized (0.04% trypsin-EDTA) at 70-80% confluency and further culture expanded in expansion medium (EM) consisting of LG-DMEM, 30% FBS, 100 IU/mL Penicillin, 0.1mg/mL Streptomycin and 2mM L-glutamine at 21% oxygen. At passage stage 2 the cells were cryopreserved in EM with 10% DMSO (Sigma-Aldrich; Oakville, ON, CAN) by overnight control-rate freezing canisters at -80°C (Nalgene® Mr.Frosty; Fischer Scientific; Markham, ON, CAN) before transfer to long-term storage in liquid nitrogen.

Cryopreserved CB-MSC were thawed and plated at a cell density of 5000 cells/cm<sup>2</sup> in EM at 21% oxygen. EM was completely changed every 2-3 days. Cells were harvested at 60-80% confluency by enzymatic digestion with trypsin-EDTA and counted with automated cell counter (Nucleocounter® NC100, Mandel, Guelph, ON, CAN) prior to chondrogenic differentiation.

## Differentiation

Chondrogenic differentiation was carried out in both pellet culture and membrane culture at 21% (referred to as normoxic conditions) and 5% (hypoxic) oxygen conditions. Refer to figure 7 for study design.



*Figure 7. Study Design*

*CB-MSCs from 5 horses were expanded at 21% oxygen and then differentiated in 21% and 5% oxygen in membrane and pellet culture. The passage number at time of differentiation was 5 or 6. Pooled samples (four pellets, three membranes) were used per each biological replicate for biochemical protein assays and gene expression quantification.*

Pellet culture: CB-MSCs were suspended in chondrogenic medium (CM) containing High Glucose Dulbecco's modified Eagle medium (HG-DMEM) (Sigma-Aldrich; Oakville, ON, CAN), 200 mM Glutamax (Invitrogen; Burlington, ON, CAN), 100 mM Sodium pyruvate (Invitrogen; Burlington, ON, CAN), 1x ABAM (Invitrogen; Burlington, ON, CAN), 0.1 mM dexamethasone (Sigma-Aldrich; Oakville, ON, CAN), 100 mg/mL ascorbic acid-2 phosphate (Sigma-Aldrich; Oakville, ON, CAN), 40 mg/mL proline (Sigma-Aldrich; Oakville, ON, CAN), 1x ITS (BD Biosciences; Mississauga, ON,

Canada), and 10 ng/mL TGF $\beta$ 3 (R&D Systems; Minneapolis, MN, USA), at a concentration of  $2.5 \times 10^5$  cells per 250 $\mu$ l (per pellet). The cell suspension was then placed in V-bottom polypropylene 96 well plates (Phenix; Candler, NC, USA) and centrifuged at 200x g for 10 minutes and incubated in either 5% or 21% oxygen. From each cell culture, 10 replicate pellets were made for each oxygen concentration.

Membrane culture: Protocol adapted from (Lee, Hurtig et al. 2011). Briefly, 12mm diameter, 0.2mm pore size PTFE membrane inserts (Millipore; Billerica, MA, USA) were coated with 100 $\mu$ l of DMEM with 10% human plasma fibronectin (Millipore; Billerica, MA, USA) and left to dry overnight. The following day coated membranes were subjected to 10 minutes of UV treatment and placed in the incubator for >6 hours. We used 400 $\mu$ l of EM containing  $2.0 \times 10^6$  cells to seed the top of the membrane, with an additional 600 $\mu$ l of EM surrounding the insert. All membranes were initially incubated overnight in 21% oxygen. The following day, EM was replaced with 1.5mLCM. Membranes designated for 5% oxygen culture was incubated for 2 hours at 21% oxygen before transfer to a low oxygen incubator for the remaining culture time. CM was changed every 2-3 days. Wet mass was determined at day 21 and adjusted for cell density, where the mass of membrane generated tissues were divided by 8 to account for the fact that the cell seeding density on the membrane was 8 times higher than pellet culture. Neocartilage from membrane culture was adjusted to reflect only generated tissue, minus the mass of membrane insert.

### **Histochemistry and Immunohistochemistry (IHC)**

Sample pellet and membrane cultures were fixed in 10% neutral formalin buffer overnight, dehydrated in isopropanol, and embedded in paraffin. Serial 5 $\mu$ m thick sections were prepared for histochemistry and IHC. Sections were deparaffinised and rehydrated prior to staining. Toluidine blue was used to visualize GAG content. The stock solution contained 1g Toluidine blue powder (Sigma-Aldrich; Oakville, ON, CAN) solubilized in 100ml of 70% EtOH, diluted to a 10% working solution with 1% NaCl (pH 2.0). Following rehydration, sections were stained in Toluidine blue working solution

for 2 minutes and washed in three changes of distilled water. To visualize overall tissue structure, a standard Hematoxylin and Eosin (H&E) staining protocol was used (Carson 1997). IHC was used to identify type I and type II collagen. Slides were deparaffinised and rehydrated, and then subject to enzymatic antigen retrieval: 20mg/mL proteinase K at room temperature for 10 minutes and 1600U/mL hyaluronidase at 37°C for 20 minutes. Slides were then blocked with 3% FBS was at room temperature for 30 minutes followed by quenching with 3% hydrogen peroxide for 15 minutes. Slides were then incubated with primary antibody, either mouse anti-type I collagen (Calbiochem, Cat# CA60801-158) or mouse anti-type II collagen (Developmental studies hybridoma bank; Iowa city, Iowa, USA Cat# II-II6B3) overnight at 4°C. Slides were then washed with PBS 3 times for 2 minutes each and incubated with an HRP-conjugated goat anti-mouse (DAKO; Burlington, ON, CAN) for 1 hour at room temperature. All slides were visualized with 3,3 diaminobenzidine chromogen (DAKO; Burlington, ON, CAN) and a hemotoxylin counterstain. Imaging was done with Axiovision 4.7.1 software.

## **Biochemistry**

Triplicate samples were snap frozen and stored at -80°C. Samples were then digested in 40mg/mL papain (Invitrogen; Burlington, ON, CAN), 20mM ammonium acetate, 1mM EDTA, and 1mM DTT (Sigma-Aldrich; Oakville, ON, CAN) for 48 h at 65°C and frozen at -20°C. Samples were then thawed for quantification using dimethylmethylene blue (DMMB) dye binding assay for GAG content, hydroxyproline assay for total collagen content and DNA quantitation kit for DNA quantification. For the DMMB assay, Chondroitin sulphate (Sigma-Aldrich; Oakville, ON, CAN) was used to generate a standard curve. Results were read at a wavelength of 525nm. Hydroxyproline was used as a proxy to estimate overall tissue collagen content with a conversion factor of 10% (Lee, Hurtig et al. 2011). In brief, the hydroxyproline assay was conducted as follows: papain digested samples were hydrolyzed with 6N HCl 110°C for 18 hours, and then neutralized with NaOH. Samples and standards were then incubated with 0.05N chloramine T for 20 minutes at RT, 3.15N perchloric acid for 5 minutes

at RT and Ehrlich's reagent for 20 minutes at 60°C. Absorbance was read at wavelength 560nm. Hydroxyproline (Sigma-Aldrich; Oakville, ON, CAN) was used as a standard. DNA content was assessed using a commercial kit: DNA quantitation kit – Fluorescence assay Cat# DNAQF-1KT (Sigma-Aldrich; Oakville, ON, CAN) in a 96 well plate. The assay was carried out according to manufacturer's instructions. GAG and collagen content was normalized to DNA content.

### **Quantitative qPCR**

Samples were homogenized using a rotor stator (Powergen 1000) (Fischer Scientific; Markham, ON, CAN). Total RNA from samples were isolated using the Ambion® mirVanamiRNA isolation kit (Invitrogen; Burlington, ON, CAN) according to manufacturer's instructions. RNA concentration and purity was assessed using Nanodrop ND-1000 spectrophotometer. RNA was treated with DNase I (Invitrogen; Burlington, ON, CAN) and reverse transcribed using Superscript®II Reverse Transcriptase (Invitrogen; Burlington, ON, CAN) according to instructions with random hexamers (Invitrogen; Burlington, ON, CAN). Quantitative PCR (qPCR) was done using Ssofast™ Evagreen® Supermix (Bio-Rad; Hercules, CA, USA) with the CFX96 Touch™ Real-Time PCR detection system (Bio-Rad; Hercules, CA, USA) with a 10 µL reaction volume and 200nm total primers. Annealing temperature was determined to be 60°C by running temperature gradients for all primers. Standard curves were generated with 4 fold serial dilution to determine primer efficiency. qPCR products were run on agarose gel, sequenced and verified by BLASTN for sequence alignment. Genes were normalized to beta-2-microglobulin (B2M) as a housekeeping gene for relative expression levels (Foldager, Munir et al. 2009). Fold difference was compared to the 21% pellet sample. See table 4 for primer information.

*Table 4. Primer sequences for quantitative qPCR*

Gene	Forward Primer	Reverse Primer	Accession Number
Collagen IA1	GAAAACATCCCAGCCAAGAA	TGATGTTTTGAGAGGCATGG	XM_001492939.1 (Berg, Koch et al. 2009)
Collagen IIA1	GACAACCTGGCTCCCAA	ACAGTCTTGCCCCACTTAC	NM_001081764B (Berg, Koch et al. 2009)
Collagen XA1	CTTGGTTCATGGCGAGTTT	GTCCAGGGCTTCCATAACT	XM_001504101.1
HIF 1a	TGCATCTCCATCTCCTACC	CGCCAGGATCTTTGGATT	XM_001493206.3
B2M	TCGGGCTACTCTCCCTGACT	ATTTCAATCTCAGGCGGATG	NM_001082502.2
Sox9	ATCTGAAGAAGGAGAGCGAG	TCAGAAGTCTCCAGAGCTTG	XM_001498424.3(Zehentner, Dony et al. 1999)

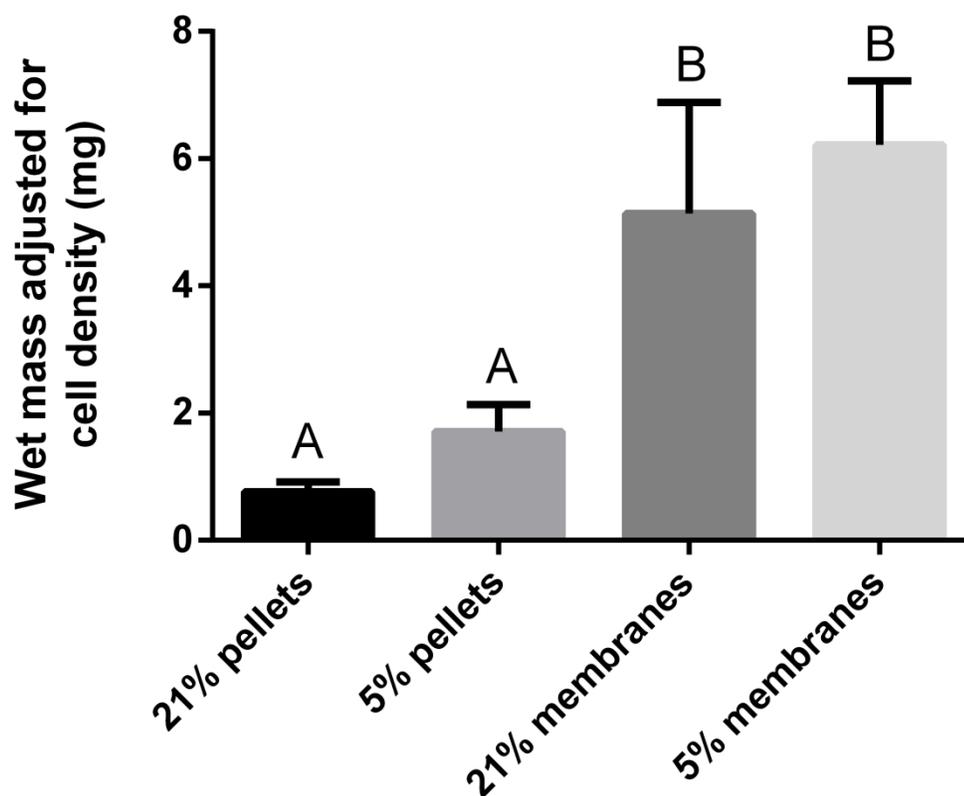
## Statistics

GraphPad Prism 6.02 Software was used to perform statistical analysis. Protein and wet mass data was analyzed by ordinary two-way ANOVA with Tukey's post hoc test to determine differences between treatment groups. Gene expression data did not pass normality tests (Shapiro-Wilk and Kolmogorov-Smirnov). Therefore, non-parametric Kruskal-Wallis ANOVA was used with Dunnett's post hoc testing for multiple comparisons. Outliers were determined and excluded based on high z scores of triplicate Ct values, or if the corresponding melting curve peak was not consistent. Results are reported as means with 95% confidence intervals and significance is assigned at  $p < 0.05$ .

## RESULTS

### Wet mass

Membrane cultures resulted in significantly greater wet mass than pellet cultures (Figure 8). Although no differences were observed between the different oxygen conditions for membrane cultures, the mean wet mass of 5% pellet culture was greater than that of 21% pellets ( $P=0.3$ ).

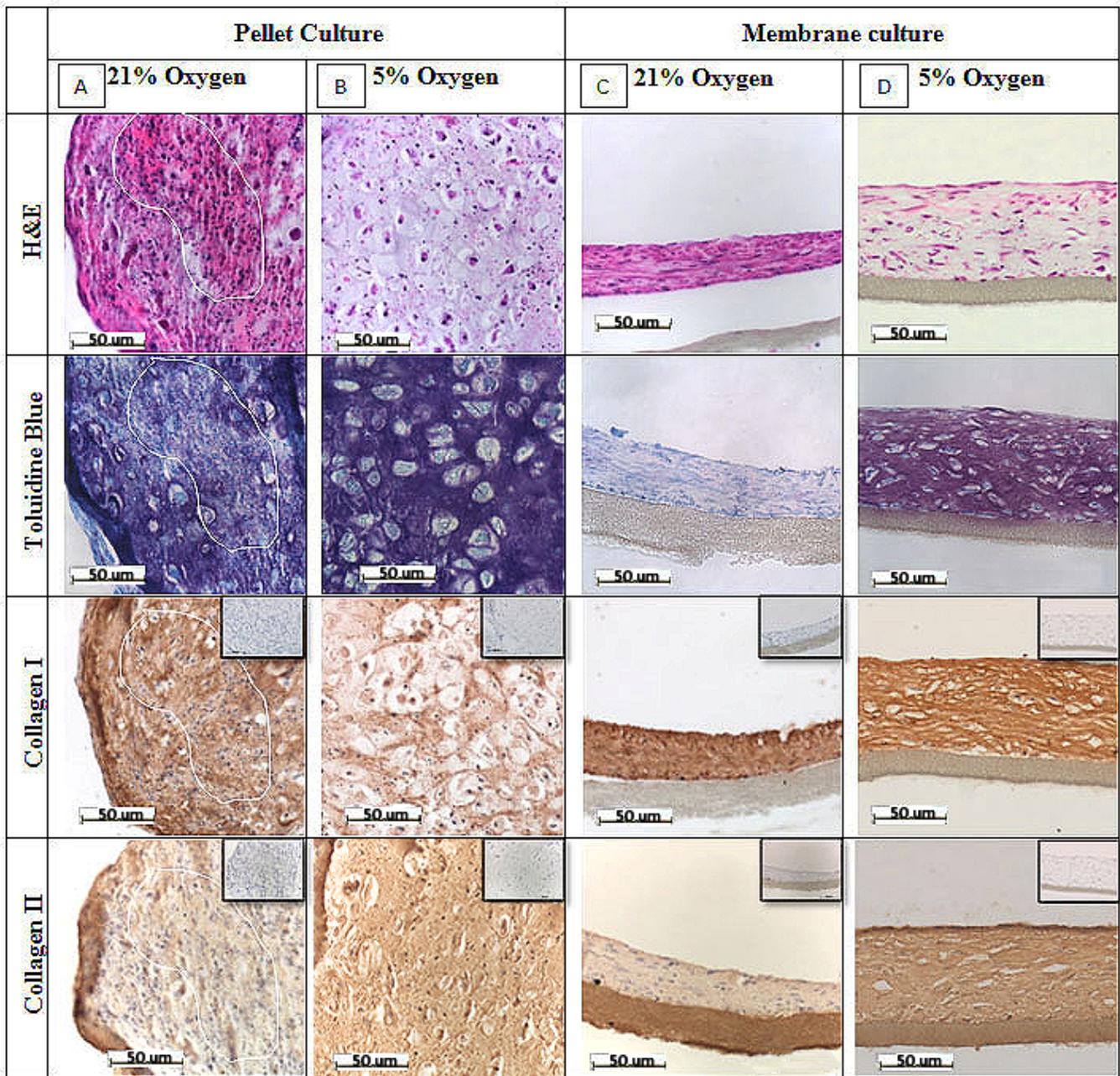


*Figure 8. Wet mass*

*Wet mass of generated tissue was adjusted for cell density, and presented as means  $\pm$  95% CI.  $N=5$  biological replicates, run in technical duplicates. To account for the fact that membrane culture uses 8 times the amount of cells pellet culture does, membrane wet mass was divided by 8. Regardless of the adjustment, membrane culture generated significantly higher tissue mass than pellet culture ( $P<0.0001$ ).*

## **Histology**

Neocartilage most closely resembling hyaline cartilage was produced at 5% membrane culture (Figure 9D). More specifically, the resulting tissue had a homogenous ECM and observed high matrix:cell ratio, that was rich in both GAG and type II collagen. In contrast, 21% pellet cultures exhibited low matrix:cell composition, heterogeneous matrix distribution with indication of necrosis or fragmented nuclei and low indication of type II collagen (Figure 9A,D). At 21% oxygen, tissue generated using both membranes and pellets yielded tissue with irregular staining for both GAGs and type II collagen. Tissue collected from all four culture conditions demonstrated immunoreactivity to type I collagen.



*Figure 9. Histological assessment*

*Histological and IHC images are from a representative cell cultures at 400 times magnification. Membrane samples were sectioned while still attached to the underlying PTFE membrane insert (brown structure next to the tissue; columns C-D). H&E staining show tissue morphology with round lacunae around cells and a high matrix to cell ratio in columns B-D. Indication of necrosis or nucleus fragmentation is found in column A, as outlined in white. Toluidine blue staining revealed lowest*

*proteoglycan content in 21% membranes (C). Presence of type I and II collagen was confirmed by IHC. Insets for collagen I and II of control stains using only secondary antibody confirm that non-specific staining did not occur.*

## **Biochemistry**

Consistent with the histological data, total collagen and GAG content was highest in tissues generated using the 5% oxygen membrane culture conditions (Figures 10 and 11). Membrane culture conditions at 21% also exhibited high total collagen content, although GAG content was significantly lower than 5% membranes. In general, total collagen content was highest in membrane cultures regardless of oxygen tension (Figure 10) and GAG content was increased in 5% oxygen cultures, with highest GAG content in 5% membrane culture (Figure 11).

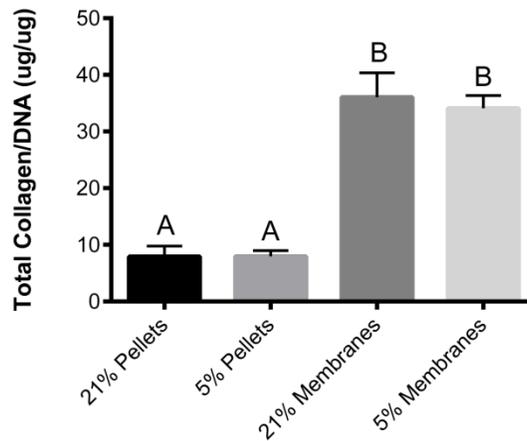


Figure 10. Total collagen content

Total collagen content normalized to DNA content is presented as mean  $\pm$  95% CI with all differences  $p < 0.0001$ .  $N=5$  biological replicates, run in technical triplicates. Both 21% membrane and 5% membranes accumulate more total collagen content than 21% pellet and 5% pellets.

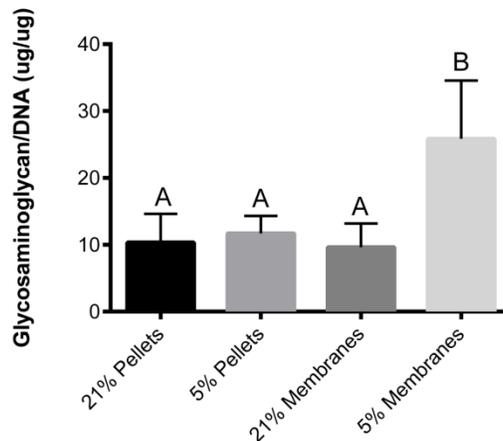
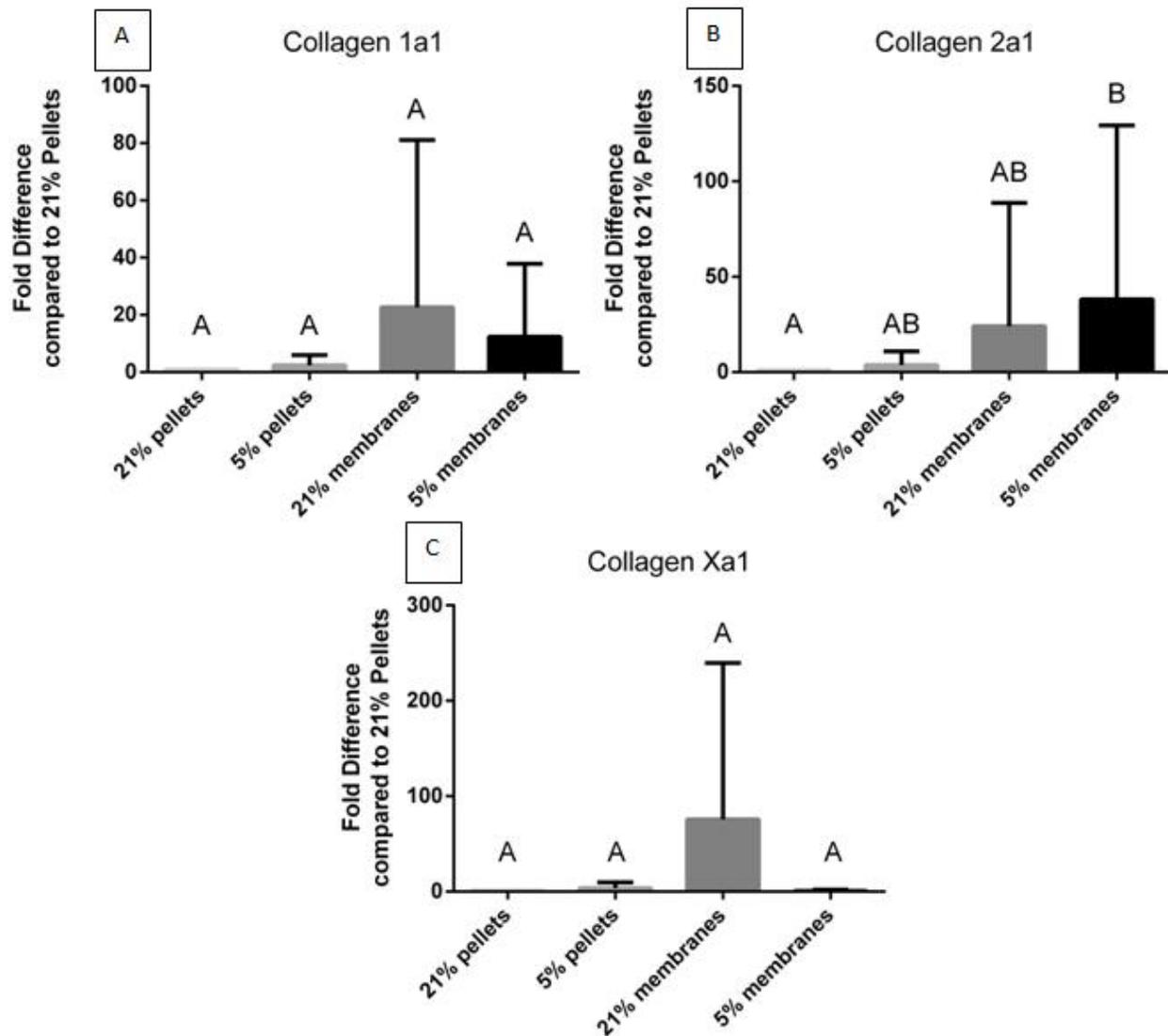


Figure 11. GAG content

GAG content normalized to DNA content is presented as mean  $\pm$  95% CI.  $N=5$  biological replicates, run in technical triplicates. Significance of 5% membranes compared to 21% pellets, 5% pellets, and 21% membranes are  $p=0.0003$ ,  $p=0.0008$ , and  $p=0.0001$ , respectively. 5% membranes show the highest accumulation of GAG content compared to other groups.

## Quantitative qPCR

Using qPCR, only collagen IIA1 demonstrated a significantly elevated expression level, and only between 5% membrane cultures and 21% pellet cultures (Figure 12). No significant differences were observed for mRNA expression of collagen IA1, collagen XA1, Sox9, HIF1 $\alpha$  (Figure 13).



*Figure 12. mRNA expression on collagen genes*

*mRNA expression showing collagen mRNA distribution as mean  $\pm$  95% CI with fold difference compared to 21% pellets. N=5 biological replicates, run in technical triplicates. Significance is observed in collagen IIA1 5% membranes to 21% pellets,  $p=0.03$ . No other significant differences were noted.*

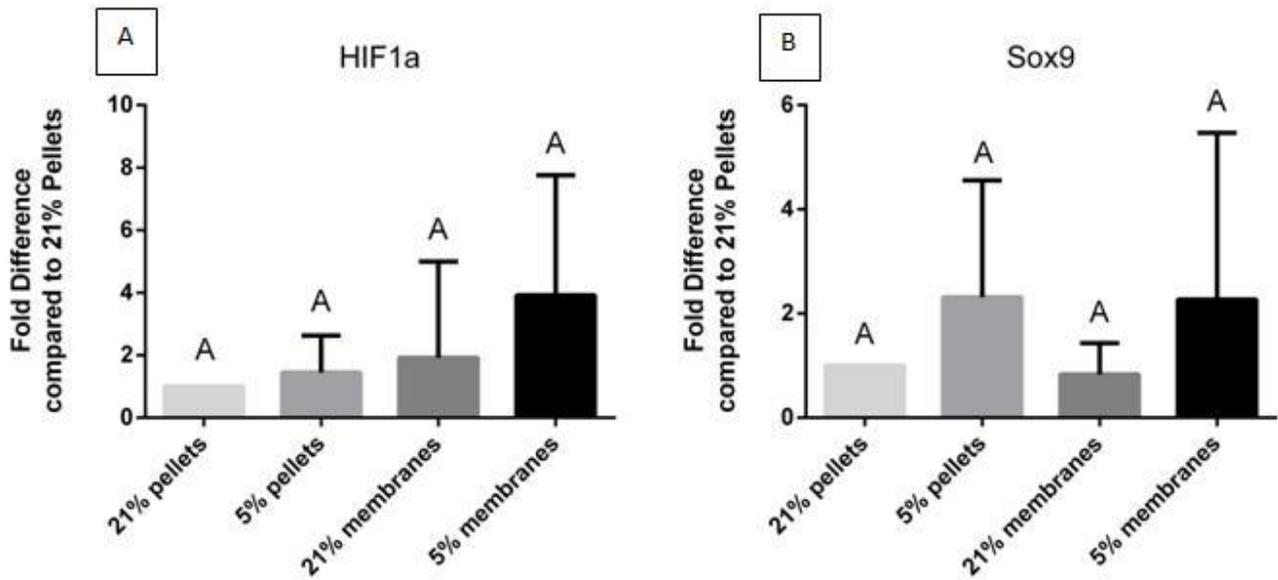


Figure 13. mRNA analysis for SOX9 and HIF1a

*mRNA expression of transcription factors HIF1a and Sox9 is presented as mean  $\pm$  95% CI with fold difference compared to 21% pellets. N=5 biological replicates, run in technical triplicates. No statistically significant differences were observed. HIF1a:*

## DISCUSSION

Here I demonstrate that mode of membrane culture and oxygen concentration are significant contributors to in vitro generated neocartilage. Under the set experimental groups, desirable cartilage phenotype was maximized in membrane culture at 5% oxygen. Cartilage phenotype from this culture condition was matrix rich with homogenous distribution of ECM and rich in GAG and collagen II content.

Porous membrane culture for in vitro chondrogenesis has been studied independently using similar techniques at 21% oxygen for the purpose of overcoming limitations of the pellet method (Murdoch, Grady et al. 2007; Lee, Hurtig et al. 2011). Consistent with our findings, previous investigations have found that membrane culture yields homogenous, hyaline-like neocartilage (Murdoch, Grady et al. 2007; Lee, Hurtig et al. 2011).

In addition to advantages over pellet culture, such as reduced surface tension and homogenous diffusion gradient, membrane culture also presents scale up possibilities. The seeding density was  $2 \times 10^6$  million cells per 12mm membrane insert in the current study, an 8 time increase in initial seeding density over pellet culture. PTFE membranes are available in larger diameter; further scale up may be possible. However, efficacy and technical feasibility will need to be verified in larger membrane inserts to determine whether an upper limit may exist.

A defining characteristic of membrane culture is disc shaped tissue formation. To manipulate the tissue to form on top of PTFE membranes as multi-cellular layers, membranes were coated with adhesion proteins. Fibronectin was used in the present study, whereas previous work employed type IV collagen (Lee, Hurtig et al. 2011). Despite this coating, tissue adherence to membranes was inconsistent, representing a possible limitation of the membrane culture method. Tissue contraction is a likely cause of tissue-membrane separation, and therefore the addition of a reversible factor Rho associated kinase (ROCK) inhibitor (e.g., Y27632) in the initial days of culture may be beneficial. Y27632 has been shown

to inhibit spheroid formation of MSC, and upregulate cartilage associated genes in the chondrogenic cell line ATDC5 (Huang, Dai et al. 2011; Kambe, Hayashi et al. 2012).

Reduced oxygen tension during chondrogenic differentiation of MSC has been investigated in human, rat and ovine BM-MSc and human as well as mouse AT- MSC (Malladi, Xu et al. 2006; Kanichai, Ferguson et al. 2008; Zscharnack, Hepp et al. 2010; Adesida, Mulet-Sierra et al. 2012). In contrast, little is known about the effect of reduced oxygen tension on the chondrogenesis of equine CB-MSc. While reduced oxygen culture conditions promote in vitro chondrogenesis of BM-MSc in various species (Kanichai, Ferguson et al. 2008; Zscharnack, Poesel et al. 2009; Markway, Tan et al. 2010; Meyer, Buckley et al. 2010), it did not have the same effect on human and murine AT-MSc (Malladi, Xu et al. 2006; Pilgaard, Lund et al. 2009). Here we report that, similar to BM-MSc, equine CB-MSc was enhanced by reduced oxygen culture. The different findings between BM-MSc, CB-MSc and AT-MSc may be attributed to differences between the MSC sources or that MSC cultures consist of heterogeneous cell population. Differential response of different sourced MSC populations to reduced oxygen levels may reflect the distinctive nature of the original niche. For example, the AT niche has an ambient oxygen concentration (10-15%) higher than that of BM (<7%) (Harrison, Rameshwar et al. 2002; Bizzarri, Koehler et al. 2006). To date, the oxygen level in the in vivo CB niche remains unknown, although it is presumed to not exceed 8% oxygen (Fischer and Bavister 1993).

HIF1 $\alpha$  mRNA transcripts were not significantly upregulated in the present study, although the mean mRNA expression level of both HIF1 $\alpha$  and Sox9 were increased in 5% culture, compared to 21% cultures. Consistent with our data, Adesida and colleagues did not observe significant difference in HIF1 $\alpha$  mRNA levels between 21% and 5% chondrogenic culture of BM-MSc (Adesida, Mulet-Sierra et al. 2012). HIF proteins are constitutively expressed and regulated at the protein level (Schipani 2006), thus possibly accounting for the non-significant differences in transcript levels of HIF genes.

Alternatively, Lafont and colleagues have proposed that HIF2 $\alpha$ , and not HIF1 $\alpha$ , is responsible for Sox9 upregulation and downstream effectors (Lafont, Talma et al. 2007).

Temporal changes in mRNA expression levels in equine CB-MSCs during *in vitro* chondrogenesis have been reported (Buechli, Lamarre et al. 2012). Similar dynamic changes in gene expression levels were observed between membrane and pellet cultures at days 7 and 14, although by day 21 these differences were not significant (Lee, Hurtig et al. 2011). In the present study, end point assessment was conducted on day 21 cultures and no significant differences were observed for gene expression analysis between treatment groups, including groups in reduced oxygen. Future assessment targeting temporal changes at multiple time points would be productive in determining changes in response to mode of culture as well as reduced oxygen.

Another plausible explanation for non-significant gene expression data may be due to continuous chemical induction with a single factor, TGF $\beta$ 3. Chondrogenic culture at high cell density with the addition of TGF $\beta$ 3 mimics the *in vivo* initiation of chondrogenesis, particularly the cellular condensation event (Yoon and Lyons 2004). However, unlike the continuous static application of TGF $\beta$ 3 in culture conditions, *in vivo* MSC differentiation is influenced by a variety of different and dynamically expressed spatiotemporal cues. The temporal administration of growth factors, such as timed addition or removal of dexamethasone or TGF $\beta$ 3 was shown to positively influence matrix components (Bahney, Hsu et al. 2011; Buxton, Bahney et al. 2011). I speculate that continuous administration of solely TGF $\beta$ 3 may cause gene dysregulation. After chondrogenesis has been initiated by TGF $\beta$ 3 in the first few days, a cascade of endogenous growth factors may come into play, possibly interacting with the exogenous source of TGF $\beta$ 3.

Future studies would benefit from quantitative analyses of collagen and HIF protein abundance. Protein quantification (or semi quantitative) assays such as western blotting or ELISA (enzyme linked

immunosorbent assay) would be a useful complement to mRNA expression data which could provide insights into the functional aspects of chondrogenesis.

Significant donor variation is observed during gene expression analysis in the present study as evidenced by large confidence intervals. Stringent determination of technical outliers was applied on raw CT values to avoid arbitrary dismissal of biological variation. A larger study group may be necessary to confirm true non significance or show trends of differential response to treatment groups.

### **Future directions**

Reduced oxygen membrane culture conditions represent an improved protocol to pre-condition CB-MSCs to chondrogenic cell fate. Interesting future experiments would include determining the contribution of hypoxia on hypertrophy as well as future experiments geared towards utilizing the pre-conditioned cells for generation of biphasic implants would be of interest.

A major limitation of using MSCs for chondrogenesis *in vitro* is the induction of hypertrophic phenotype. Oxygen is reportedly involved in the progression to hypertrophy (Morita, Miyamoto et al. 2007; Kim, Kim et al. 2008). Other than type X collagen, additional markers of interest for documenting the transition to hypertrophy include RunX2, a potent transcription factor for endochondral ossification, osterix (OSX), a downstream factor controlling osteoblasts differentiation (Nakashima, Zhou et al. 2002), and Parathyroid related protein (PTHrP), which has been shown to down regulate collagen type X and RunX2 gene expression (Kim, Kim et al. 2008).

A biphasic osteochondral construct is an implant consisting of cartilage and bone. Generation of this construct involves seeding pre-conditioned chondroprogenitor cells on top of an osteoconductive scaffold. Previous evaluations of biphasic scaffolds *in vitro* have been done using BM-MSCs in an ovine model, and implantation into non-loading locations have demonstrated success (Kandel, Grynblas et al.

2006; Lee, Hurtig et al. 2011). Similar evaluation in horses, and in a location which experiences high impact loading is warranted before clinical application in horses is possible.

Membrane culture conditions represent an intermediate step to pre-condition MSC to chondrogenic cell fate. Following pre-conditioning, cells are released from their matrix for transplantation or seeding on to an osteoconductive scaffold. My preliminary data indicates that collagenase digestion is able to release cells while maintaining cell viability (Appendix 1, Figure 1). However, more work is warranted to increase cell recovery after digestion, as maximally achieved recovery was only approximately a quarter of the initial seeded cells (Appendix 1, Figure 2).

My preliminary data has shown that chondrocytes derived from pre-conditioned eCB-MSC and seeded on top of osteoconductive scaffolds are able to secrete ECM without the application of exogenous induction factors (such as TGF $\beta$ -3)(Appendix 2, Figure 1). Detailed characterization of the cartilage formed from these osteochondral constructs is warranted prior to application *in vivo*.

Work by Lee and colleagues demonstrated feasibility and initial success of such biphasic constructs *in vitro* using ovine BM-MSC (Lee, Hurtig et al. 2011). However, the resulting cartilage was unable to withstand the loading and shear force *in vivo*, possibly due to the lack of a zone of transitional calcification at the interface between cartilage and scaffold (David W. Lee, personal communication). It is possible that the scaffold material, calcium polyphosphate (CPP), may inhibit calcification (Lee, Hurtig et al. 2011). In this respect, the use of alternative scaffold materials should be considered. Moreover, FDA approved osteoconductive scaffolds are available commercially such as proOsteon® (Parsippany, NJ, USA).

Another suggestion to improve integration would be to seed and induce MSC in two layers. The first layer would be intentionally induced to hypertrophy by 21% culture, TGF $\beta$  and Dexamethasone withdrawal or administration of Thyroid hormone (Mueller and Tuan 2008)) to form the transitional

calcified layer. For the second layer, MSC would be seeded on top of this ‘hypertrophic’ layer and induced to chondrogenesis and prevented from hypertrophy (in low oxygen culture). Since the process of endochondral ossification occurs progressively, it is unlikely that the hypertrophic chondrocytes from the initial seeding would revert to a pre-hypertrophic state.

In vivo cartilage repair in horses is difficult for various reasons. In particular, heavy loading and shear movement of the animal during the repair process can lead to loss of the implanted construct. Confinement or use of slings are options to reduce movement in cooperative horses. Another option may be use of KineSpring® technology (Hayward, CA, USA), where an extra-articular, spring-loaded implant unloads and alleviates joint pain in humans. Modification to this technology to withstand much heavier loading such as that experienced in the horse joint would be useful to reduce the likelihood of implanted cartilage to shear off. Ideally, the degree of unloading could be controlled to allow for progressive re-loading and therefore experience in vivo mechanical stimulation.

## **Conclusion**

In conclusion, my data demonstrates that mode of culture and oxygen availability are significant contributors to the phenotype of neocartilage generated in vitro. Membrane culture and reduced oxygen produced neocartilage with more hyaline-like phenotype with homogenous morphology, high GAG and type II collagen content. Relative quantification by mRNA analysis were non significant between culture groups. Temporal assessment of mRNA levels or protein quantification of types I,II and X collagen, and HIF1 $\alpha$  may provide insight in the observed non significance. The work presented here is an important step towards using equine CB-MSC for cartilage replacement strategies to treat articular cartilage damage in horses. Future studies should aim to evaluate these preconditioned cells in tissue engineered implants or cytotherapeutic strategies.

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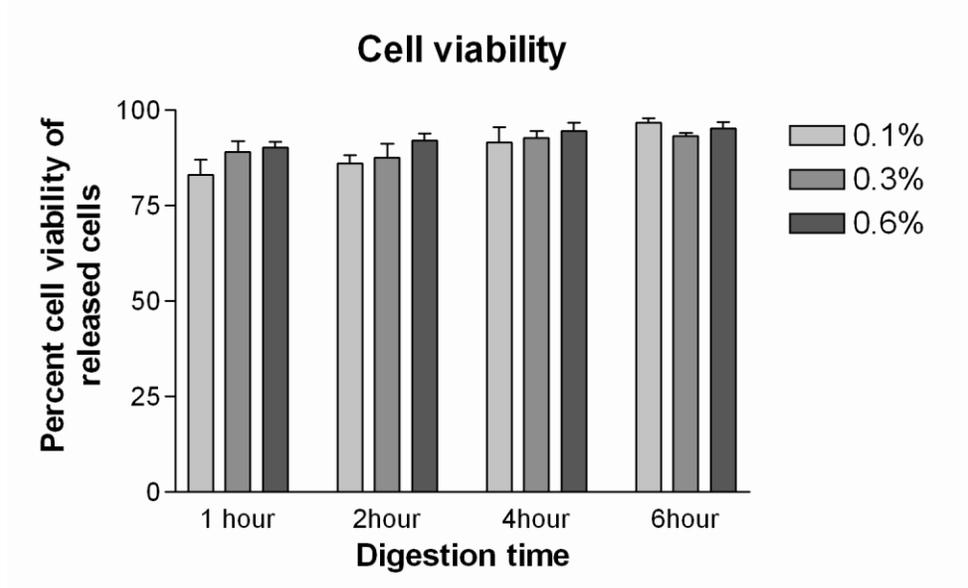
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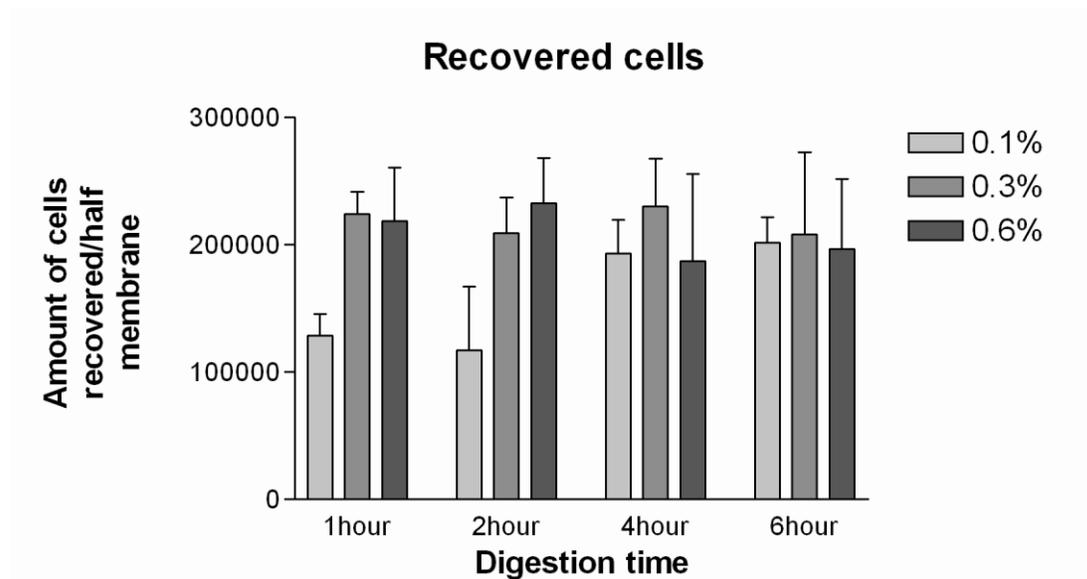
## APPENDIX 1

### Cellular release preliminary data



*Figure A1. Cell viability of cells released from membrane culture by collagenase digestion*

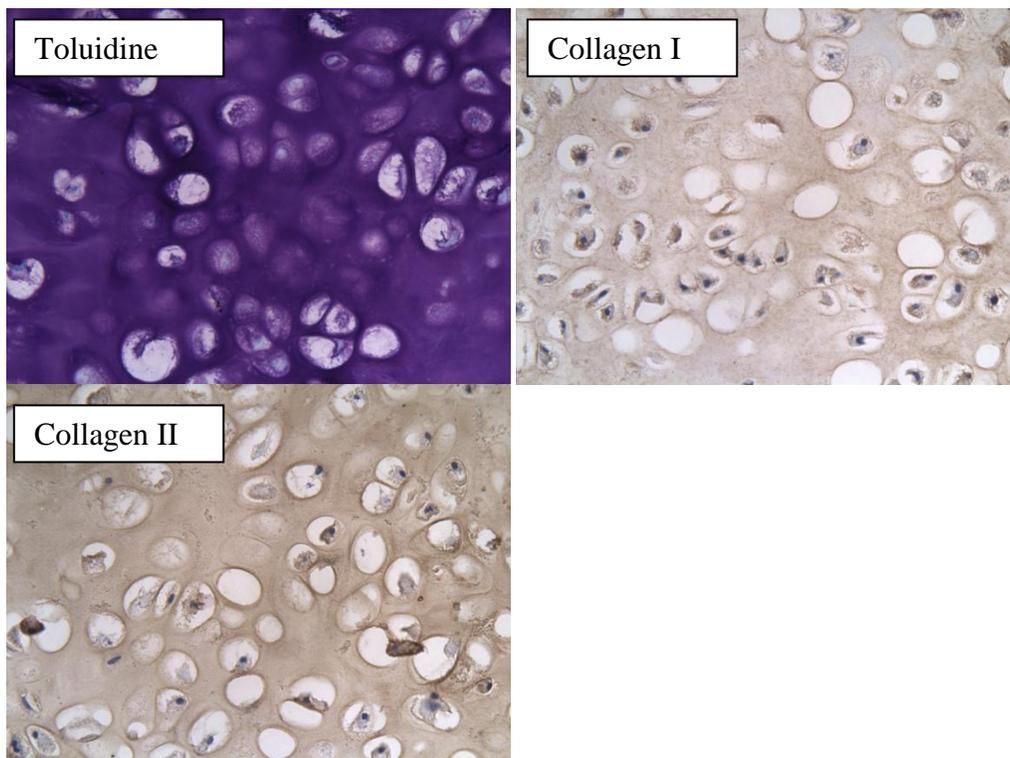
*Preconditioned cells from membrane culture are successfully released by collagenase digestion with high viability.*



*Figure A2. Recovered cells following collagenase digestion.*

*Following cellular release from collagenase digestion, cell recovery was about one quarter of the initial amount of cells seeded (2 million cells per membrane, where half a membrane was used per treatment group).*

Brief summary of methods: Membrane culture was set up as described in materials and methods and was in chondrogenic culture for two weeks. At termination, neocartilage was removed from the membrane insert and sliced in half for collagenase digestion study. Collagenase (Sigma-Aldrich; Oakville, ON, CAN) was mixed with CM (minus TGF $\beta$ 3) to the set concentrations (0.01%, 0.03%, 0.06%). Digestion was then carried out in 1.5ml PCR tubes in heated blocks with agitation. After designated time, cells were washed with CM and counted with nucleocounter. Cell recovery is therefore representative of cells recovered per half membrane, or  $1 \times 10^6$  of the initially seeded cells.



*Figure 3. Histological examination of cartilage tissue generated on top of CPP.*

*Neocartilage formed from preconditioned cells display hyaline like phenotype despite lack of induction agent (TGF $\beta$ ). Presence of GAG by toluidine blue and collagens type I and II are observed.*

**Brief summary of methods:** Pre conditioned chondrocytes generated from 5% membrane culture for 2 weeks was released with 2 hours of 0.06% collagenase digestion as described above. CPP scaffolds (Courtesy David W.Lee) were neutralized in PBS and subsequently seeded with  $2 \times 10^6$  of the released cells and cultured in chondrogenic media without TGF $\beta$ 3 in 5% oxygen and cultured for an additional 2 weeks. Media for both steps were changed every 2 days. Histological materials and methods were identical to those listed in chapter 2.