CHONDROGENIC CAPACITIES OF EQUINE SYNOVIAL PROGENITOR POPULATIONS

BY

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DISSERTATION

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ABSTRACT

Osteoarthritis is linked to approximately 60% of equine lameness problems. This disease involves compromised homeostasis of articular cartilage and eventual loss of this tissue in affected joints. Mesenchymal stem cells (MSCs) provide promise for cell-based therapies for articular cartilage repair; however, MSCs from specific tissues exhibit source-based differences in phenotypic and biosynthetic activities, influencing the utility of specific MSC populations for clinical applications. The objective of this research program is to provide strategies using synovial progenitor populations for articular cartilage repair in horses.

The synovial fluid samples were added directly to culture medium and maintained until confluence. The primary cultures were passage twice to generate sufficient cell numbers for differentiation. Monolayer cells were treated with osteogenic and adipogenic medium for 14 days and confirmed by cytochemical analysis. For chondrogenesis, cells were maintained in pellet culture with chondrogenic medium for 20 days. Expression of chondrocytic phenotypes was assessed by quantitative RT-PCR of chondrocyte-specific genes, DMMB assays for deposition of sulfated glycosaminoglycans (sGAG), an ELISA assay for collagen type II (Coll II) deposition and the alkaline phosphatase (ALP) bio-assay. The results showed that equine synovial fluid-derive MSCs is an alternative cell source for articular cartilage repair and might be more appropriate source than MSCs isolated from bone marrow.

To compare the chondrogenic capacities of MSCs isolated from equine synovium (SYN), bone marrow (BM) and adipose (FAT) tissue, six healthy adult horses were used in this study. BM

aspirates were collected from tuber coxae, SYN tissues were collected from right radiocarpal joint, and FAT tissues were collected from the subcutaneous space adjacent the tail. Synovium and fat were minced and digested in 0.2% collagenase for 3 hr. Primary cell isolates were seeded at low density and expanded through two passages. After expansion, cells were transferred to pellet cultures in DMEM with ITS, ascorbic acid, sodium pyruvate, dexamethasone and TGF-β1 for 20 days. Chondrogenesis was assessed by Q-PCR of chondrocytic gene expression, measurement of Col II and sGAG secretion, and by ALP activity. The results showed that SYN cells are phenotypically more appropriate for articular chondrogenesis than MSCs from bone marrow or fat.

We also have developed equine BMP-2 adenoviral expression system that can induce chondrogenesis in equine synovium-derived cells. The putative equine BMP-2 sequence was generated by blasting the human and murine BMP-2 cDNA sequences across the equine genome data base. The open reading frame of equine BMP-2 was obtained from equine articular chondrocyte RNA by gene specific RT-PCR, following by two rounds of nested PCR amplification. The nested PCR product was sub-cloned into pCMV-SPORT to generate the eqBMP-2 expression vector and into the VQAd CMV K-NpA shuttle plasmid to generate the equine BMP-2 adenoviral expression system. The results showed that the mature eqBMP-2 protein contained 114 amino acids and was completely consistent with the amino acid sequences of the mature human and murine BMP-2 proteins, and the adenovirus, when applied to cells at an MOI of 100, generated BMP signaling activity equivalent to approximately 25 ng BMP-2/ml. The eq-BMP2-infected cells deposited significantly higher sGAG and formed larger size of pellets than did control and LacZ-infected groups.

To my parents

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CHAPTER 1: INTRODUCTION

Osteoarthritis is linked to approximately 60% of equine lameness problems. This disease involves compromised homeostasis of articular cartilage and eventual loss of this tissue in affected joints. Articular cartilage is a highly specialized connective tissue with several unique characteristics; it is a hypocellular, alymphatic and avascular tissue with little or no intrinsic regenerative capacity. Current clinical treatments for articular cartilage injuries, including arthroscopic debridement/lavage and microfracture surgery, are less than satisfactory and rarely produce a fully functional restoration of the articular surface.

Currently, autologous chondrocyte transplantation is the most effective method for cartilage repair. However, there are limited sources of autologous, fully differentiated chondrocytes and in vitro expansion of these cells inevitably compromises the specialized phenotype of these cells. MSC populations represent an attractive alternative source of chondrogenic cells, given their chondrogenic potential, capacity for self-renewal, ease of access, and lack of immunogenic or tumorigenic activities. Under appropriate culture conditions with stimulation of growth factors, MSCs can be induced to undergo chondrogenic differentiation.

The objective of this research program is to provide strategies using synovial progenitor populations for articular cartilage repair in horses.

This thesis is composed by a literature review and three original research chapters. The literature review covers the topics of characteristics of general stem cells and mesenchymal stem cells,

development of synovial joints, adult stem cells within and around joints, as well introducing stem cell-based cartilage repair.

The first study (chapter 3) addressed the chondrogenic potential of equine synovial fluid-derived stem cells. In this chapter, the experiments were focus on determining whether equine synovial fluid-derived stem cells are capable of expanding to provide experimentally useful number of cells, of multi-lineages differentiation, and of endochondral or permanent/ articular chondrogenesis. We also compared the CFU-forming capacities of synovial fluid-derived stem cells from equine arthritic and normal joints.

The second study (chapter 4) investigated the comparative chondrogenic capacities of equine synovium-, bone marrow-, and adipose-derived stem cells under identical culture conditions and the endochondral differentiation potential among three sources. We also optimized the culture requirements for *in vitro* chondrogenic differentiation of equine synovium-derived stem cells in pellet cultures.

The third study (chapter 5) focused on the development of equine BMP-2 adenoviral expression system that can be used for clinical application in horses. This study was conducted to clone and characterize equine BMP-2, as well introducing chondrogenesis of synovium-derived stem cells by adenoviral expression of equine BMP-2.

CHAPTER 2: LITERATURE REVIEW

Characteristics of stem cells

Stem cells are characterized as cells able to self-renew and differentiate into multiple cell types. Stem cells are categorized on the breadth of their differentiation ability.

(1) **Totipotent stem cells** have the potential to differentiate into all cell types in an organism, including extra-embryonic cell types. Totipotent cells include spores and zygotes.
(2) **Pluripotent stem cells** are derived from inner cell mass of the blastocyst. They are able to differentiate into three germ layers: endoderm, mesoderm and ectoderm. Inner mass cells are pluripotent.
(3) **Multipotent stem cells** can give rise to multiple cell types in limited lineages, such as hematopoietic cells and mesenchymal stem cells.
(4) **Oligopotent stem cells** are able to give rise to a few cell types within a tissue, such as corneal stem cells and vascular stem cells.
(5) **Unipotent stem cells** have the capacity of differentiating into a single specialized cell type, such as hepatocytes and epithelium stem cells.

Embryonic stem cells (ES cells) are derived from inner cell mass of the blastocyst. ES cells can differentiate into the three germ layers and propagate indefinitely under defined conditions. The characteristics of plasticity and unlimited replication of embryonic stem cells provide useful tools for clinical and regenerative research. As examples, in 2009, the first human ES trial using oligodendrocytes derived from human ES cells for spinal cord injury was approved by U.S. Food

and Drug Administration. In 2011, Advanced Cell Technology received the approval from U.K. Medicines and Healthcare Products Regulatory Agency for a human clinical trial using retinal pigment epithelium derived from human ES cells for the treatment of Stargardt's Macular Dystrophy. Further, intra-lesional injection of an equine ES cell line improved tendon healing in an equine collagenase-induced tendinopathy model (Watts *et al*., 2011).

Adult stem cells are multipotent stem cells residing within tissues among differentiated cells. They are required for tissue repair and homeostasis. It has been reported that stem cells are found in almost every tissue in the body and are less likely to initiate rejection after transplantation (Bradley, Bolton, & Pedersen, 2002; Duffy *et al*., 2011; Pilat & Wekerle, 2010; Sordi & Piemonti, 2011). To maintain stem cell characteristics, adult stem cells reside in microenvironmental niches, in which stem cell activity is regulated by adjacent supporting cells. Stem cell niches have been characterized for hematopoietic stem cells (HSC), myosatellite cells, central nervous system stem cells, spermatogonial stem cells and epithelial stem cells (Aziz, Sebastian, & Dilworth, 2012; Goldstein & Horsley, 2012; Hugnot & Franzen, 2011; Oatley & Brinster, 2012; Suarez-Alvarez, Lopez-Vazquez, & Lopez-Larrea, 2012).

In the absence of exogenous stressors, stem cells remain quiescent in their niches interactions between stem cells and surrounding cells and among stem cells themselves via adherent junction and soluble signals. With stress, stem cells undergo symmetrical and asymmetrical division to give rise to daughter cells committed to specialized cells while maintaining stem cell numbers (Morrison & Kimble, 2006). In symmetrical division, a stem cell gives rise to two daughter cells

identical to the original cell. In asymmetrical division, a stem cell divides to produce an identical stem cell and another daughter cell committed to differentiate into more specialized cell types. In epithelial stem cells (EpSCs), the strength of contacts between the stem cell and basement membrane or between stem cells determines whether these cells will undergo asymmetrical or symmetrical division. With the strong inter-cellular contacts, EpSCs undergo symmetrical division and give rise to two identical stem cells. When contacts between EpSCs and the underlying basement membrane dominate, the mitotic spindle becomes aligned perpendicularly to the basement membrane, generating a stem cell and a lineage-committed daughter cell (Yamashita, Jones, & Fuller, 2003).

Induced pluripotent stem cells (iPSCs) are differentiated cells reprogrammed to embryonic stem cell-like state by inducing expression of transcription factor genes important for maintaining characteristics of embryonic stem cells. In 2006, murine iPSCs were produced by introducing Oct3/4, Sox2, c-Myc, and Klf4 expression constructs into mouse fibroblasts, and maintaining the transduced cells under ES cell culture conditions (Takahashi & Yamanaka, 2006). The mouse iPSCs not only created teratomas after injection into nude mice, but also contributed to embryonic development after injection into blastocysts. A year after the initial publication, human IPSCs were generated by introducing the same four genes into human dermal fibroblasts (Takahashi *et al*., 2007). These cells acted as human embryonic stem cells and could differentiate into all three germ layer cell types in vitro and form teratomas. In light of the substantial ethical and legal issues surrounding ESC research and clinical applications, iPSCs provide a valuable alternative resource for ongoing stem cell research.

Characteristics of mesenchymal stem cells

Mesenchymal stem cells (MSCs), originally referred to as 'colony-forming unit-fibroblast' (CFU-Fs), were first isolated from bone marrow by Friedenstein and colleagues (Friedenstein, Gorskaja, & Kulagina, 1976). Many subsequent studies demonstrated that MSCs are multipotent, non-hematopoietic stem cells present in low numbers (estimated at 1 in 100,000-500,000 nucleated cells in bone marrow aspirates) which are capable of differentiating into mesenchymal tissues, including muscle, bone, cartilage, tendon, ligament and adipose (Nitahara-Kasahara *et al*., 2012; Wei *et al*., 2011). However, unlike ESCs, MSCs exhibit finite life spans under culture expansion, and become less prevalent with age, indicating that replicative senescence is active under both in vivo and in vitro conditions (Beausejour, 2007; Colleoni *et al*., 2009; Fehrer *et al*., 2007; Liu *et al*., 2009; Sethe, Scutt, & Stolzing, 2006). These deficits have stimulated many studies addressing in vitro culture techniques and alternative MSC sources to ensure sufficient number of competent MSCs can be generated for clinical needs.

The isolation and in vitro expansion of MSC populations is best characterized for bone marrow-derived cells. MSCs are routinely isolated from aspirates of bone marrow, and are cultured on culture plates with 10% fetal Bovine Serum at low initial seeding densities. Cells capable of adhering to the plates are more likely to be MSCs, whereas hematopoietic cells and other cells from bone marrow do not attach and are washed away over time. Stem cell 'purity' develops as the comparatively greater proliferative capacity of the MSCs dominates those of co-isolated non-stem cells that remain after washing. MSC purification has been improved by size-dependent filtering and cell sorting with microbeads or FACS (Xu *et al*., 2010; Folch *et al*., 2009; Pina-

Aguilar *et al*., 2009; Nelson *et al*., 2009). Single or combinatorial of growth factor 'cocktails', including transforming growth factor β1 (TGF-β1), basic fibroblast growth factor (bFGF), epidermal growth factor (EGF) and platelet-derived growth factor(PDGF), can be administered to the initial bone marrow-derived cell cultures, to accelerate proliferation and generate more homogenous population of MSCs (Ahn *et al*., 2009; Hall *et al*., 2001; Long *et al*., 1995; Sun *et al*., 2003).

Definitive identification of MSCs is still challenging. MSCs exhibit a number of surface markers; however, none of them are exclusively expressed on MSCs. Stro-1 is the best-known cell surface epitope, but it is progressively lost during culture expansion and is not exclusively expressed by MSCs (Gronthos *et al*., 2003). Co-expression of Stro-1, CD73 (ecto-5'-nucleotidase), and CD106 (vascular cell adhesion molecule) are recommended as the most useful marker profile for MSCs (Kolf, Cho, & Tuan, 2007). Negative markers can also be applied to eliminate contaminants from MSC preparations. CD11b (an immune cell marker), glycophorin-A (an erythroid lineage marker), CD31 (a marker of endothelial and hematopoietic cells), CD45 (a marker of all hematopoietic cells), or CD117 (a marker of hematopoietic cells) are consensually not expressed on MSCs (Colter, Sekiya, & Prockop, 2001; Gronthos *et al*., 2001; Meirelles Lda & Nardi, 2003). In 2006, the International Society for Cellular Therapy (ISCT) proposed three criteria to define human MSCs. Human MSCs must be plastic-adherent cells, express CD105, CD73 and CD90, and lack expression of CD45, CD34, CD14 or CD11b, CD79α or CD19 and HLA-DR surface molecules. Human MSCs must be capable of differentiating to osteoblasts, adipocytes and chondroblasts in vitro (Dominici *et al*., 2006). To date, similar immuno-

phenotypes have not been established for equine MSCs, due to a lack of species-specific antibodies and validation of epitope expression and/or recognition by existing antibodies.

Despite the fact that bone marrow is the most accessible and enriched source of MSCs, and the most studied, MSCs and MSC-like cells have been isolated from a number of other tissues and body fluids including muscle, adipose tissue, periosteum, dermis, pericytes, blood, trabecular bone and synovial membrane (De Bari, Dell'Accio, & Luyten, 2001; De Bari, Dell'Accio *et al*., 2001; Dowthwaite *et al*., 2004; Jones *et al*., 2004; Park *et al*., 2012; Wickham *et al*., 2003), providing promising alternative sources of MSCs for tissue engineering and regenerative medicine. It is noteworthy that MSCs in tissues other than bone marrow demonstrated more limited capacity of differentiation, and appear to be adapted to the tissues in which they reside.

Chondrogenesis

"Chondrogenesis" refers to the process whereby cartilage is generated from pre-chondrogenic cell populations. Chondrogenesis involves mesenchymal cell condensation, chondrocyte differentiation, proliferation, hypertrophy and ossification, resulting in the formation of cartilage (Khan *et al*., 2007). In the embryo, undifferentiated mesenchymal cells aggregate and undergo condensation to form skeletal anlage, or templates at the sites of future bones. Postnatally, chondrogenesis occurs at site of bone repair where healing occurs by endochondral ossification.

During skeletogenesis, mesenchymal cells undergo further condensation at joint initiation sites to generate an interface and cavitation zone. The synovial cavity is then formed by physical

separation of the skeletal anlaga and the interlocking structure of the joint is molded by morphogenetic processes and biomechanical stimulation (Archer, Morrison, & Pitsillides, 1994; Craig, Bentley, & Archer, 1987; Ito & Kida, 2000). Ultimately, the mature synovial joint is formed, composed of the synovial cavity, articular cartilage, synovial membrane and joint capsule.

The rate and extent of chondrogenesis and chondrocyte differentiation are controlled by a network of interacting regulatory pathways, driven by paracrine and autocrine growth factors. Transforming growth factor-βs (TGF-β), Indian Hedgehog (IHH), parathyroid hormone related peptide (PTHrP) and fibroblast growth factor 18 (FGF-18) regulate proliferating chondrocyte transition to the hypertrophic phase of differentiation. In proliferating chondrocytes, TGF-β and C-terminal IHH upregulate the expression of PTHrP, which not only stabilizes the proliferative chondrocyte phenotype but also inhibits the transition of proliferating chondrocytes into the hypertrophic phase of differentiation (Ballock *et al*., 1993; Deckelbaum *et al*., 2002; Serra, Karaplis, & Sohn, 1999). PTHrP mutation results in Jansen-type metaphyseal chondrodysplasia, manifesting as delayed skeletal maturation (Minagawa *et al*., 1997; Suda, 1997). PTHrPR -/- chondrocytes in chimeric mice exhibit premature hypertrophy and influence the proliferation rate of surrounding wild-type cells (Verheijen *et al*., 1999). Similarly, activating mutations in fibroblast growth factor receptor 3 (FGFR3), the receptor expressed by chondrocytes that responds to the FGF ligand, resulting in chondrodysplasias characterized by aberrant chondrocyte differentiation (Sahni *et al*., 1999).

Ballock *et al* showed that TGF- β1 inhibits hypertrophic differentiation in vitro (Ballock *et al*., 1993a) and Serra *et al* demonstrated that expression of dominant negative transforming growth factor-β receptor (TGF-βR II) results in increased hypertrophic differentiation (Serra *et al*., 1997), which provide evidence that hypertrophic differentiation is inhibited by TGF-β. Serra *et al* also demonstrated that TGF-β1 induces PTHrP expression in a metatarsal rudiment organ culture, indicating that these signaling pathways interact to regulate chondrocyte differentiation (Serra *et al*., 1999).

IHH is upregulated in early hypertrophic chondrocytes. Loss of IHH expression results in a significant reduction in growth plate cell proliferation and loss of perichondrial PTHrP expression (St-Jacques, Hammerschmidt, & McMahon, 1999). Deckelbaum *et al* identified distinct and antagonistic activities of N-terminal and C-terminal fragments of IHH (Deckelbaum *et al*., 2002). The amino terminal fragment remains membrane-bound and stimulates hypertrophic differentiation. However, the carboxyl terminal fragment is released from the pericellular environment and stimulates PTHrP expression in the perichondrium and proximal chondrocytes, which inhibits hypertrophic differentiation. Kameda *et al* demonstrated that BMP-2 induces IHH expression in an in vitro model of hypertrophic differentiation. BMP signaling is necessary for the maintenance of the chondrocytic phenotype and for the induction of hypertrophy (Kameda *et al*., 1999). These data indicate that IHH is critical for the negative regulation of chondrocyte entry into hypertrophy and the stimulation of hypertrophic differentiation by chondrocytes that have already entered into this stage of differentiation.

FGF is an inhibitor of chondrocyte terminal differentiation (Inoue *et al*., 1989). Iwamoto *et al* demonstrated that hypertrophic differentiation is coupled to down-regulation of FGF receptor type 3 (Iwamoto *et al*., 1991). FGFR3 is preferentially expressed in early hypertrophic chondrocytes. FGFR3 signaling induces the transition to hypertrophic chondrocytes, mediated by the FGF 18, a ligand expressed by perichondrial cells immediately adjacent the growth plate (Sahni *et al*., 1999).

Downstream of these ligand-mediated signaling pathways, several transcription factors conspire to regulate chondrogenesis and determine the fate of chondrocytes (de Crombrugghe, Lefebvre, & Nakashima, 2001). Sox9 is required for mesenchymal condensation and is expressed throughout chondrogenesis until hypertrophy. Sox5 and 6 are also required for the synthesis of a cartilaginous extracellular matrix. Runx2 is mandatory for bone formation, but also stimulates the transition to hypertrophic chondrocytes. Collectively, these growth factor ligands and transcription factors coordinate the chondrogenic differentiation of MSCs and the subsequent phenotypic status of committed chondrocytes during embryonic skeletogenesis, post-natal bone growth and fracture callus maturation.

Stem cell populations in joints

Synovium-derived stem cells. Multipotent mesenchymal stem cells from synovium (SM-MSCs) were first identified by De Bari *et al* in 2001 (De Bari, Dell'Accio, Tylzanowski *et al*., 2001). Synovial tissue was collected from human knees of donors (range 18-65 years with mean 41), either within 12 hours of death or at the time of knee replacement. SM-MSCs were able to

expand over 10 passages with similar proliferation activity and limited senescence, even in cryopreserved cells. They were positive for CD44, CD 90, CD105, and were negative for CD45 (Harvanova *et al*., 2011). Donor age, cell passaging, and cryopreservation did not affect the multipotent capacity of SM-MSCs. It has been shown that a subpopulation of CD105+ SM-MSCs exhibits more uniformity in spindle shape (Harvanova *et al*., 2011), compared to non-selected cells, and showed similar chondrogenic potential from OA and normal synovium (Arufe *et al*., 2009; Harvanova *et al*., 2011). SM-MSCs express high levels of proline arginine-rich end leucine-rich repeat protein (PRELP), a common characteristic of MSCs from intraarticular tissues and articular cartilage.

SM-MSCs exhibit greater chondrogenic and proliferation potential than MSCs from bone marrow, periosteum, skeletal muscle, and adipose tissue, in vitro and in vivo (Koga *et al*., 2008; Mochizuki *et al*., 2006; Sakaguchi *et al*., 2005; Shirasawa *et al*., 2006; Yoshimura *et al*., 2007). Even though MSCs from bone marrow and from periosteum also show considerable chondrogenic capacity, SM-MSCs pellets are larger and deposit more cartilage matrix, indicating they are superior in chondrogenesis. Undifferentiated SM-MSCs in collagen gel were transplanted into full thickness osteochondral defects of adult rabbits at 5×10^7 cells /ml. After 24 weeks, the repair tissue integrated into cartilage without the expression of type I or type X collagen, and, in the deep zone, transplanted SM-MSCs differentiated into hypertrophic chondrocyte-like cells and bone (Koga *et al*., 2008). In an in vitro model, autologous human serum is predominant in proliferation and chondrogenic capacity of SM-MSCs, compared to fetal bovine serum. However, the in vivo chondrogenic potential of rabbit SM-MSCs is similar in autologous serum and FBS (Nimura *et al*., 2008).

Differentiation-predictive markers have been studied in synovium-derived mesenchymal stem cells. Arufe *et al* analyzed the chondrogenic potential of CD73+, CD106+, and CD271+ subpopulations of MSCs derived from human synovium (Arufe *et al*., 2009; Arufe *et al*., 2010). The CD106+ subpopulation had the poorest chondrogenic potential, while it was less osteogenic and more adipogenic than the CD73+ subpopulation. The CD271+ subpopulation had the greatest chondrogenic potential, followed by CD73+ subpopulation. The CD73+ subpopulation exhibited greater osteogenic capacity than the other two immune-phenotypes.

Synovial fluid-derived stem cells. The first paper to evaluate presence of mesenchymal stem cells in synovial fluid was published in 2004. Jones *et al* collected synovial fluid samples from 100 arthritic patients. MSCs in synovial fluid were clonogenic and multipotent to differentiate into chondrocytes, osteoblasts, and adipocytes, and the number of MSCs was significantly higher in osteoarthritis (OA) than in rheumatoid (RA) (Jones *et al*., 2004). In 2008, the same group showed MSCs were present in normal joint and were increased 7 fold in early OA (Jones *et al*., 2008). Morito *et al* compared the synovial fluid derived mesenchymal stem cells (SF-MSCs) from patients with anterior cruciate ligament injury and healthy volunteers. SF-MSCs from ligament injury patients yielded 100 times more total colony numbers than SF-MSCs from healthy volunteers. The epitope profiles of SF-MSCs were similar to the MSCs derived from bone marrow and synovium. However, the gene profiles in SF-MSCs were more similar to those in MSCs from synovium than in bone marrow MSCs (Morito *et al*., 2008). SF-MSCs were

negative for CD14, CD34, CD45, and positive for CD44, CD73, CD90, CD105 and CD166 (Lee *et al*., 2012; Morito *et al*., 2008).

The origin of SF-MSCs remains unclear. They are likely derived from synovium, in terms of the morphology, colony size, and gene expression profiles similar between synovium MSCs and SF-MSCs (Morito *et al*., 2008). The positive correlation between the number of SF MSCs and the number of synovial tissue fragment also supports the possibility that the synovium might be the origin of SF-MSCs. The other local origin of SF-MSCs is the superficial zone of cartilage (Lee *et al*., 2012). It has been showed that MSCs reside in the superficial zone of cartilage (Dowthwaite *et al*., 2004). In early OA, the superficial zone of cartilage is disrupted and might release the MSCs into synovial fluid, contributing to the number of MSCs increasing in early OA. Subchondral bone marrow may be another origin of SF-MSCs. It has been reported that synovial fluid from OA and normal joints have similar ability to stimulate migration of MSCs from subchondral bone marrow in vitro (Gerter, Kruegel, & Miosge, 2012), while the migration rate was significantly reduced in SF from RA joints (Kruger *et al*., 2012). The subset of chemokines, CCL25, CXCL10, and XCL1, present in synovial fluid may contribute to the recruitment of human mesenchymal stem cells from subchondral bone marrow (Endres *et al*., 2010).

Intra-articular adipose stem cells. Recent studies suggest that mesenchymal stem cells are present in intra-articular fat pad. In the first human study, intra-articular fat pads were obtained from patient with mean ages of 68 ± 11.1 years. Stromal cells were separated from floating adipocytes by centrifugation at 300 g for 5 minutes. Fat pad derived stem cells were constantly

positive for CD9, CD10, CD13, CD29, CD44, CD49e, CD59, CD105, CD106, and CD166. These cells were also capable of differentiating into osteoblast, chondrocytes, and adipocytes, under appropriate culture conditions (Wickham *et al*., 2003). English *et al* compared the chondrogenic capacity of human cartilage-, infrapatellar fat pad-, and bone marrow-derived cells. Infrapatellar fat pad derived stem cells were superior in stable chondrogenic capacity even after 16 passages, compared to stem cells isolated from healthy cartilage, OA cartilage, and bone marrow (English *et al*., 2007). It also been reported that intra-articular fat stem cells from OA and RA patients have similar chondrogenic potential (Skalska *et al*., 2011).

In an in vivo model, rabbit fat pad derived stem cells were intra-articular injected into OA joints, induced by unilaterally anterior cruciate ligament transection, after 12 week from surgery, at 1 million cells per ml of medium in each joint. After 16 and 20 weeks of surgery, all knees were examined by radiology, macroscopic observation, and histo-pathological evaluations. Rabbits injected with fat pad derived stem cells exhibited a lower degree of articular cartilage degeneration, osteophyte formation, and subchondral sclerosis, with a marked better quality of cartilage compared to control group (Toghraie *et al*., 2011). In a bovine model, infrapatellar fat pads were obtained from 3 month old claves. Lubricin, also called superficial zone protein, was expressed in bovine infrapatellar fat pad. Neither BMP-7 nor TGF-β1 alone stimulated the accumulation of lubricin, but together these two growth factors had an additive action on lubricin expression. The combination of BMP-7 and TGF-β1 also significantly upregulated the expression of the chondrocytic markers, collagen type II, aggrecan and Sox9, in infrapatellar fat pad-derived cells (Lee, Nakagawa, & Reddi, 2008).

Cartilage-derived stem cells. A unique progenitor population isolated from superficial zone of articular cartilage has been reported. Archer *et al* collected chondrocytes from superficial, middle, and deep zones of articular cartilage of 7 day-old calves, and initially isolated a progenitor population by differential adhesion to fibronectin (Dowthwaite *et al*., 2004). Superficial zone chondrocytes initially cultured on fibronectin for 20 minutes formed significantly bigger and larger number of colonies, compared to chondrocytes from middle and deep zones cultured for 40 minutes on fibronectin. The chondrocytes were also selected for Notch-1 expression and were cultured on fibronectin for 20 minutes. Eighty six percent of superficial zone chondrocytes were Notch-1 positive, significantly higher than cells from middle and deep zones, with 10% and 34%, respectively. Notch-1 positive superficial zone chondrocytes had a higher affinity to fibronectin and generated larger number of colonies compared to non-selected cells and chondrocytes from middle and deep zones. In an embryonic chick system, superficial zone chondrocytes were capable of differentiating into bone, tendon, perimysium, and articular fibrocartilage.

In normal cartilage, Notch-1, Stro-1, and VCAM-1, are expressed in a large number (45%) of chondrocytes. The number of Notch-1, Stro-1, and VCAM-1 positive cells is higher in the superficial zone of normal cartilage; however, Notch-1, Stro-1, and VCAM-1 positive cells are increased in middle zone of OA cartilage. It might not be appropriate to identify stem cells in articular cartilage by exclusive expression of Notch-1, Stro-1, and VCAM-1 (Grogan *et al*., 2009). Alsalameh *et al* also showed the percentage of multipotent CD105+/CD166+ cells isolated from OA cartilage were significantly higher than normal cartilage, and those cells did

not express chondrocytic markers. However, when cultured in micromass system with chondrogenic medium, CD105+/CD166+ cells expressed chondrocytic markers and deposited cartilaginous extracellular matrix (Alsalameh *et al*., 2004).

Subchondral bone-derived stem cells. In human late-stage osteoarthritis, a unique progenitor cells population distinct from chondrocytes and osteoblasts, termed chondrogenic progenitor cells (CPCs), has recently been identified (Koelling *et al*., 2009). CPCs are elongated cells located alongside breaks in the tidemark that delineate the interface between non-calcified and calcified articular cartilage. Koelling *et al* obtained 8-15 mm^3 specimens from areas adjacent to the main defect in human OA joints. These explants were cultured in DMEM supplemented with FBS and L-glutamine. After 10 days, the specimens were removed and the outgrown CPCs were cultured in monolayer. CPCs are able to migrate out of cartilage tissue taken from human OA, but this behavior is not found in healthy cartilage specimens. These cells are also positive for stem cell-relevant markers, STRO-1 and CD29, CD44, CD73, or CD90, but negative for hematopoietic markers. A later study reported that CPCs are positive for CD9, CD90, and CD166. CPCs are able to undergo adipogenic differentiation, osteogenic differentiation, and chondrogenic differentiation. Worthy of notice, CPCs are capable of chondrogenic differentiation in 3D-alginate culture without requiring stimulation by growth factors. Moreover, knockdown of the osteogenic transcription factor runx-2, enhances the expression of sox-9, resulting in an increase in collagen type II and aggrecan mRNA expression in CPCs (Gerter *et al*., 2012).

The gender of patients influences the differentiation potential of CPCs (Koelling & Miosge, 2010). It has been reported that sex hormone receptor proteins were abundant in CPC populations and physiological concentrations of estrogen and testosterone upregulated Sox9 and collagen type II in CPCs, from women and men, respectively. Runx2 expression was decreased by estrogen and high physiological concentrations of testosterone in CPCs from men. A decreased concentration of estrogen increased the expression of collagen type I in CPCs, more pronounced in men. Sex hormone adjustment in the late stages of OA patients might have beneficial effects on regenerative potential of CPCs.

Stem cell-based cartilage repair. Articular cartilage is a highly specialized connective tissue with several unique characteristics; it is a hypocellular, alymphatic and avascular tissue with little or no intrinsic regenerative capacity. Current clinical treatments for articular cartilage injuries, including arthroscopic debridement/lavage and microfracture surgery, are less than satisfactory and rarely produce a fully functional restoration of the articular surface. Chondrogenically-committed stem cell transplantation represents another alternative strategy for repairing articular cartilage.

Tissue engineering strategies utilize synthetic and natural scaffolds in conjunction with pre-seeded cells or host cells to replace or repair dysfunctional tissues due to congenital abnormalities or disease. Three main elements contribute to successful strategy for cell-based tissue engineering in articular cartilage repair. Firstly, a proper cell source to express the appropriate chondrocytic phenotype and generate a functional cartilaginous extracellular matrix.

Secondly, the appropriate stimulus of growth factors and hormones are necessary to stimulate chondrogenic differentiation and persistent matrix synthesis. Thirdly, a scaffold is required that retains or attracts the reparative cells and supports functional tissue growth in a three-dimensional context, that resists compression and shearing forces inside the joint.

Currently, autologous chondrocyte transplantation is the most effective method for cartilage repair. However, there are limited sources of autologous, fully differentiated chondrocytes and in vitro expansion of these cells inevitably compromises the specialized phenotype of these cells. MSC populations represent an attractive alternative source of chondrogenic cells, given their chondrogenic potential, their capacity for self-renewal, ease of access, and lack of immunogenic or tumorigenic activities. Under appropriate culture conditions with stimulation of growth factors, MSCs can be induced to undergo chondrogenic differentiation. Transforming growth factor-β (TGF-β) (Bian *et al*., 2011; Buxton *et al*., 2011; Park *et al*., 2009; Zheng *et al*., 2010), bone morphogenetic proteins (BMPs) (Badlani *et al*., 2008; Sellers, Peluso, & Morris, 1997; Tamai *et al*., 2005; van Beuningen *et al*., 1998), and insulin-like growth factors (IGFs) (Longobardi *et al*., 2006; Mierisch *et al*., 2002) have the ability to stimulate chondrogenesis in vitro and in vivo. These growth factors can be used during in vitro chondrogenesis, prior to implantation, or following implantation of chondrogenic cells, to drive the requisite phenotype and matrix biosynthesis. As an example, in a recent study, pre-differentiated MSCs seeded into a collagen gel were implanted into cartilage defects in sheep knees. After 12 months, no sign of cartilage degradation was detected in vivo and the histological results of the MSC-seeded defects were superior to those of the autologous chondrocyte group (Marquass *et al*., 2010).

MSCs can also be delivered directly into joints. Agung *et al* demonstrated the localization of bone marrow-derived stem cells into injured tissue after intra-articular injection. In this study, intra-articular injections of one million MSCs localized to injured anterior cruciate ligaments, while injections of 10 million MSCs resulted in MSC delivery to injured meniscus and cartilage lesions (Agung *et al*., 2006). Murphy *et al* demonstrated intra-articular injection of bone marrow-derived stem cells into injured caprine joints stimulated regeneration of meniscal tissue without evidence of repair of ligament or cartilage (Murphy *et al*., 2003). In a porcine model, bone marrow-derived stem cells suspended in hyaluronic acid were injected intra-articularly after the creation of partial-thickness cartilage defects. The cell-treated group showed significantly improved cartilage healing both histologically and morphologically, compared to the control defects, and injected MSCs were found in the defect neo-cartilage (Lee *et al*., 2007). McIlwraith *et al* demonstrated a statistically significant enhancement in cartilage regenerate volume and stiffness in horses subject to microfracture of distal femoral condylar defects and intra-articular injection of bone marrow-derived stem cells, compared to microfracture alone, after a 12-month training program (McIlwraith *et al*., 2011), although there were no significant differences in clinical signs of lameness in these horses. More recently, it has been shown that entire articular surfaces regenerate without direct cell transplantation, using a lapine proximal humerus model. TGF-β3-infused bio-scaffolds were fully covered with hyaline cartilage at the articular surface, after 4 months implantation following humeral head excision, indicating regeneration of complex tissue is possible by directed recruitment of endogenous stem cells (Lee *et al*., 2010). The outcome of this study provides clear support for the application of exogenous and intrinsic stem cell populations for cartilage repair.

In light of the major impact that articular cartilage pathology exerts on equine athletic performance and wastage, the experiments in this dissertation were conducted to assess the utility of equine synovial cell populations for chondrogenic applications.

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CHAPTER 3: CHONDROGENIC CAPACITY OF EQUINE SYNOVIAL FLUID-DERIVED CELLS

Introduction

Mesenchymal stem cells (MSCs) were first isolated from bone marrow by Friedenstein in 1976 (Friedenstein, Gorskaja, & Kulagina, 1976). These cells are characterized on the basis of their ability to form colonies from single 'founder' cells, maintain extensive proliferative capacity over multiple passages and differentiate along several lineages, including muscle (Nitahara-Kasahara *et al*., 2012; Wei *et al*., 2011), bone (Bruder *et al*., 1998; Turgeman *et al*., 2002), cartilage (Koga *et al*., 2009; Richter, 2009), tendon (Alberton *et al*., 2012; Okamoto *et al*., 2010), ligament (Kanaya *et al*., 2007) and adipose tissue (Scott *et al*., 2011). As a consequence, MSCs hold considerable promise for tissue engineering and regenerative medicine applications to repair musculoskeletal tissues.

Although much of the initial MSC research was focused on bone marrow MSC populations, it is now well established that MSCs are present in most tissues and body fluids (De Bari *et al*., 2001; Dowthwaite *et al*., 2004; Jones *et al*., 2004; Park *et al*., 2012; Wickham *et al*., 2003). Despite similarities in isolation protocols, MSCs derived from different tissues demonstrate significant variation in their ability to adopt specific phenotypes and synthesize functional extracellular matrices (Al-Nbaheen *et al*., 2012; Viero Nora *et al*., 2011). As examples, a number of studies have demonstrated the superiority of bone marrow-derived MSCs over adipose-derived progenitors in osteogenesis and chondrogenesis assays (Danisovic *et al*., 2009; Hayashi *et al*., 2008; Sakaguchi *et al*., 2005; Vidal *et al*., 2008; Wickham *et al*., 2003; Yoshimura *et al*., 2007),

whereas adipose-derived cell populations appear to have specific immuno-modulatory activities that might prove to be therapeutically beneficial (Bochev *et al*., 2008; Gonzalez-Rey *et al*., 2010).

Articular cartilage is a dense connective tissue synthesized and maintained by chondrocytes that covers joint surfaces, serves to minimize friction during motion, and transmits loads to the 1subchondral bone and surrounding periarticular tissues. Articular cartilage is an avascular, aneural and alymphatic tissue, with a comparatively low cellular density (3-5% of wet weight) and little intrinsic healing capacity. As a consequence, degenerative arthritis frequently develops secondarily to cartilage injury that is not adequately repaired. A number of techniques have been developed to overcome the limited reparative capacity of articular cartilage, including arthroscopic debridement, subchondral microfracture, chondrocyte implantation, osteochondral graft transplantation and, most recently, MSC transplantation (Hunziker, 2002; Schindler, 2011; Seo & Na, 2011). These techniques are particularly relevant to the treatment of joint disease in equine athletes, since arthritis is a major cause of reduced performance and wastage in these animals (Bailey *et al*., 1999; Olivier, Nurton, & Guthrie, 1997; Perkins, Reid, & Morris, 2005; Rossdale *et al*., 1985; Wilsher, Allen, & Wood, 2006).

Although, by definition, MSCs from all sources are capable of chondrogenic differentiation, progenitor cells from differing sources show clear quantitative differences in their ability to synthesize functional cartilage matrices (Koga *et al*., 2008; Sakaguchi *et al*., 2005; Yoshimura *et al*., 2007). Further, optimal articular cartilage repair/regeneration requires that stem cells adopt a permanent, non-hypertrophic phenotype, as opposed to the phenotype of chondrocytes committed to endochondral differentiation. A number of studies have demonstrated that MSCs

isolated from bone marrow enter the endochondral lineage with chondrogenic stimulation (Bosnakovski *et al*., 2006; Pelttari *et al*., 2006; Sheehy, Buckley, & Kelly, 2012; Winter *et al*., 2003; Yoo *et al*., 1998); a phenotypic state that is less than optimal for cartilage regeneration.

The presence of MSCs in synovial fluids was first reported by Jones *et al*, in 2004. In this study, cells expanded from pathological human synovial fluid aspirates had immuno-phenotypes consistent with MSCs and were capable of chondrogenic differentiation (Jones *et al*., 2004). Several subsequent studies have linked the number of MSCs in synovial fluid to joint pathology (Jones *et al*., 2008; Sekiya *et al*., 2012). The specific origin(s) of synovial fluid MSCs remains unclear. They are likely derived from synovium, superficial zone of cartilage, or the subchondral bone space (Jones *et al*., 2008; Lee *et al*., 2012; Morito *et al*., 2008). Given that cell populations within and around joints share developmental and spatial proximity to articular chondrocytes, we hypothesized that cells within synovial fluid will be capable of chondrogenic differentiation and express characteristics more representative of an articular phenotype than the reported phenotypic markers of bone marrow-derived MSCs.

The experiments in this study were conducted to address the following objectives: (1) To determine whether cells from equine synovial fluid can be expanded to provide experimentally "useful" numbers of cells; (2) To determine whether there are more CFU-forming cells in synovial fluid of joints with established pathology than in synovial fluid of clinically normal joints; (3) To determine whether cells expanded from equine synovial fluid are capable of multi-lineage differentiation; (4) To determine whether cells expanded from equine synovial fluid are

capable of chondrogenic differentiation, and (5) to determine whether these cells express an endochondral or permanent/articular chondrocytic phenotype.

Materials and Methods

Synovial fluid collection

Synovial fluid samples were aspirated from diarthrodial joints of adult horses immediately prior to arthroscopic surgery, or immediately after euthanasia for the reasons other than musculoskeletal disease. In seven cases, matched synovial fluids were collected from joints with arthritic change established by clinical and radiographic findings, and the contralateral clinically normal joints. One ml of synovial fluid was diluted in 10 ml of Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (basal medium) and seeded into 100 mm culture plates.

Colony-Forming Unit Assay

In the seven matched normal and arthritic synovial samples, 1 ml of synovial fluid was diluted into 10 ml of basal medium and seeded into 100 mm culture plates (a nominal seeding density of 20-50 cells /cm^2). The cultures were monitored on a daily basis for the formation of colonies. Colony-forming units (CFU) were defined as focal clusters of at least of 25 cells and these were counted across the entire surface of the culture dishes at day 14.

Monolayer expansion

To select for proliferation-competent progenitor cells and to generate sufficient numbers of cells for subsequent chondrogenesis experiments, the synovial fluid-derived cells were expanded in monolayer cultures with basal medium through two passages. The culture media were renewed every 3 days. At 80-90% confluence, the cell monolayers were lifted with 0.05% Trypsin/EDTA buffer and the resultant cell suspension was assessed for cell number and viability by trypan blue exclusion. The cells were re-seeded at 10,000 cells per cm^2 and were expanded through two passages.

Differentiation culture conditions

Chondrogenesis. After the second passage of monolayer expansion, synovial fluid-derived cells were trypsinized and the cells were counted and then pelleted (2.5×10^5 cells per pellet) by centrifugation at 390 rfu in 1.5 ml microcentrifuge tubes. At Day 5, pellets were gently aspirated from the microcentrifuge tubes and transferred to 6-well ultra-low attachment culture plates. Pellets were maintained in DMEM, supplemented with insulin/transferrin/selenous acid (ITS), 37.5 μg/ml ascorbic acid, 100 μg/ml sodium pyruvate, and 1% penicillin-streptomycin.

The initial experiments were conducted to define the in vitro conditions necessary for equine synovial-derived cell chondrogenesis. To determine the requirement for glucocorticoid supplementation, the synovial fluid-derived pellet cultures were maintained in the presence or absence of dexamethasone for up to 20 days, to determine the temporal need for this agent and the effect of dexamethasone supplementation on phenotype. Further, the synovial fluid-derived pellets were also treated with one of two 'chondrogenic growth factor' regimes; 10 ng/ml of

transforming growth factor β1 (TGF-β1) for 20 days, or 10 ng/ml of TGF-β1 for the first 10 days of culture, followed by 100 ng/ml of BMP-2 for the final 10 days of culture. Treatment with BMP-2 was substituted for TGF-β1 in the second group to provide a robust hypertrophic stimulus during the later stages of chondrogenic differentiation.

On days 10 and 20, pellets from each treatment group were aspirated from the culture medium, snap-frozen in liquid nitrogen and stored at -20 degrees Celsius for biochemical assessment of chondrogenic phenotype. The remaining pellets were snap-frozen in liquid nitrogen and stored at -80 degrees Celsius for RNA isolation using the phenol-based dissociation agent, TRIzol® (Invitrogen Corporation, Carlsbad, CA). Complementary DNA was generated using a commercial reverse transcription kit (Superscript ™ First-Strand Synthesis System® for RT-PCR, Invitrogen Corporation, Carlsbad, CA). Chondrogenesis was assessed by measuring the expression of chondrocyte-specific genes, collagen type II and aggrecan protein secretion, and alkaline phosphatase activity, as described below.

Osteogenesis. After two passages of monolayer expansion, synovial fluid-derived cells were seeded in 12-well plates, at 5,000 cells per cm^2, cultured in basal medium supplemented with 100 nM dexamethasone, 50 μg/ml ascorbic acid, 10 mM glycerol-2-phosphate (β-GP) for up to 14 days. At day 14, osteogenesis was assessed by Alizarin Red staining and ALP staining, as described below.

Adipogenesis. Following monolayer expansion, synovial fluid-derived cells were seeded in 12-well plates at 5,000 cells per cm^2, cultured in basal medium supplemented with 10 μg/ml insulin,

10^{-6} M dexamethasone, 100 µM indomethacin, and 0.5 mM isobutylmethylxanthine (IBMX) for 14 days. At day 14, adipogenesis was assessed by Oil Red O staining of the monolayers to assess the accumulation of lipoproteins.

Phenotypic assessments

Chondrogenesis assays. Quantitative real time-PCR (qPCR) was used to measure the induction of collagen type II, aggrecan and collagen type X mRNAs. Collagen type II and aggrecan are routinely used to identify the chondrogenic phenotype. Collagen type X is a biomarker of endochondral chondrogenesis, and equine growth plate mRNA was used as a positive control for this gene. RNA isolated from primary chondrocyte pellets was used as a reference control for chondrocyte-specific gene expression. The primers used for qPCR analyses are listed in **Table 1**.

QPCR was performed using 5 µL of diluted cDNA template (1:10 dilution) combined with 20 µL of a mixture composed of 12.5 µL 1 x SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA), 1 µL each of the 10 µM forward and reverse primer stocks and 5.5 µL DNase/RNase-free water in a 96-well microplate. Each sample was run in duplicate. The reactions were performed in a BioRad iCycler iQ^{TM} using the following conditions: initial denaturation for 3 minutes at 95 °C, 40 cycles of denaturation at 95 °C for 10 seconds, annealing temperature of 62.1 °C for 30 seconds and polymerase extension at 72 °C for 20 seconds. The samples were denatured at 95 °C for 1 minute before starting the melting curve protocol which consisted of increasing the temperature from a starting point of 55 °C for 1 minute followed by increments of 0.5 °C every 10 seconds until 95 °C was reached. The presence of a single PCR

product was monitored by melting curve analyses. Sterile water was used as a 'no template' negative control for each of the PCR reactions to monitor the possibility of contamination.

The qPCR data were normalized to expression of the reference gene, elongation factor -1 alpha (EF1-α) which was selected after comparisons with expression profiles of β-Actin and -glyceraldehyde 3-phosphate dehydrogenase (GAPDH), with particular regard for inter-sample consistency. The level of expression for each target gene was calculated as $2^{\Delta Ct}$ and the comparative ΔCt method was used to determine relative gene expression levels.

A collagen type II ELISA assay (Chondrex) was used to measure collagen type II protein within pellets in control and chondrogenically-induced cultures, using three pellets from each sample. Pellets were dissolved in 0.05M acetic acid (pH 2.8-3.0 with formic acid) and were digested in 1/10 the starting volume of pepsin solution at 4°C overnight with mixing on a rotator. The following day, 1/10 the starting volume of 10X TBS was added and the pH was adjusted to 8.0 with 1N sodium hydroxide. To digest intra- and inter-crosslinkages within collagen molecules, 1/10 of the starting volume of pancreatic elastase (1mg/mL dissolved in 1XTBS, pH7.8-8.0) was added with mixing on a rotator at 4°C overnight. Each well of a 96-well plate was added 100 µl of the capture antibody solution and incubated at 4°C overnight. The following morning, the 96-well plate was washed 6 times in wash buffer. The samples (100 µl) and type II collagen standard were added to the plate wells and incubated at room temperature for 2 hours. The plate was then washed with wash buffer six times. The detection antibody solution (100 µl) was added to each well and incubated at room temperature for 2 hours, following by rinsing with wash

buffer at least six times. Streptavidin peroxidase solution (100 µl) was then added and incubated at room temperature for 1 hour. After washing, 100 µl of OPD-Urea H_2O_2 solution was immediately added to each well. After 30 min, 50 µl of stop solution (2N sulfuric acid) was added and the Optical Density (OD) values were measured spectrometrically at 405nm wavelength using a FLUOstar Optima Microplate Reader (BMG LABTECH, Durham, NC).

The dimethyl methylene blue dye-binding (DMMB) assay was used to measure the secretion and accumulation of sulfated glycosaminoglycans (sGAGs) in triplicate samples of control and chondrogenically-induced cultures, using three pellets from each sample. This assay is based on the ability of sulfated GAGs to bind the cationic dye 1, 9-dimethylmethylene blue. Pellets were digested in 250 µl of papain digestion buffer (SIGMA Chemical Cp., St. Louis, MO) at 65 °C overnight. Fifty µl of lysate and 200 µl of DMMB reagent were added to 96-well plates and Optical Density (OD) values was measured spectrometrically at 530 nm by a FLUOstar Optima Microplate Reader (BMG LABTECH, Durham, NC).

An alkaline phosphatase (ALP) bioassay was used to measure alkaline phosphatase activity in triplicate samples of chondrogenically-induced cultures, using three pellets from each sample. ALP is a cell membrane-associated enzyme that is routinely used as a biomarker of endochondral chondrogenesis. The ALP assay was based on ALP-mediated conversion of p-nitrophenol phosphate to p-nitrophenol. Nitrophenol has a maximal absorbance at 405nm. Pellets were homogenized in 2% Trition-X100 on ice, and then were centrifuged at 2500 rpm for 15 minutes at 4°C to remove insoluble debris. ALP reagent (100 µl; Sigma 104 phosphatase substrate) and

100 µl of lysate were added to 96-well plate wells. After 10 minutes incubation, the P-nitrophenol in each well was measured spectrometrically at 405nm wavelength by a FLUOstar Optima Microplate Reader (BMG LABTECH, Durham, NC). Two percent Triton-X 100 alone was used as the negative control and murine growth plate lysate was used as the positive control.

Pico green fluorescence assay for DNA quantification was assessed for the cell number in pellet culture during chondrogenesis. Total double-stranded DNA was quantified by measuring fluorescence of an ultra-sensitive nucleic acid stain, PicoGreen (Invitrogen Life Technologies, Carlsbad, CA). Samples were diluted 1:5 in 1X TE buffer (10mM Tris-HCl, 1mM EDTA, pH 7.5). Serially diluted calf thymus DNA aliquots were used as a standard curve. Duplicate 100 µl aliquots of each sample and the standard were transferred to a black 96-well microplate. On the day of the experiment, the Pico Green reagent was prepared, based on the 100 µl volume required for each well (1 µl Pico Green reagent diluted in 200 µl of 1X TE buffer) and added to every sample and standard. The microplate was placed in the dark to prevent reagent photo-degradation. Following 5 minutes incubation, the fluorescence was measured at 485 nm wavelength (FLUOstar OPTIMA, BMG, Lab Technologies).

Osteogenesis assays. Alizarin Red staining was used for calcium deposit. Cells were fixed with 10% formalin for 30 minutes, and were washed three times with distilled water. One ml of fresh 2% Alizarin Red (Sigma-Aldrich) solution (pH 4.1) was added to each well at room temperature for 20 minutes. The stain was removed and washed with distilled water until the rinsed solution was clear. Mineral deposits within the cell layers were stained bright red. Representative images

of stained monolayers were obtained (Leica Microsystems, Leica Application Suite -LAS- version 2.6.R1).

Alkaline phosphatase staining was used for detecting alkaline phosphatase activity in osteogenic cell monolayers. Cell layers were fixed with 10 % formaldehyde for 1 minute and were washed three times with distilled water. An alkaline dye (Procedure No. 86 AP, leukocyte; Sigma Aldrich) was added to each well at room temperature for 15 minutes, and the cell layers were washed three times with distilled water. The neutral red solution was added for 5 minutes. Cells clusters exhibiting alkaline phosphatase activity were stained blue. Representative images of stained monolayers were obtained (Leica Microsystems, Leica Application Suite -LAS- version 2.6.R1).

Adipogenesis assays. Oil Red O staining was used for detecting accumulation of lipoproteins. Cells were fixed with 10% formalin for 30 minutes, and were washed 3 times with 1X PBS. 0.36% Oil Red O solution in 60% isopropanol was added to each well at room temperature for 50 minutes. Oil Red O solution was removed and washed 3 times with distilled water. Hematoxylin solution was added for 15 minutes, and the stained was removed. Lipid droplets were stained red by Oil Red O and the cell nucleus were stained dark blue by hematoxylin. Representative images of stained monolayers were obtained (Leica Microsystems, Leica Application Suite -LAS- version 2.6.R1).

Chondrocyte isolation and culture

To generate reference values for the chondrogenesis assays, fully differentiated articular chondrocytes were isolated and cultured as pellets, similarly to the pellet cultures of MSCs, above. Articular cartilages were collected from two young adult horses with no clinical signs or joint disease. The cartilage explants were diced and digested with 0.2% collagenase type II overnight. The number of viable cells was determined by counting with a hematocytometer and by trypan blue exclusion. Primary chondrocytes were pelleted by centrifugation at 390 rfu in 1.5 ml microcentrifuge tubes at the density of 2.5×10^5 cells per pellet. The primary chondrocyte pellets were maintained in DMEM, supplemented with insulin/transferrin/selenous acid (ITS), 37.5 μg/ml ascorbic acid, 100 μg/ml sodium pyruvate, 10^{-7} M dexamethasone, and 1% penicillin-streptomycin for 20 days. These samples were assessed using the chondrogenesis protocols detailed above, to provide comparative "primary chondrocyte" benchmarks for the synovial fluid-derived cell activities.

Statistical Analyses

The assays detailed above were routinely run using three replicates from each cell isolate. All quantitative differences in phenotype or biosynthesis were assessed by one-way ANOVA. Statistical analyses were performed by Prism 5.0. Data were expressed as the mean ± standard error, and a p value < 0.05 was considered to be statistically significant.

Results

Monolayer expansion. There was considerable variation in the number of CFUs in both normal and arthritic synovial fluid samples, ranging from 30 to 120 colonies per ml of fluid. There was no statistical difference between the CFUs in normal and arthritic synovial fluid aspirates (Fig 1A). Primary isolates required 4-6 weeks to reach confluence, whereas subsequent passages reliably achieved confluence within 7-12 days (Fig 1B). Nucleated cell counts were not determined at the time of synovial aspirate collection but, assuming a nominal nucleated cell count of 5×10^3/ml in the primary aspirates (five times higher than the upper normal value), there was more than 1100-fold increase in cell number by the stage of passage 2 confluence, indicative of substantial proliferative capacity of these cells.

Osteogenic and adipogenic capacities. Synovial fluid-derived cells cultured in osteogenic medium for 14 days formed characteristic multicellular nodules. These cellular nodules exhibited a marked increase in ALP-positive cells, compared with cells cultured in basal medium (Fig 2A-B). After 14 days, synovial fluid-derived cells also secreted a mineralized matrix, confirmed by Alizarin Red staining, whereas there was negligible stain accumulation in control cultures (Fig 2C-D). Synovial fluid-derived stem cells cultured in adipogenic medium for 14 days developed intracellular lipid droplets, identified by Oil Red O accumulation, whereas cells cultured without adipogenic medium showed no evidence of intracellular lipid formation (Fig 2E-F).

Synovial fluid-expanded cell chondrogenesis

Dexamethasone dependence. Initial experiments conclusively demonstrated that dexamethasone supplementation was mandatory for the survival of cells after transfer to the chondrogenic pellet cultures. With dexamethasone supplementation, the pellets maintained DNA content throughout the 20 days of culture, independent of TGF-β1 or BMP-2 administration (Fig 3A). Consistent with the DNA data, Dexamethasone-supplemented pellets were noticeably larger than pellets maintained without Dexamethasone (Fig 3B). Our preliminary experiments indicated that supplementing dexamethasone to the culture medium for the first 10 days, for the final 10 days or for the 20-day duration of the experiment did not significantly affect collagen type II and aggrecan expression. Therefore, dexamethasone administration was maintained throughout the 20 days of culture in subsequent experiments.

Synovial fluid-derived cell chondrocytic phenotype. Synovial fluid-derived cells treated with TGF-β1 or BMP-2 for 20 days expressed collagen type II mRNA at levels 3-5 fold above those seen in primary chondrocyte pellets (Fig 4A). In addition, collagen type II protein translation, secretion and pericellular deposition were efficient. Fully differentiated articular chondrocytes accumulated 0.05 µg/pellet of collagen type II. Synovial fluid-derived cells deposited collagen type II in the pericellular matrix at levels approximately 3-5 folds of that measured in primary chondrocyte pellets (Fig 4C). Synovial fluid-derived cell pellets maintained in chondrogenic medium and treated with TGF-β1 or BMP-2 for 20 days expressed aggrecan mRNA at levels markedly above those seen in primary chondrocyte pellets (Fig 4B). Fully differentiated articular chondrocytes accumulated 1.6 µg /pellet of sGAG, while synovial fluid-derived cell pellets

deposited 2.6-3.5 fold more sGAG than the articular chondrocytes under the same culture conditions (Fig 4D).

Endochondral phenotype. Of particular importance to the objective of this study, there was no significant ALP induction and negligible levels of collagen type X mRNA in synovial fluid-derived cell pellets, indicating that synovial fluid-derived cells undergo chondrogenesis but do not express markers characteristic of the transient, hypertrophic chondrocytic phenotype engaged in endochondral ossification (Fig 5A, B).

Discussion

This study was conducted to determine whether cells with the characteristics of MSCs are present in equine synovial fluid. Cells capable of sufficient clonal expansion to generate colonies were present in all synovial fluid aspirates assessed in these experiments. The initially small numbers of cells in the aspirates were successfully expanded through multiple passages, demonstrating substantial proliferative capability. Further, the expanded cell populations were capable of differentiating under standard in vitro culture conditions to express osteogenic, adipogenic and chondrogenic phenotypes, satisfying the multi-lineage potential requirement of MSCs.

Of particular importance to the objective of this study, equine synovial fluid MSCs were capable of cartilaginous matrix biosynthesis to a degree comparable with fully differentiated articular chondrocytes maintained under similar culture conditions. Further, synovial fluid-derived cells

did not express biomarkers characteristic of the hypertrophic phenotype (collagen type X mRNA and ALP induction). The absence of hypertrophic marker up-regulation was evident under standard chondrogenesis conditions driven by TGF-β1, and was not altered when the hypertrophy-inducing growth factor, BMP-2, was substituted during the final 10 days of the differentiation protocol. These results suggest that synovial fluid MSC chondrogenesis is phenotypically distinct from chondrogenic differentiation in MSC populations isolated from bone marrow and adipose tissue and supports the use of synovial MSCs for articular cartilage repair applications.

There are several potential sources of synovial fluid MSCs. Progenitor cell populations have been identified in synovial membrane (De Bari *et al*., 2001), intra-articular adipose tissues (Lee, Nakagawa, & Reddi, 2008), articular cartilage itself (McCarthy *et al*., 2011), the subchondral bone marrow space (Koelling *et al*., 2009), and it is also possible that circulating MSCs (Caplan & Dennis, 2006; Dar *et al*., 2005; Wynn *et al*., 2004) can localize to the synovial cavity with appropriate chemotactic stimulation. Given that the synovial fluid MSCs express a non-hypertrophic chondrogenic phenotype, a synovial and/or cartilaginous source seems more likely, since bone marrow- and adipose-derived MSCs express hypertrophic characteristics during chondrogenic differentiation. Further, the joints sampled in these experiments did not have sufficient cartilage loss to provide direct communication with the subchondral marrow compartment and equine tibiotarsal and metacarpo/metatarso-phalangeal joints do not have prominent intra-articular adipose depots.

Accepting these potential phenotypic advantages, the in vitro expansion of primary synovial fluid cells from 1 ml aspirates required several weeks; a clinically non-viable process. The time required for the generation of clinically useful cell numbers could be easily reduced by collecting larger volumes of synovial fluid and by sampling several joints from individual donors. Arthrocentesis is a minimally invasive procedure, compared to bone marrow and adipose tissue harvesting, Further, in vitro expansion of bone marrow-derived MSC populations is accelerated by mitogens such as fibroblast growth factor 2 (FGF-2). Of particular relevance to cartilage repair applications, FGF-2 also improves subsequent chondrogenic differentiation (Solchaga *et al*., 2005; Stewart *et al*, 2007). MSC populations from synovial fluid might be similarly influenced by FGF administration, reducing the time required to produce sufficient cells for clinical applications.

Several studies using human samples have demonstrated a positive association between joint pathology and synovial fluid MSC numbers (Jones *et al*., 2008; Morito *et al*., 2008; Sekiya *et al*., 2012), although the numbers of CFUs vary widely within and between studies. A similar association was not observed in the current study; however, the matched samples used to compare pathological and normal joints were collected from young adult horses with radiographically confirmed osteochondrosis lesions. Apart from the focal osteochondral lesions present in these joints, there were minimal signs of overt arthritis or synovitis. In comparison to the intra-articular pathology in human knee joints with cruciate ligament tears (Morito *et al*., 2008; Sekiya *et al*., 2012) or with advanced osteoarthritis (Jones *et al*., 2004; Kurose *et al*., 2010; Sekiya *et al*., 2012), the intra-synovial pathology in the equine aspirates might have been insufficient to induce significant increases in MSC numbers. Ongoing assessments of synovial

fluid MSC numbers from overtly arthritic equine joints will determine whether joint pathology in horses is associated with similar increases in progenitor cell concentrations.

Figures and Tables

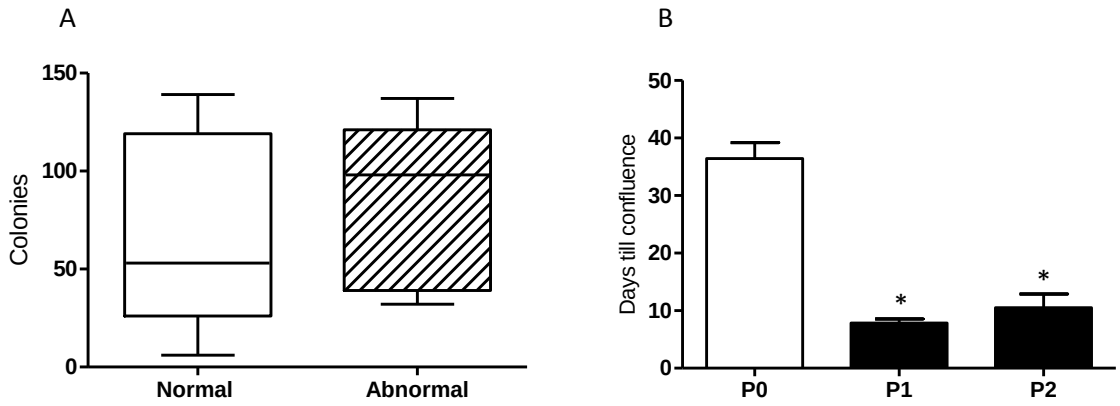

Figure 1. Colony-forming and proliferative capacity of synovial fluid -derived cells. In left panel, the colony forming assay was conducted on synovial fluids from normal and pathological equine joints. The median number of colonies was 53 and 98 in normal and arthritic joints, respectively. There was no significant difference in colony-forming potential of arthritic and normal joint fluids. In the right panel, primary synovial fluid-derived cells required an average of 36 days to reach confluence. In subsequent passages, time to confluence required 7-12 days.

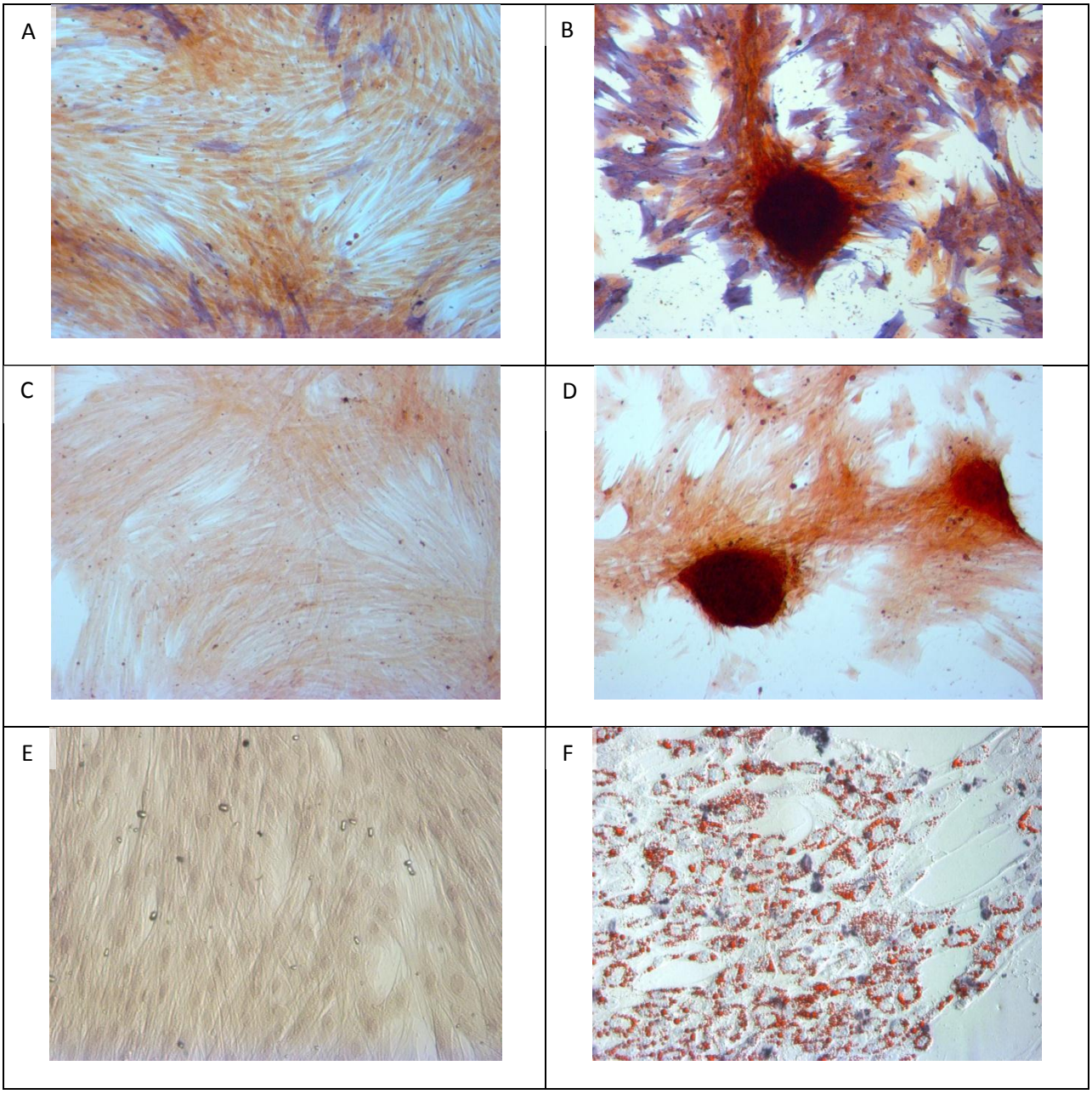

Figure 2. Histochemical analyses of osteogenic and adipogenic capacities of synovial fluid-derived cells. Synovial fluid-derived cells were cultured in control medium (A, C and E) or in osteogenic (B, D) or adipogenic (F) medium for 14 days. (A, B) The cultures were stained with p-nitrophenol for alkaline phosphatase (A and B; magnification 100X), with Alizarin Red for mineralized matrix (C and D; magnification 100X)., and with Oil Red O for intracellular lipid accumulation (E and F; magnification 200X).

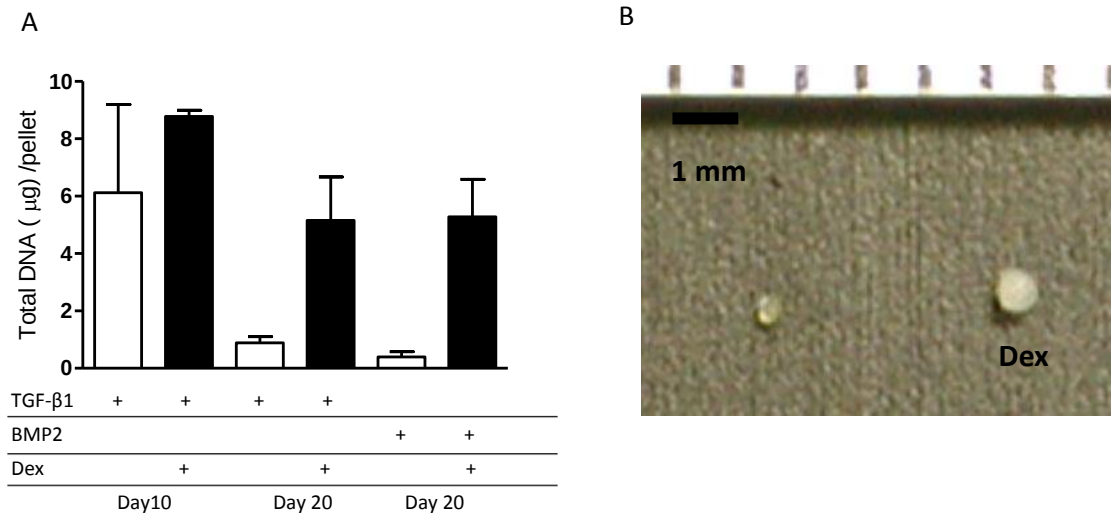

Figure 3. The effect of dexamethasone on synovial fluid-derived cell pellet cultures. In the left panel, pellets treated with dexamethasone (black columns) maintained DNA content throughout the 20-day culture interval. In contrast, DNA content fell significantly in un-supplemented cultures (open columns). In the right panel, the size of pellet treated with dexamethasone for 20 days was marked larger than the pellet without dexamethasone supplementation, consistent with the DNA measurements.

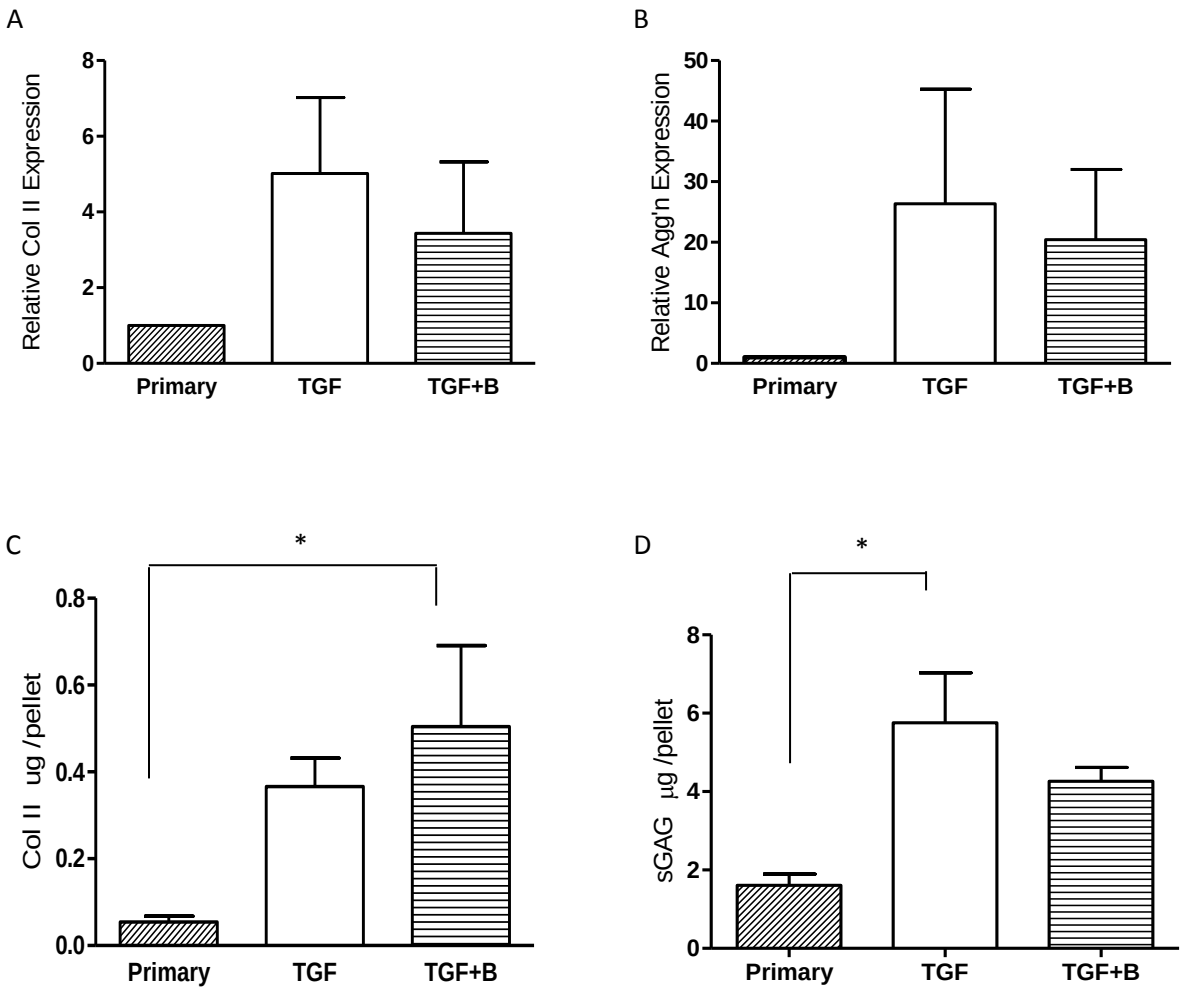

Figure 4. Expression of cartilaginous extracellular matrix by synovial fluid-derived cells undergoing chondrogenesis. For mRNA expression, primary chondrocyte pellets were used as a reference. Synovial fluid-derived cells expressed higher levels of collagen type II (Col II; A) and aggrecan (Aggn; B) mRNA than did primary chondrocyte pellets. Synovial fluid-derived cell pellets also accumulated more collagen type II (Col II) protein (C) and sulfated glycosaminoglycans (sGAG) than did primary chondrocyte pellets (C, D).

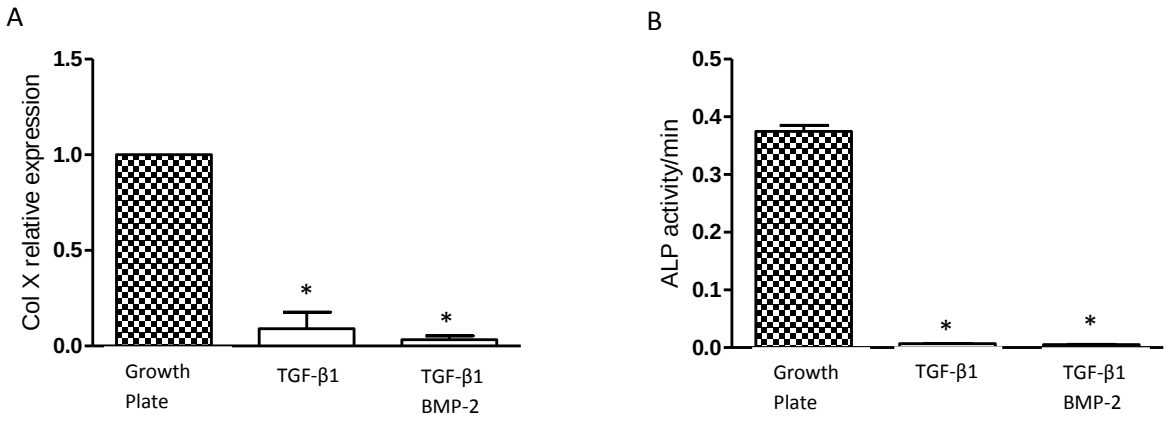

Figure 5. Expression of hypertrophic markers by synovial fluid-derived cells. Synovial fluid-derived cells maintained in pellet cultures for 20 days expressed relatively low levels of collagen type X (Col X) mRNA (A) and had negligible alkaline phosphatase (ALP) activity (B), compared to endochondral chondrocytes from equine growth plate.

Table 1: Primers utilized in the qPCR reactions

Gene (size)	Sense Primer Antisense Primer	Annealing temperature
Col II (223 bp)	5’ AGCAGGAATTTGGTGTGGAC (4325) 5’ TCTGCCCAGTTCAGGTCTCT (4548)	62.1 °C
Col X (244 bp)	5’ TGCCAACCAGGGTGTAACAG (39) 5’ ACATTACTGGGGTGCCGTTC (283)	62.1 °C
Aggrecan (202 bp)	5’ GACGCCGAGAGCAGGTGT (33) 5’ AAGAAGTTGTCGGGCTGGTT (235)	62.1 °C
EF1-alpha (328 bp)	5’ CCCGGACACAGAGACTTCAT (48) 5’AGCATGTTGTCACCATTCCA (376)	62.1 °C

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CHAPTER 4: COMPARATIVE CHONDROGENESIS OF EQUINE SYNOVIUM-, BONE MARROW-, AND ADIPOSE-DERIVED PROGENITOR POPULATIONS

Introduction

Articular cartilage is a highly specialized connective tissue that serves to distribute load across joint surfaces and minimize friction during motion. Articular cartilage is a hypocellular, avascular and alymphatic tissue and the resident chondrocytes constitute only 3-5% of the tissue wet weight. As a consequence, articular cartilage has little intrinsic regenerative capacity. This issue is critical to the progressive pathophysiology of osteoarthritis. Although osteoarthritis involves pathological changes to several intra- and peri-articular tissues, degeneration and loss of articular cartilage is the hallmark of the disease (Adatia, Rainsford, & Kean, 2012). In order to restore the articular cartilage, a number of techniques have been developed to overcome the limited reparative capacity of articular cartilage, including arthroscopic debridement, subchondral microfracture, chondrocyte implantation, osteochondral graft transplantation and, most recently, mesenchymal stem cell (MSC) transplantation (Hunziker, 2002; Schindler, 2011; Seo & Na, 2011). These techniques are particularly relevant to the treatment of joint disease in equine athletes, since arthritis is a major cause of reduced performance and wastage in these animals (Bailey *et al*., 1999; Olivier, Nurton, & Guthrie, 1997; Perkins, Reid, & Morris, 2005; Rossdale *et al*., 1985; Wilsher, Allen, & Wood, 2006).

Mesenchymal stem cells hold great potential for cartilage repair, in light of their multipotentiality, self-renewal capacity, easy accessibility, and immuno-modulatory activities (Bailey *et al*., 1999; Olivier *et al*., 1997; Perkins *et al*., 2005; Rossdale *et al*., 1985; Wilsher *et al*., 2006). Under

appropriate culture conditions, MSCs can be induced to undergo chondrogenic differentiation. Transforming growth factor-β (TGF-β) (Bian *et al*., 2011; Buxton *et al*., 2011; Park *et al*., 2009; Zheng *et al*., 2010), bone morphogenetic proteins (BMPs) (Badlani *et al*., 2008; Sellers, Peluso, & Morris, 1997; Tamai *et al*., 2005; van Beuningen *et al*., 1998), and insulin-like growth factors (IGFs) (Longobardi *et al*., 2006; Mierisch *et al*., 2002) are able to stimulate chondrogenesis and regulate cartilaginous tissue synthesis. TGF-β stimulates MSC chondrogenesis and prevents fully differentiated chondrocytes from hypertrophy. BMPs act as key regulators in endochondral chondrocyte differentiation and in articular cartilage homeostasis and repair. IGF-1 stimulates chondrogenic differentiation by up-regulating the expression of collagen type II and aggrecan, stimulating proliferation, and regulating cell apoptosis (Longobardi *et al*., 2006). Prior to in vitro chondrogenesis, the addition of basic fibroblastic growth factor (bFGF) to culture medium during monolayer expansion of MSCs stimulates cell proliferation, reducing the time in required for progenitor population expansion and retaining subsequent differentiation potential (Bianchi *et al*., 2003; Mastrogiacomo, Cancedda, & Quarto, 2001; Stewart *et al*., 2007; Tsutsumi *et al*., 2001). The optimization of culture conditions for MSCs chondrogenesis is critical for future clinical application in stem cell-based therapy of articular cartilage repair.

Even though most of the early work in MSC biology was focused on bone marrow-derived progenitor populations, it is now apparent that MSCs reside in most mesenchymal tissues and body fluids (Nitahara-Kasahara *et al*., 2012; Wei *et al*., 2011). However, a number of studies show that MSCs derived from specific tissues exhibit source-based differences in phenotypic and biosynthetic activities (Al-Nbaheen *et al*., 2012; Viero Nora *et al*., 2011). As examples, several studies have demonstrated the superiority of bone marrow-derived MSCs over adipose-derived

progenitors in osteogenesis and chondrogenesis assays (Danisovic *et al*., 2009; Hayashi *et al.*, 2008; Sakaguchi *et al*., 2005; Vidal *et al*., 2008; Wickham *et al*., 2003; Yoshimura *et al*., 2007), whereas adipose-derived cell populations appear to have specific immuno-modulatory activities that might prove to be therapeutically beneficial (Bochev *et al*., 2008; Gonzalez-Rey *et al*., 2010). More recently, comparative analyses in rodent and human cell populations suggest that synovial progenitor cells are more capable of chondrogenic differentiation than other MSC sources, although the specific phenotypic characteristics of these cells are unclear (Sakaguchi *et al*., 2005; Yoshimura *et al*., 2007). These differences will almost certainly influence the utility of specific MSC populations for clinical applications. Given that cell populations within and around joints share developmental and spatial proximity to articular chondrocytes, we hypothesized that synovium-derived cells will be more appropriate for *in vitro* articular chondrogenic differentiation than bone marrow- and adipose-derive cells, under identical culture conditions.

The experiments in this study were conducted to address the following objectives: (1) To determine whether cells from equine synovium can be expanded to provide experimentally " useful" numbers of cells; (2) To determine whether cells expanded from equine synovium capable of multi-lineage differentiation; (3) To determine whether cells expanded from equine synovium are capable of chondrogenic differentiation; (4) To determine whether these cells express an endochondral or permanent/articular chondrocytic phenotype; (5) To determine the culture requirements for in vitro chondrogenic differentiation of equine synovium-derived cells in pellet cultures, and (6) to compare the chondrogenic capacities of cells isolated from equine synovium (SYN), bone marrow (BM) and adipose tissue (FAT).

Materials and Methods

Collection of samples

The use of the animals in this study was approved by the Institutional Animal Care and Use Committee of the University of Illinois. Bone marrow, adipose tissue and synovium were obtained aseptically from six healthy two-year-old horses. Horses were sedated with 0.5-1.0 mg /kg of xylazine IV. Anesthesia was induced with 2.2 mg/ kg of ketamine and 0.1 mg /kg of diazepam, and was maintained with a combination of 5% guaifenisin solution with 1,000 mg /L of ketamine and 1,000 mg /L of xylazine. Following collection of bone marrow aspirates, all horses were euthanized with 104 mg /kg of sodium pentobarbital given IV. The adipose tissue and synovium were collected immediately following euthanasia.

Bone marrow collection. Bone marrow aspirates were collected from tuber coxae. A 5cm x 5cm area over the tuber coxae was clipped and aseptically prepared. A stab incision was made with a #11 scalpel blade at the site of collection. The bone marrow aspirate was obtained through a bone marrow biopsy needle. The 10-15 ml of marrow aspirate was collected into 30 ml syringes containing 1,000 IU of heparin to prevent coagulation.

Synovium collection. Synovium was collected from right radiocarpal joint. The skin over the right radiocarpal joint was clipped and aseptically prepared. An incision was made into the dorsal aspect of the joint and through the synovial membrane over the dorsal surface of the joint

cavity was exposed. Two-three grams of synovium was collected and placed in a 50 ml polypropylene tube containing sterile PBS solution.

Adipose tissue collection. Adipose tissues were collected from the subcutaneous fat depot directly adjacent the tail head. The skin lateral to the tail head was clipped and aseptically prepared. A 10-15 cm skin incision was made and 8-10 grams of adipose tissue were collected and placed in a 50 ml polypropylene tube containing sterile PBS solution.

Cell isolation

Bone marrow derived-cell isolation. Bone marrow aspirates were diluted with an equal volume of sterile PBS and centrifuged at 300g for 15 minutes. The supernatant was discarded, and the cell pellet was washed with PBS and re-centrifuged as described. The supernatant was discarded and the cell pellet was suspended in 0.8% ammonium chloride at room temperature for 20 minutes to lyse the red blood cells, and the centrifuged as described above. The supernatant was removed and cell pellet was re-suspended in 10 ml of PBS.

Synovium derived-cell isolation. Synovium tissue was diced and digested in 10-fold volume of 0.2% collagenase type II (Worthington Biochemical Corporation) in Dulbecco modified Eagle medium (DMEM) supplemented with 2% fetal bovine serum (FBS) and 1% penicillin-streptomycin (P/S) for 3 hours to release the cells. After digestion, cells were filtered through a 40 μm mesh.

Adipose derived-cell isolation. Adipose tissue was diced and digested in 10-fold volume of 0.2% collagenase type II (Worthington Biochemical Corporation) in Dulbecco modified Eagle medium (DMEM) supplemented with 2% fetal bovine serum (FBS) and 1% penicillin-streptomycin (P/S) for 3 hours to release the cells. After digestion, the cells were filtered through a 40 µm mesh.

The cells isolated from each source were collected by centrifugation. The cell numbers of primary adipose and synovial cells were determined by using a hematocytometer. Additionally, cellular viability was assessed by trypan blue exclusion followed by cryo-preservation until further use for monolayer expansion and chondrogenic induction. Primary bone marrow-derived cells were plated and cultured until the primary monolayers reached 80% confluence. The cells were then trypsinized, seeded at 5 x 10^3 cells per cm^2 and expanded for one passage before cryo-preservation. For all three cell types, the medium used for cryopreservation contained 50 % DMEM, 40 % FBS and 10 % Dimethyl sulfoxide (DMSO). The cryovials were placed in an isopropanol freezing chamber at -80 ° C for 24 hours before being transferred to liquid nitrogen.

Monolayer Expansion

To select for proliferation-competent progenitor cells and to generate sufficient numbers of cells for chondrogenesis experiments, the cells were expanded in monolayer cultures with Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS) and 1%

penicillin/streptomycin, at a seeding density of 5×10^3 cells per cm^2 in 100 mm plates (55 cm^2). Additionally, 100 ng /ml basic fibroblastic-growth factor (bFGF) was added to the culture medium to stimulate cell proliferation and shorten the period of time required for monolayer expansion. The culture media were renewed every 3 days. The cells were incubated at 37 °C, 5 % CO_2 and 90 % humidity. At 80-90% confluence, the cell monolayers were lifted with 0.05% Trypsin/EDTA buffer and the resultant cell suspensions were assessed for cell number and viability by trypan blue exclusion. The cells were re-seeded at 5,000 cells per cm^2 and were expanded through two passages.

Osteogenic differentiation

After two passages of monolayer expansion, synovium-derived cells were seeded in 12-well plates, at 5,000 cells per cm^2, cultured in basal medium supplemented with 100 nM dexamethasone, 50 μg/ml ascorbic acid, 10 mM glycerol-2-phosphate (β-GP) for up to 14 days. At day 14, osteogenesis was assessed by Alizarin Red staining and ALP staining, as described below.

Alizarin Red staining was used to identify pericellular calcium deposition. Cells were fixed with 10% formalin for 30 minutes, and were washed three times with distilled water. One ml of fresh 2% Alizarin Red (Sigma-Aldrich) solution (pH 4.1) was added to each well at room temperature for 20 minutes. The stain was removed and washed with distilled water until the rinse solution was clear. Mineral deposits within the cell layers were stained bright red. Representative images of stained monolayers were obtained (Leica Microsystems, Leica Application Suite -LAS- version 2.6.R1).

Alkaline phosphatase (ALP) staining was used for detecting cell-associated ALP activity in osteogenic cell monolayers. Cell layers were fixed with 10 % formaldehyde for 1 minute and were washed three times with distilled water. An alkaline dye (p-nitrophenol; Procedure No. 86 AP, leukocyte; Sigma Aldrich) was added to each well at room temperature for 15 minutes, and the cell layers were washed three times with distilled water. Neutral red solution counter-stain was added for 5 minutes. Cells clusters exhibiting alkaline phosphatase activity were stained blue. Representative images of stained monolayers were obtained (Leica Microsystems, Leica Application Suite -LAS- version 2.6.R1).

Adipogenic differentiation

Following monolayer expansion, synovium-derived cells were seeded in 12-well plates at 5,000 cells per cm^2, cultured in basal medium supplemented with 10 µg/ml insulin, 10^{-6} M dexamethasone, 100 µM indomethacin, and 0.5 mM isobutylmethylxanthine (IBMX) for 14 days. At day 14, adipogenesis was assessed by Oil Red O staining of the monolayers to assess the intracellular accumulation of lipoproteins. Cells were fixed with 10% formalin for 30 minutes, and were washed 3 times with 1X PBS. 0.36% Oil Red O solution in 60% isopropanol was added to each well at room temperature for 50 minutes. Oil Red O solution was removed and washed 3 times with distilled water. Hematoxylin solution was added for 15 minutes, and the stained was removed. Lipid droplets were stained red by Oil Red O and the cell nucleus were stained dark blue by hematoxylin. Representative images of stained monolayers were obtained (Leica Microsystems, Leica Application Suite -LAS- version 2.6.R1).

Chondrogenic differentiation

To determine the in vitro conditions required for SYN cell chondrogenesis, pellet cultures were maintained in DMEM, supplemented with insulin/transferrin/selenous acid (ITS), 37.5 μg/ml ascorbic acid, 100 μg/ml sodium pyruvate, and 1% penicillin-streptomycin for 20 days. The SYN pellet cultures were maintained in dexamethasone for the first 10 days of culture, the final 10 days of culture, or entire 20 days of culture, to determine the need for this supplement and the effect of restricted supplementation on phenotype. The SYN pellets were also treated with one of three 'chondrogenic growth factor' regimes; 10 ng/ml of transforming growth factor β1 (TGF-β1) for 20 days, 100 ng/ml of BMP-2 for 20 days, or TGF-b1 for the first 10 days in culture, followed by BMP-2 for the final 10 days, to determine the effects of these factors on the chondrogenic phenotype expressed by SYN cells. BMP-2 induces a hypertrophic phenotype in chondrocytes committed to the endochondral lineage, but does not do so in articular chondrocytes.

To assess the comparative chondrogenic capacities of BM, SYN and FAT cells, second passage monolayers of each cell type were trypsinized and pelleted (2.5×10^5 cells per pellet) by centrifugation at 390 rfu in 1.5 ml microcentrifuge tubes. The pellets were maintained in standard chondrogenic medium comprising DMEM, supplemented with insulin /transferrin/ selenous acid (ITS), 37.5 μg/ml ascorbic acid, 100 μg/ml sodium pyruvate, 10^{-7} M dexamethasone, 1% penicillin-streptomycin, and 10 ng/ml of TGF-β1 for 20 days. At Day 5, pellets were gently aspirated from the microcentrifuge tubes and transferred to 6-well ultra-low attachment culture plates for the remainder of the culture period.

To provide reference values for the chondrogenic phenotype analyses, fully differentiated articular chondrocytes were maintained as pellets in chondrogenic culture medium for 20 days, analogously to the MSC cell pellet cultures.

RNA isolation

On days 10 and 20, 20 pellets from each source were aspirated from the culture medium, snap-frozen in liquid nitrogen and stored at -80 degrees Celsius for RNA isolation using the phenol-based dissociation agent, TRIzol® (Invitrogen Corporation, Carlsbad, CA) and the manufacturer's recommended high salt protocol to exclude proteoglycans. One milliliter of TRIzol® was added into each tube containing 20 pellets from each source. An Ultra Turrax-T 25 homogenizer (Janke & Kunkel, IKA-Labortechnik, Staufen, Germany) was used to disrupt the pellets. Following a 3-minute centrifugation, the cell lysates were transferred to 1.5 ml microcentrifuge tubes and 200 µL chloroform added. Samples were vortexed and incubated for 2-3 minutes at room temperature. Following centrifugation at 12,000 rpm for 30 minutes at 4°C (Centrifuge 5415 R, Ependorf), the upper aqueous phase (approximately 400 µL) containing the RNA was transferred to a new 1.5 ml microcentrifuge tube. During this process, great care was taken to leave the insoluble material at the interface undisturbed. This interface material contains insoluble proteins, lipids and DNA.

The RNA within the aqueous phase was precipitated by adding 250 µL of isopropanol and 250 µL of 0.8 M sodium citrate/1.2 M sodium chloride; the 'high salt' variation of the basic TRIzol protocol, used for samples containing high levels of proteoglycans. The samples were stored

overnight at -20 °C to facilitate RNA precipitation. The microcentrifuge tubes were then centrifuged at 10,000 rpm for 20 minutes at 4°C, resulting in the formation of a translucent pellet. The supernatant was removed and the pellet was washed in 500 µL of 70% ethanol (to reduce the salt content of the pellet). The samples were vortexed, incubated for 10 minutes at room temperature and centrifuged 5 minutes at 10,000 rpm. The supernatant was removed and the pellet was allowed to air-dry for 5-10 minutes. A small volume of diethyl pyrocarbonate (DEPC)-treated water was added to dissolve the pellet. The samples were kept on ice for 5 minutes, vortexed, spun down to bring contents to the bottom and incubated at 65 °C for 10 minutes. The concentration of RNA (1:40 aliquot of each sample) was determined by measuring the absorbance at 260 nM (A260) and 320nM (A320) in a spectrophotometer (Smart Spec™ 3000, BioRad, Hercules, CA). Total RNA was calculated as follows:

$$\text{RNA in } \mu g/\mu L = \frac{\text{OD at 260 nM} - \text{OD at 320 nM x 40 (dilution factor) x 40 (coefficient)}}{1000}$$

Quantitative PCR assessment

Complementary DNA was generated using the commercial reverse transcription kit, Superscript ™ First-Strand Synthesis System® for RT-PCR (Invitrogen Corporation, Carlsbad, CA). Quantitative real time-PCR (qPCR) was used to measure the expression of collagen type II, aggrecan and collagen type X mRNAs. Collagen type II and aggrecan are routinely used as markers of the chondrogenic phenotype. Collagen type X is a biomarker of endochondral chondrogenesis. Equine growth plate mRNA was used as a positive control for this gene, while RNA isolated from primary chondrocyte pellets was used as a reference control for chondrocyte-

specific gene expression. Ten million undifferentiated cells from BM, SYN, and FAT were collected before transfer to chondrogenic culture conditions as T^0 controls. The primers used for qPCR analyses are listed in **Table 2**. QPCR was performed using 5 µL of diluted cDNA template (1:10 dilution) combined with 20 µL of a mixture composed of 12.5 µL 1 x SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA), 1 µL each of the 10 µM forward and reverse primer stocks and 5.5 µL DNase/RNase-free water in a 96-well microplate. Each sample was run in duplicate. The reactions were performed in a BioRad iCycler iQ^{TM} using the following conditions: initial denaturation for 3 minutes at 95 °C, 40 cycles of denaturation at 95 °C for 10 seconds, annealing temperature of 62.1 °C for 30 seconds and polymerase extension at 72 °C for 20 seconds. The samples were denatured at 95 °C for 1 minute before starting the melting curve protocol which consisted of increasing the temperature from a starting point of 55 °C for 1 minute followed by increments of 0.5 °C every 10 seconds until 95 °C was reached. The presence of a single PCR product was monitored by melting curve analyses. Sterile water was used as a 'no template' negative control for each of the PCR reactions to monitor the possibility of contamination. The qPCR data were normalized to expression of the reference gene, elongation factor -1 alpha (EF1-α) which was selected after comparisons with expression profiles of β-Actin and -glyceraldehyde 3-phosphate dehydrogenase (GAPDH), with particular regard for inter-sample consistency. The level of expression for each target gene was calculated as $2^{\Delta Ct}$ and the comparative ΔCt method was used to determine relative gene expression levels.

Pellet collagen type II content

A collagen type II ELISA assay (Chondrex) was used to measure collagen type II protein within pellets in control and chondrogenically-induced cultures, using three pellets from each sample. Pellets were dissolved in 0.05M acetic acid (pH 2.8-3.0 with formic acid) and were digested in

1/10 the starting volume of pepsin solution at 4°C overnight with mixing on a rotator. The following day, 1/10 the starting volume of 10X TBS was added and the pH was adjusted to 8.0 with 1N sodium hydroxide. To digest intra- and inter-crosslinkages within collagen molecules, 1/10 of the starting volume of pancreatic elastase (1mg/mL dissolved in 1XTBS, pH7.8-8.0) was added with mixing on a rotator at 4°C overnight. Each well of a 96-well plate was added 100 µl of the capture antibody solution and incubated at 4°C overnight. The following morning, the 96-well plate was washed 6 times in wash buffer. The samples (100 µl) and type II collagen standard were added to the plate wells and incubated at room temperature for 2 hours. The plate was then washed with wash buffer six times. The detection antibody solution (100 µl) was added to each well and incubated at room temperature for 2 hours, following by rinsing with wash buffer at least six times. Streptavidin peroxidase solution (100 µl) was then added and incubated at room temperature for 1 hour. After washing, 100 µl of OPD-Urea H_2O_2 solution was immediately added to each well. After 30 min, 50 µl of stop solution (2N sulfuric acid) was added and the Optical Density (OD) values were measured spectrometrically at 405nm wavelength using a FLUOstar Optima Microplate Reader (BMG LABTECH, Durham, NC).

Total pellet sulfated glycosaminoglycan (sGAG) content

The dimethyl methylene blue dye-binding (DMMB) assay was used to measure the secretion and accumulation of sulfated glycosaminoglycans (sGAGs) in triplicate samples of control and chondrogenically-induced cultures, using three pellets from each sample. This assay is based on the ability of sulfated GAGs to bind the cationic dye 1, 9-dimethylmethylene blue. Pellets were digested in 250 µl of papain digestion buffer (SIGMA Chemical Cp., St. Louis, MO) at 65 °C

overnight. Fifty µl of lysate and 200 µl of DMMB reagent were added to 96-well plates and Optical Density (OD) values was measured spectrometrically at 530 nm by a FLUOstar Optima Microplate Reader (BMG LABTECH, Durham, NC).

Alkaline phosphatase (ALP) bioactivity

An alkaline phosphatase (ALP) bioassay was used to measure alkaline phosphatase activity in triplicate samples of chondrogenically-induced cultures, using three pellets from each sample. ALP is a cell membrane-associated enzyme that is routinely used as a biomarker of endochondral chondrogenesis. The ALP assay is based on ALP-mediated conversion of p-nitrophenol phosphate to p-nitrophenol. Nitrophenol has a maximal absorbance at 405nm. Pellets were homogenized in 2% Trition-X100 on ice, and then were centrifuged at 2500 rpm for 15 minutes at 4°C to remove insoluble debris. ALP reagent (100 µl; Sigma 104 phosphatase substrate) and 100 µl of lysate were added to 96-well plate wells. After 10 minutes incubation, the P-nitrophenol in each well was measured spectrometrically at 405 nm wavelength by a FLUOstar Optima Microplate Reader (BMG LABTECH, Durham, NC). Two percent Triton-X 100 alone was used as the negative control and murine growth plate lysate was used as the positive control.

Total pellet DNA content

Pico green fluorescence assay for DNA quantification was assessed for the cell number in pellet culture during chondrogenesis. Total double-stranded DNA was quantified by measuring fluorescence of an ultra-sensitive nucleic acid stain, PicoGreen (Invitrogen Life Technologies,

Carlsbad, CA). Samples were diluted 1:5 in 1X TE buffer (10mM Tris-HCl, 1mM EDTA, pH 7.5). Serially diluted calf thymus DNA aliquots were used as a standard curve. Duplicate 100 µl aliquots of each sample and the standard were transferred to a black 96-well microplate. On the day of the experiment, the Pico Green reagent was prepared, based on the 100 µl volume required for each well (1 µl Pico Green reagent diluted in 200 µl of 1X TE buffer) and added to every sample and standard. The microplate was placed in the dark to prevent reagent photo-degradation. Following 5 minutes incubation, the fluorescence was measured at 485 nm wavelength (FLUOstar OPTIMA, BMG, Lab Technologies).

Statistical Analyses

The assays listed above were routinely run in three replicates within each cell isolate. The quantitative differences in phenotype of synovium-derived cells between treatment groups were assessed by one-way ANOVA. All quantitative differences in phenotype or biosynthesis between cells isolated from bone marrow, synovium and adipose tissue were assessed by two-way repeated measures ANOVA to control for differences among horses. When group differences for chondrogenic induction were detected, pairwise multiple comparisons were made by use of the Holm-Sidak nonparametric test. A commercially available statistical program (Sigma Stat® version 11.0) was used to perform statistical analysis. Data were expressed as the mean ± standard error, and a p value < 0.05 was considered to be statistically significant.

Results

Monolayer expansion

Synovium primary isolates required around 9 days to reach confluence in monolayer culture, and subsequent passages reliably achieved confluent within 6 days, indicative of substantial proliferative capacity in these cell populations. No statistical difference was seen in days required for confluence among primary cultures and passages (Fig 6).

Osteogenic and adipogenic capacities

Synovium-derived cells cultured with osteogenic medium for 14 days exhibited the nodule formation and marked increase of ALP positive cells, compared to cells cultured without osteogenic medium (Fig 7A, B). After 14 days culturing with osteogenic medium, synovium-derived cells not only formed nodules but also secreted moderate amounts of mineralized matrix, confirmed by Alizarin Red staining, whereas cells cultured without osteogenic medium showed little or no Alizarin Red up-take (Fig 7C, D). Synovium-derived cells cultured in adipogenic medium for 14 days contained the intracellular lipid droplets. Cells cultured without adipogenic medium showed no evidence of intracellular lipid formation (Fig 7E, F).

Chondrogenic phenotype assessment

Comparative analyses of cartilaginous matrix gene expression and protein deposition with differing dexamethasone supplementation protocols, indicated that the expression of collagen

type II and aggrecan mRNAs was not significantly affected by continuous or partial (days 1-10 or 10-20) dexamethasone administration (Figures 8A and 8B). However, pellets treated with dexamethasone only in the final 10 days of culture deposited significantly less collagen type II protein than the other two groups (Fig 8C). The three groups deposited similar amounts of sGAG in the pericellular matrix (Fig 8D). On the basis of these experiments, dexamethasone was added to the culture medium throughout the 20 days of culture in subsequent experiments.

SYN cell pellet cultures treated with 100 ng BMP-2/ml accumulated more collagen type II (Fig 9A) and sGAG (Fig 9B) than pellets treated with TGF-β1, or pellets sequentially exposed to TGF-β1 and BMP-2. There was minimal ALP activity in all treatment groups (Fig 9C). Collagen type X mRNA levels were substantially lower than expression levels in hypertrophic cartilage and were not significantly different from negative control (Fig 9D), indicating that synovium-derived cells undergo chondrogenesis but do not express markers characteristic of the transient, endochondral chondrocytic phenotype.

Comparative chondrogenic capacities of SYN cells

Monolayer expansion time. BM primary isolates required around 20 days to reach confluence in monolayer culture. SYN and FAT primary isolates reached confluence around 10-13 days, less days than BM primary cells. Subsequent passage cultures reached confluence in 5-6 days among BM, SYN, and FAT cells (Fig 10).

Chondrogenic capacities. No obvious differences were apparent in the chondrocytic gene expression of undifferentiated SYN, BM or AD cells after monolayer expansion through two passages (data not shown). The transcriptional assays demonstrated that SYN and BM pellets significantly up-regulated chondrocyte-specific genes, at levels that exceeded levels of expression in undifferentiated cells. In contrast, there was minimal up-regulation of chondrogenic genes by FAT cells (Figure 11A-B). BM and SYN pellets secreted significantly more Coll II and sGAG proteins than the FAT pellets. Fully differentiated articular chondrocytes accumulated 0.05 µg/pellet of collagen type II. BM pellets deposited significantly more collagen type II in the pericellular matrix than primary chondrocyte pellets (Fig 11C). Fully differentiated articular chondrocytes accumulated 1.6 µg /pellet of sGAG, while BM and SYN pellets deposited 2.7 fold more sGAG than primary chondrocyte pellets (Fig 11D).

Expression of hypertrophic markers. BM cells significantly increased Col X mRNA expression (Fig 12A) and transiently increased ALP activity (Fig 12B), both cardinal markers of a hypertrophic chondrocytic phenotype. These changes were not seen in SYN and FAT cells. These findings suggest that BM MSCs enter the endochondral chondrogenic lineage, as opposed to a non-hypertrophic phenotype/articular phenotype.

Discussion

The experiments in this study were conducted to address several aspects of equine synovium-derived cell in vitro proliferation characteristics, multi-potency, chondrogenic capacity and chondrocytic phenotype after chondrogenic differentiation. We found that equine synovial cells

could be expanded in monolayer cultures with proliferation rates very similar to those of bone marrow- and adipose-derived cells. Under standard osteogenic culture conditions, SYN cells were formed the multicellular nodules that characterizes this differentiation process. The nodules stained strongly for ALP activity but the Alizarin Red up-take indicated that mineralized matrix deposition was only moderately positive. Although direct comparisons of osteogenic capacity were not conducted in the current study, similar experiments carried out with bone marrow-derived MSCs in a separate series of experiments indicate that the osteogenic capacity of SYN cells is considerably less than that of BM cells (data not shown). Characteristic lipid-filled vacuoles were present in SYN cells maintained under adipogenic conditions for 14 days. Collectively, these data indicate that SYN cells express the cardinal features of both osteogenic and adipogenic lineages with appropriate stimuli.

As expected, SYN cells were also able to differentiate along the chondrogenic lineage with appropriate non-adherent culture conditions and stimuli. In the standard chondrogenesis assay, driven by TGF-β1, BM cells expressed significantly higher levels of collagen type II (approximately 50-fold) and aggrecan (approximately 8-fold) mRNAs than SYN cells. BM cells also deposited more collagen type II protein than SYN cells; however the relatively modest four-fold difference in collagen type II protein deposition is far less than the transcriptional disparity between BM and SYN cells and suggests that post-transcriptional activities are comparatively inefficient in BM cells undergoing chondrogenesis. Further, the optimization experiments (Figure 9A and B) demonstrated that SYN cells respond more potently to BMP-2 stimulation (as opposed to TGF-β), with 2-3 fold increases in both collagen type II and sGAG secretion, consistent with the findings of several previous studies (Shintani & Hunziker, 2007; Shirasawa *et*

al., 2006; Yoshimura *et al*., 2007). These levels of matrix secretion by SYN cells compare favourably with BM cell cartilage matrix secretion in response to TGF-β1 stimulation. Given that BMP-2 supports the differentiated phenotype of articular chondrocytes *in vitro* and is required for phenotypic stability and cartilage matrix protein expression *in vivo* and *vitro* (Oshin, *et al*., 2007), it is likely that SYN cells are comparatively more responsive to BMP ligands than other MSC sources.

Of particular relevance to articular cartilage repair, neither BMP-2 nor TGF-β1 induced a hypertrophic phenotype in SYN pellets. Given that SYN cells cultured under osteogenic conditions up-regulated ALP activity substantially within 14 days (Figure 7B), this outcome suggests that the absence of any ALP up-regulation under chondrogenic conditions is a specific phenotypic aspect of these cells, rather than a more generic inability to activate ALP expression and activity under all conditions. Collectively, these findings suggest that SYN cells are phenotypically more appropriate for articular chondrogenesis than MSCs from bone marrow or fat, since the development of a stable, non-hypertrophic chondrocytic phenotype is critical for articular cartilage development and maintenance, and aberrant expression of the hypertrophic phenotype is considered to be central to the pathogenesis of osteoarthritis and associated degeneration of articular cartilage in affected joints.

Although adipose-derived cells were not the focus of these studies, the very poor chondrogenic activity of FAT cells in this study was remarkable, considering that a number of published studies advocate the use of these cells for cartilage tissue engineering applications. It has been

shown that FAT cells do not express the type I TGFβ-receptor under standard chondrogenic culture conditions, and these cells also down-regulate expression of the chondrogenic BMPs-2,-4, and-6 (Hennig *et al*., 2007). FAT cells require BMP-6 treatment to induce TGFβ-receptor-I up-regulation, restore TGF-β responsiveness and increase chondrogenic potential. It is highly likely that equine FAT cells require similar combinatorial treatment to exhibit robust chondrogenic activity.

Figures and Tables

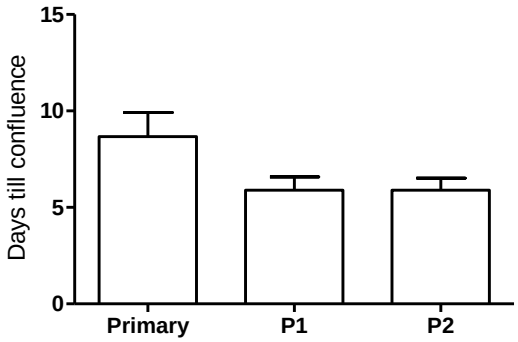

Figure 6. Proliferative capacity of synovium-derived cells in monolayer cultures. Primary synovium-derived cells required about 9 days to reach confluence, no significant difference from P1 and P2 cultures. P1: passage 1 of synovium derived cells; P2: passage 2 of synovium derived cells.

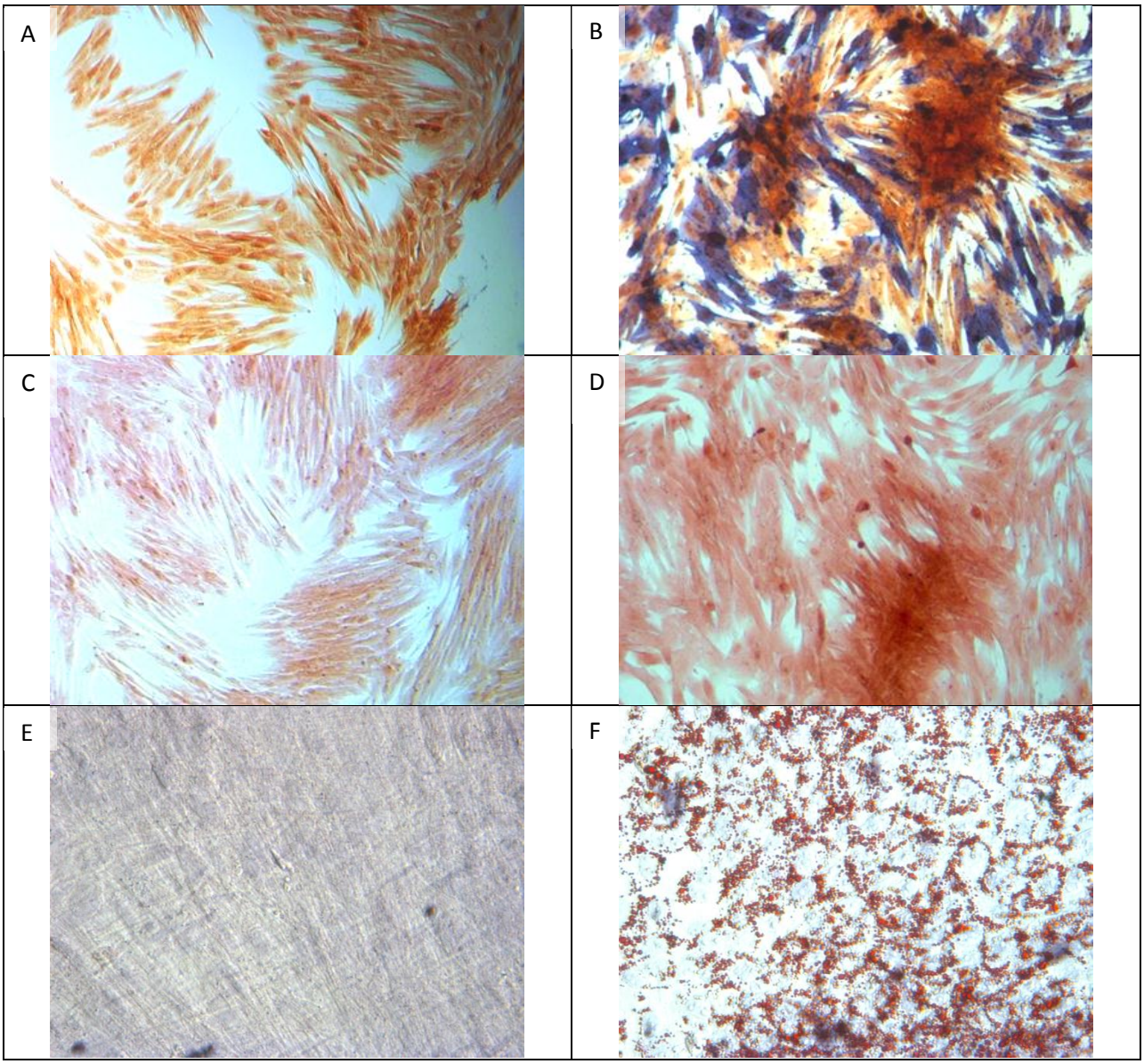

Figure 7. Osteogenic and adipogenic differentiation of synovium-derived cells. Synovium-derived cells were cultured in control (A, C and E), osteogenic (B, D) or adipogenic (F) for 14 days, and stained for alkaline phosphatase activity (A and B; magnification 100X), matrix mineralization (Alizarin Red, C and D; magnification 100X) or intracellular lipid accumulation (Oil Red O, E and F; magnification 200X).

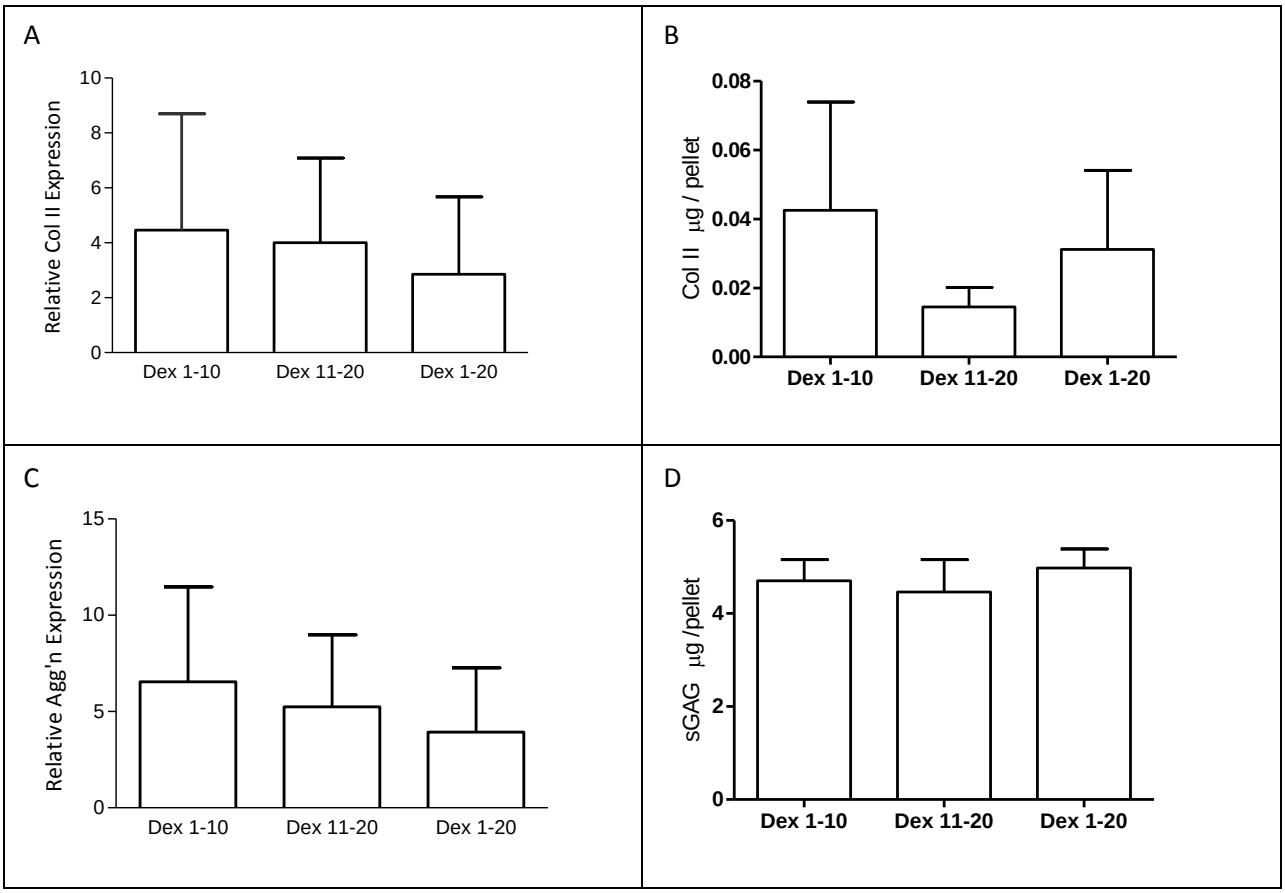

Figure 8. The temporal effect of Dexamethasone (DEX) administration on SYN cell chondrogenesis. Dexamethasone was administered to SYN pellet cultures for the first 10 days of culture (Dex 1-10), the final 10 days of culture (Dex 11-20) or for the duration of the experiment (Dex 1-20). There was no significant effect on the expression of collagen type II mRNA (A). DEX administration for days 11-20 reduced collagen type II protein secretion, although this did not reach statistical significance. Aggrecan mRNA (C), and sGAG levels (D) were not significantly affected by intermittent or continuous DEX administration. For mRNA expression, articular cartilage from equine 2 year old horses was used as reference. All treatment groups were also stimulated with BMP-2.

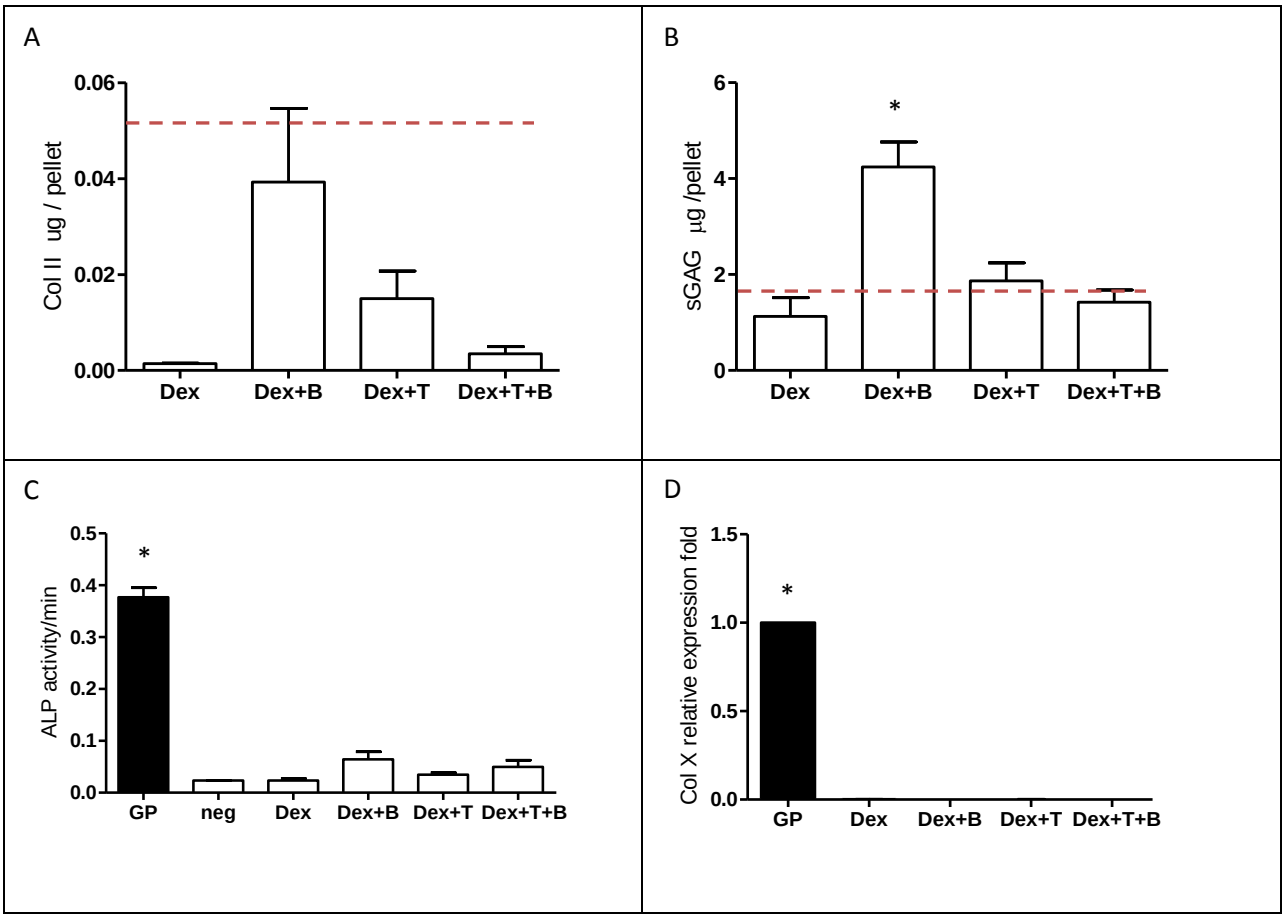

Figure 9. The effects of chondrogenic growth factors on chondrogenesis of equine synovium-derived cell pellets. Synovium-derived pellets stimulated with BMP-2 (B) accumulated more collagen type II (A) and sGAG (B) than groups treated with dexamethasone (Dex) alone, with TGF-β1 (T) and with sequential exposure to TGF-β1 and BMP-2. Pellets treated with BMP-2 secreted these matrix components at levels comparable to those in fully differentiated chondrocyte pellets, indicated by the dashed red lines. All treatment groups expressed significantly lower ALP activity (C) and collagen type X mRNA (D) than the growth plate chondrocyte (GP) positive controls.

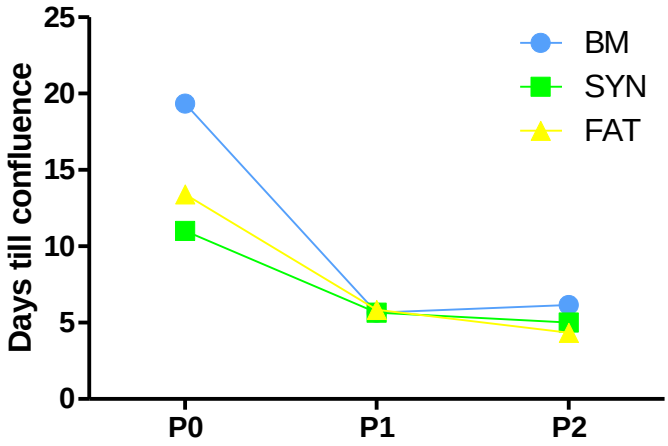

Figure 10. Monolayer expansion times. Primary BM cells required more days to reach confluence than SYN and FAT cells. Subsequent passage cultures reached confluence in 5-6 days among BM, SYN, and FAT cells.

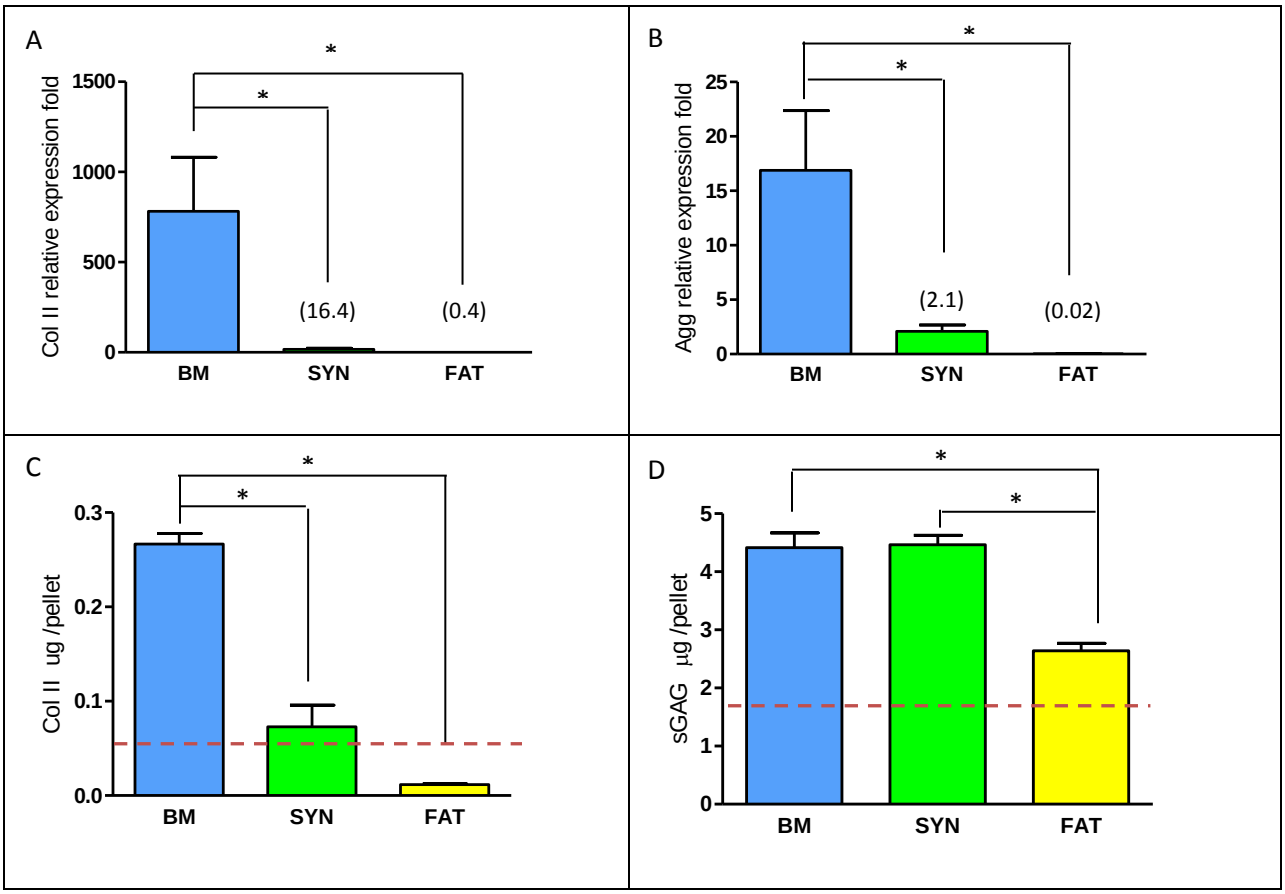

Figure 11. **Chondrogenic capacities of BM, SYN, FAT pellet cultures**. Both bone marrow- (BM) and synovial- (SYN) derived cells significantly increased collagen type II (Col II; A) and aggrecan (Agg; B) mRNA expression after 20 days in chondrogenic medium. In contrast, Col II and Agg up-regulation in FAT cells was minimal. Day 10 SYN pellets were used as transcriptional baseline references, designated as "1". BM and SYN cells deposited significantly more Coll II protein (C) and sulfated glycosaminoglycans (sGAG; D) into the pericellular matrices than FAT cells. BM pellet Col II accumulation significantly exceeded that of SYN and FAT pellets. Both BM and SYN cell pellet cultures exceeded the Col II and sGAG deposition of primary chondrocyte pellets, indicated by the dashed red lines.

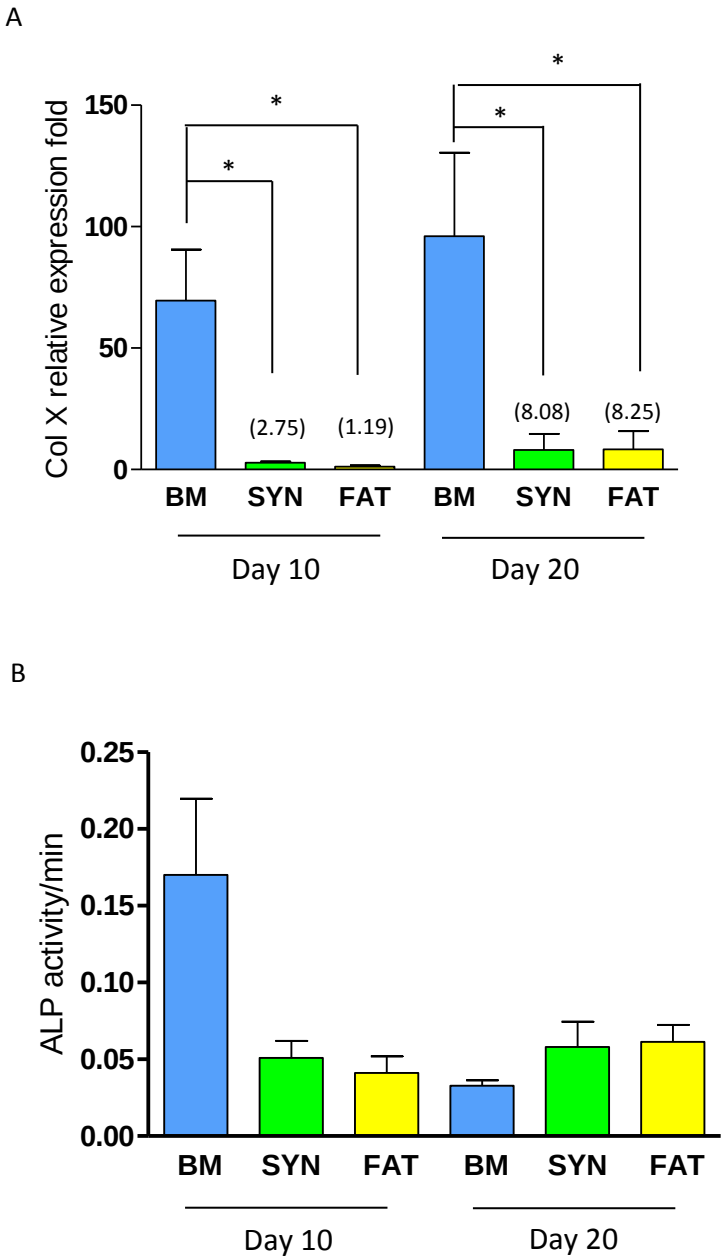

Figure 12. Endochondral phenotype assessment. A. BM cells expressed significantly higher levels of collagen type X (Col X) mRNA than SYN or FAT cell pellets at day 20. Undifferentiated 'passage 2' cells from each source were used as transcriptional baseline references. **B.** BM cells transiently increased ALP activity at Day 10 (D10), in contrast to SYN or FAT cell pellets, but the ALP activities of the three groups by day 20 (D20) were very similar.

Table 2: Primers utilized in the qPCR reactions

Gene (size)	Sense Primer Antisense Primer	Annealing temperature
Col II	5’ AGCAGGAATTTGGTGTGGAC (4325)	62.1 °C
(223 bp)	5’ TCTGCCCAGTTCAGGTCTCT (4548)	
Col X	5’ TGCCAACCAGGGTGTAACAG (39)	62.1 °C
(244 bp)	5’ ACATTACTGGGGTGCCGTTC (283)	
Aggrecan	5’ GACGCCGAGAGCAGGTGT (33)	62.1 °C
(202 bp)	5’ AAGAAGTTGTCGGGCTGGTT (235)	
EF1-alpha	5’ CCCGGACACAGAGACTTCAT (48)	62.1 °C
(328 bp)	5’AGCATGTTGTCACCATTCCA (376)	

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CHAPTER 5: CHONDROGENIC INDUCTION OF EQUINE SYNOVIUM-DERIVED CELLS BY ADENOVIRAL EXPRESSION OF EQUINE BMP-2

Introduction

BMPs (bone morphogenetic proteins) were originally discovered by Marshall Urist in the 1960s as an activity in demineralized bone matrix that was capable of stimulating ectopic bone formation in soft tissues (Urist, 1965; Urist *et al*., 1967). Since Urist's seminal discovery, more than 20 members in BMP family have been identified, and it is now established that BMPs play pivotal roles in cellular proliferation, differentiation and apoptosis, organogenesis and homeostasis in a wide range of tissues (Edgar *et al*., 2007; Katagiri *et al*., 1997; Liu & Niswander, 2005; Owens *et al*., 2008; Robert, 2007; Schulz & Tseng, 2009). BMPs belong to the TGF-β superfamily of secreted homodimeric ligands, consisting of a signal peptide, a pro-domain and mature peptide. The tertiary structure of mature BMPs is the result of interactions between six highly conserved cysteine residues that stabilize the 'finger and heel' cysteine knot structure that is characteristic of all members of the TGF-β ligand superfamily.

Given the ability of BMPs to stimulate osteogenesis, recombinant human BMP-2 and BMP-7 are approved for treatment of orthopedic trauma in USA and Europe, and have been evaluated in open tibial fractures, segmental bone defects, tibial nonunions, and spinal fusion. BMP implantation has been shown to be as effective as autologous bone grafting in augmenting bone repair, while avoiding the complications associated with bone graft collection. The use of BMPs enhances union rate, reduces the rate of inflammation in fresh fractures/nonunions, and decreases the number of secondary interventions of open tibial fractures (Boden *et al*., 2000; Cook *et al*.,

1994; Friedlaender *et al*., 2001; Govender *et al*., 2002; Johnsson, Stromqvist, & Aspenberg, 2002). However, there are several limitations of the clinical use of BMPs, including the high cost of recombinant BMPs, the rapid elution and redistribution of recombinant protein from the implantation site, which necessitates delivery of supra-physiological quantities of BMPs, and the increasingly recognized risk of bone overgrowth or heterotopic ossification at implantation sites (Carlisle & Fischgrund, 2005; De Biase & Capanna, 2005; Shimer, Oner, & Vaccaro, 2009; Villavicencio *et al*., 2005).

BMPs are also active in maintaining articular cartilage homeostasis and facilitating cartilage repair. Mice lacking BMP-2 expression in limb elements develop a fibrotic degeneration of articular cartilage within a few weeks of life (Tsuji *et al*., 2006). In post-natal contexts, BMP-2 is transiently increased following blunt articular cartilage damage, and is the only member of the TGF-β superfamily identified as being critical for the capacity of monolayer chondrocytes to regain the differentiated phenotype following in vivo implantation (Dell'Accio, De Bari, & Luyten, 2001; Dell'Accio *et al*., 2006; Fukui *et al*., 2006; Oshin *et al*., 2007). Conversely, mice with a hemi-deficiency of the BMP inhibitor, Noggin, were significantly resistant to cartilage loss in an immune-mediated inflammatory arthritis model (Lories *et al*., 2006). Both BMP-2 and BMP-7 are capable of stimulating cartilage matrix synthesis (Badlani *et al*., 2008; Fortier *et al*., 2011). BMP-7 has a protective effect against OA progression by decreasing the activities of aggrecanase, interleukins-1,-6, and -8, and metalloproteinases 1 and 13 (Badlani *et al*., 2008; Fortier *et al*., 2011}.

Musculoskeletal disease is a major cause of wastage and reduced performance in horses. Long bone fracture repair is a highly challenging problem in adult horses, since lower limb injuries are usually associated with substantial soft tissue damage, currently available orthopedic hardware is not sufficiently strong to re-establish the weight-bearing bone column and horses are not able to bear weight on three limbs for long periods of time. BMPs are potent stimulators of osteogenesis and could be applied to accelerate bone repair in equine fracture patients (Ishihara *et al*., 2008). Osteoarthritis is linked to approximately 60% of equine lameness problems. This disease involves compromised homeostasis of articular cartilage and eventual loss of this tissue in affected joints. BMP-2 is able to support the differentiated phenotype of articular chondrocytes and stimulates cartilage matrix synthesis by chondrocytes (Oshin *et al*., 2007). Given the clinical importance of articular and osseous injuries in horses, and the potent activities of BMPs in cartilage and bone repair, this study was conducted to clone and characterize equine BMP-2, and to generate equine BMP-2 expression constructs that can be developed for clinical applications in horses.

Materials and Methods

Characterizing equine BMP-2

The putative equine BMP-2 sequence was generated by blasting the human and murine BMP-2 cDNA sequences across the equine genome data base [ncbi.nlm.nih.gov/genome/equus]. The extracted equine BMP-2 sequence was aligned to the published human, murine, bovine, feline and canine BMP-2 sequences to verify the conservation of the equine sequence and definitively identify the sequences spanning the open reading frame of the gene.

Cloning of equine BMP-2

The open reading frame of equine BMP-2 was obtained from equine articular chondrocyte RNA by gene specific RT-PCR. The gene-specific anti-sense primer (5'-TTCATGTGCTGGGGTTGAA-3') was used to prime the reverse transcription reception, following by two rounds of nested PCR amplification. The first pair of primers used were forward primer (5'- CTGCGGTCTCCTAAAGGTC-3') and reverse primer (5'-TTGTTTCCCAACTTCTTTTCG-3'). The nested primers were forward primer (5'-CATGGTGGCCGGGAC-3') and reverse primer (5'-TTTTTCTCCATTCCATTCCA-3'). The nested PCR product was cloned into TOPO PCRII vector (Invitrogen, USA), which was transformed into DH5α-T1 E. *coli*, following manufacturer's instructions. The identity and correct orientation of the PCR product was confirmed by sequencing (ABI 3730XL capillary sequencer at UIUC DNA Sequencing Core). The cDNA was then sub-cloned in pCMV-SPORT using the BamH I and Not I restriction sites, to generate the eqBMP-2 expression vector.

Equine BMP-2 adenoviral constructs

The open reading frame of eqBMP-2 was sub-cloned into the VQAd CMV K-NpA shuttle plasmid (ViraQuest Inc, North Liberty IA) using the BamH I and Kpl I polylinker sites. The shuttle plasmid and adenovirus backbone were linearized with PacI and transfected into HEK293 cells. Viral foci became evident on day 8 and the plate was harvested 12 days after transfection. The initial lysate was seeded into 150 mm plates of ~80% confluent HEK293 cells for 46 hours to generate the primary lysate. The primary lysate was amplified on 150 mm plates of HEK293 cells for 30 hours. This viral lysate was purified over two rounds over CsCl gradients. The virus

particles were dialyzed against and formulated in A195 buffer, aliquoted and frozen at –80C°. Plaque-forming unit (PFU) titer was determined by plaque assay in HEK 293 cells with a final titer of 5×10^{10} PFU/ml.

Equine BMP-2 Bioactivity tests

The HepG BRA cell line was generously provided by Dr. Daniel Rifkin from New York University (Zilberberg, ten Dijke, Sakai, & Rifkin, 2007). The HepG BRA cell line is stably transfected with a BMP response element derived from the murine ID1 promoter, fused to firefly luciferase cDNA. HepG BRA cells were seeded into 6-well plates at 3×10^4 cells/ cm^2 and cultured overnight to achieve 50-80% confluence. The pSPORT-eqBMP-2 expression vector was transduced into the reporter cells using FuGENE 6 transfection reagent (Roche, USA), according to manufacturer's instructions. GFP and antisense BMP-2 expression vectors were also transduced into reporter cells as controls. Luciferase activity was measured after 36 hours, using the Bright-Glo luciferase assay (Promega, USA).

For the assessment of eqBMP-2 adenoviral bioactivity, HepG2 BRA cells were added to 24-well plates at 1×10^5 cells/ cm^2, incubated overnight. The HepG2 BRA cells were infected with the eqBMP-2 adenovirus at 1, 2, 4, 8, 10, 16, 20, 50, and 100 of multiplicities of infection (MOI). After 12 hours, recombinant human BMP-2 was added to HepG2 BRA cells at varying concentrations (1, 2, 4, 8, 10, 16, 20, 50, and 100 ng /ml), as positive control references. Luciferase activities were measured 36 hours after the exposure of the reporter cells to the virus, using the Bright-Glo luciferase assay, as above.

Chondrogenesis of synovium-derived MSCs

Synovial membrane samples were obtained from the dorsal surface of the radiocarpal joints of 2-year old healthy horses. Synovium was diced and digested with 0.2% collagenase type II overnight. Cells were seeded into 100-mm culture plates at the density of 10,000 cells per cm^2, and cultured in 10 ml of Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (basal medium). At confluence, the cells were lifted with trypsin-EDTA and re-seeded into 100-mm culture plates at 5,000 cells per cm^2 for two passages.

When the second passage cells reached 80% confluence, eqBMP-2 adenovirus was added to the monolayers at MOIs of 20 and 100. Lac-Z adenovirus was transduced into cells at 100 MOI to serve as negative controls. Non-transduced cells were maintained for use as controls, as detailed below. After a further 16 hours, the cells were trypsinized and transferred to pellet cultures (2.5 x 10^5 cells per pellet) by centrifugation at 390 rfu in 1.5 ml microcentrifuge tubes. Pellets were maintained in DMEM, supplemented with insulin/transferrin/selenous acid (ITS), 37.5 μg/ml ascorbic acid, 100 μg/ml sodium pyruvate, 10^{-7} M dexamethasone, and 1% penicillin-streptomycin. The positive control cells were pelleted, as above, and were stimulated with rhBMP-2 at 20 and 100 ng /ml. Half of the Lac-Z transduced cell pellets were stimulated with 100 ng /ml of rhBMP2. Synovium-derived cell pellets cultured in DMEM, supplemented with ITS, ascorbic acid, sodium pyruvate, dexamethasone, and 1% penicillin-streptomycin served as negative controls. At Day 5, the pellets were gently aspirated from the micro-centrifuge tubes and transferred to 6-well ultra-low attachment culture plates, and the medium was renewed every 3 days. At day 14, pellets were collected and analyzed for the secretion and accumulation of sulfated glycosaminoglycans (sGAGs) using the DMMB assays (Stewart, Byron, Pondenis, &

Stewart, 2008). Pellets were stained with toluidine blue for cartilage proteoglycan deposition (Stewart, Byron, Pondenis, & Stewart, 2007).

Statistical Analyses

The assays listed above were routinely run in three replicates within each group. All quantitative differences in phenotype or biosynthesis between cells experimental groups were assessed by one-way ANOVA. Statistical analyses were performed by Prism 5.0. Data were expressed as the mean ± standard error, and a p value < 0.05 was considered to be statistically significant.

Results

Cloning and characteristics of equine BMP-2

The equine BMP-2 gene is located on chromosome 22. Blasting the human and murine BMP-2 cDNA sequences across the equine genome identified the second and third exons, but the non-coding first exon is located within a poorly characterized sequence of the equine genome and was not located. The open reading frame of equine BMP-2 spans 1185 base pairs, which are contained entirely within exons 2 and 3. The open reading frame codes for a 69-base signal peptide, a 744-base pro-domain and a 342-base mature peptide. Overall, the equine coding sequence is 95.7% and 91.4% homologous to the human and murine BMP-2 sequences, respectively. The equine BMP-2 protein comprises a 23-amino acid signal peptide followed by a 258 amino acid pro-peptide domain. The mature protein contains 114 amino acids (Figure 13)

and is completely consistent with the amino acid sequences of the mature human and murine BMP-2 proteins.

Equine BMP-2 bioactivity

HepG BRA cells were transfected with equine BMP-2 TOPO PCR II expression vector using FuGENE 6 transfection reagent. The sense eBMP-2 vector stimulated significantly higher luminescence than the 'no vector', 'GFP vector' and 'antisense eBMP-2' groups (Figure 14). These results confirmed that equine BMP-2 TOPO PCR II expression vector expresses functional BMP-2 protein that is able to transduce signaling activity in responsive cell types.

The response of the HepG BRA cells to Ad-eqBMP-2 was similar. The reporter cells' response to rhBMP-2 reached saturation at 20 ng/ml, while the response to Ad-eqBMP-2 was linear across the MOIs tested. A comparison of luciferase activities indicated that an MOI of 80 was equivalent to 20 ng/ml of rhBMP-2 in this bio-assay (Figure 15).

Chondrogenesis driven by adenovirus eBMP-2

Chondro-progenitor cells isolated from equine synovium (SYN MSCs) that were infected with Ad-eqBMP2 deposited significantly higher sGAG than did control and LacZ-infected groups, although sGAG deposition did not reach the levels of pellets treated with 100 ng/ml of rhBMP-2 protein. Infection with the LacZ virus did not compromise SYN MSC responsiveness to BMP-2 stimulation. The pellet sizes corroborated the sGAG data in that the eq-BMP2-infected pellets were substantially larger than the Control and LacZ pellets but somewhat smaller than the rhBMP-2 pellets (Figure 16-17).

Discussion

The experiments in this study have demonstrated that the mature equine BMP-2 peptide is highly similar to the human protein. Further, induced expression of equine BMP-2 activates BMP signaling pathways and stimulates chondrogenic differentiation of synovial chondro-progenitors comparably to recombinant BMP-2 protein administration. The stimulation of sGAG deposition by adenoviral eqBMP-2 delivery was approximately 80% of that in pellets treated continuously with 100 ng rhBMP-2/ml (Figure 16). This is not surprising, given that data from the reporter cell line indicated that the adenovirus, when applied to cells at an MOI of 100, generated BMP signaling activity equivalent to approximately 25 ng BMP-2/ml. Further, it is likely that adenoviral transcriptional activity would have peaked well before the 14-day end-point of the experiment. In light of these preliminary findings, there are several potential applications of equine BMP-2 in the management of equine musculoskeletal diseases.

Accepting this, optimal strategies for delivering BMP-2 expression constructs are still unclear. The current FDA-approved device for BMP-3 delivery to bone repair sites employs a collagen-based bio-scaffold. This implantation strategy has been proven to be effective for resolution of tibial non-unions and for vertebral fusion, but the recombinant protein is rapidly eluted from the scaffold, within 3-4 days, and there is a recent growing awareness of excessive bone formation that, in cervical vertebral fusions, can lead to complications associated with osseous compression of adjacent neural and vascular structures sites (Carlisle & Fischgrund, 2005; De Biase & Capanna, 2005; Shimer, Oner, & Vaccaro, 2009; Villavicencio *et al*., 2005). Percutaneous injection of BMP adenoviral vectors has been shown to accelerate bone repair in an experimental

splint bone osteotomy model (Ishihara *et al*., 2008), but excessive new bone formation, beyond the confines of the bone defect, was also evident in this study.

It is possible that lower quantities of BMP proteins (milligram quantities of recombinant BMP-2 protein are delivered to human fracture and fusion sites), over longer periods of time, could stimulate significant clinical benefits, without the prohibitive costs issues with excessive bone formation associated with recombinant protein application. In support of this, the relatively modest adenoviral delivery of equine BMP-2 at MOI of 20 (approximately equivalent to 5ng BMP-2/ml) stimulated 50% of the sGAG deposition that occurred in pellets treated continuously with 100ng recombinant BMP-2/ml. Controlled delivery of BMP expression vectors and/or recombinant protein, for extended periods of time (Hosseinkhani *et al*, 2008), might prove to be more effective, in terms of cost and biological responses, than applying milligram quantities of recombinant protein.

Figures

MVAGTHCLLALLLPQVLLGGAAGLIPELGRRKFAASTGRSSSQPSDDV
LSEFELRLLSMFGLKQRPTPSRDAVVPPYMLDLYRRHSGQPDAPAPDH
RLERAASLANTVRSFHHEESLEELPEMSGKTTRRFFFNLTSIPTEEFI
TSAELQVFREQMQDPWENNSNFHHRINIYEIIKPATANSKFPVTRLLD
TRLVNQNASRWERFDVTPAVMRWTAQGLANHGFVVEVAHLEENRGASK
RHVRISRSLHQDEHSWSQIRPLLVTFGHDGKGHPLHKREKRQAKHKQR
KRLKSSCKRHPLYVDFSDVGWNDWIVAPPGYHAFYCHGECPFPLADHL
NSTNHAIVQTLVNSVNSKIPKACCVPTELSAISMLYLDENEKVVLKNY
QDMVVEGCGCR

Figure 13. The amino acid sequence of equine BMP-2. The purple-shaded sequence indicates the 23-amino acid signal peptide. The green-shaded sequence indicates the 258-amino acid pro-domain and the blue-shaded sequence represents the 114-amino acid mature peptide.

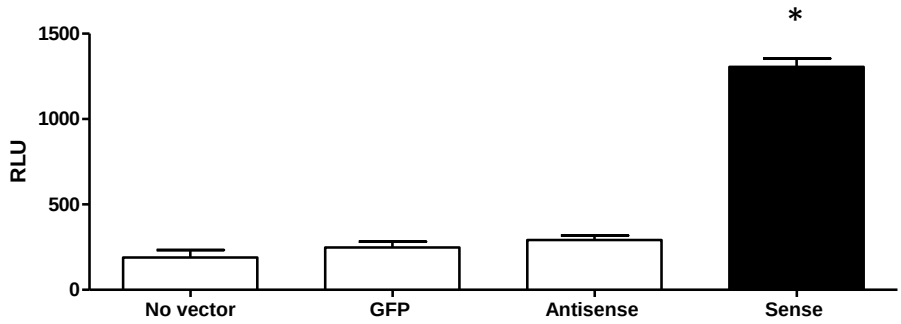

Figure 14. The bioactivity of equine BMP-2 expression vector. HepG BRA cells were transfected with expression vectors coding for GFP, equine BMP-2 in the sense orientation (Sense) or in the antisense orientation (Antisense). The Sense-transfected cells expressed significantly higher relative light units (RFUs) than the corresponding control groups, 36 hours after transfection. The sense BMP-2 expression vector induced significantly more luciferase activity than the control vectors (indicated by asterisk).

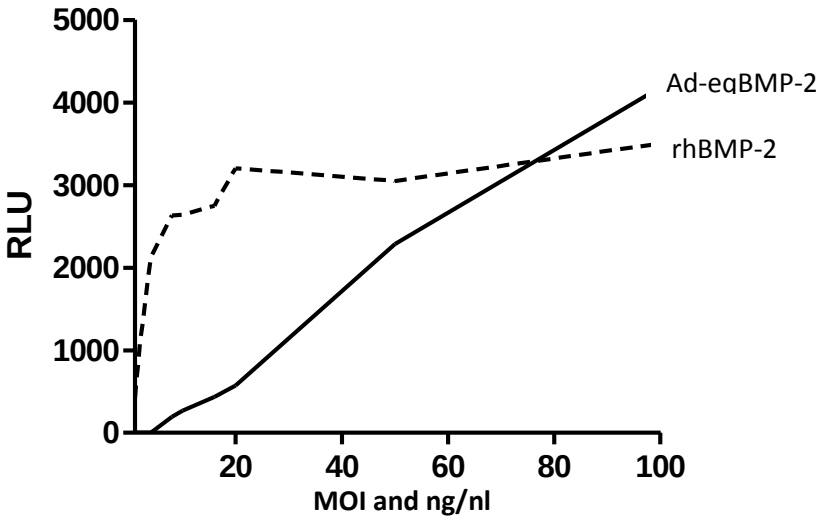

Figure 15. The response of HepG BRA cells Ad-BMP-2. The response of HepG BRA cells to recombinant human BMP-2 (rhBMP-2; dashed line) plateaued at 20 ng/ml. HepG BRA cells infected with eqBMP-2 adenovirus (AD-eqBMP-2; solid line) showed a linear dose-response increase in luciferase expression (RLU). Based on these data, an MOI of 80 corresponds to 20 ng rhBMP-2/ml.

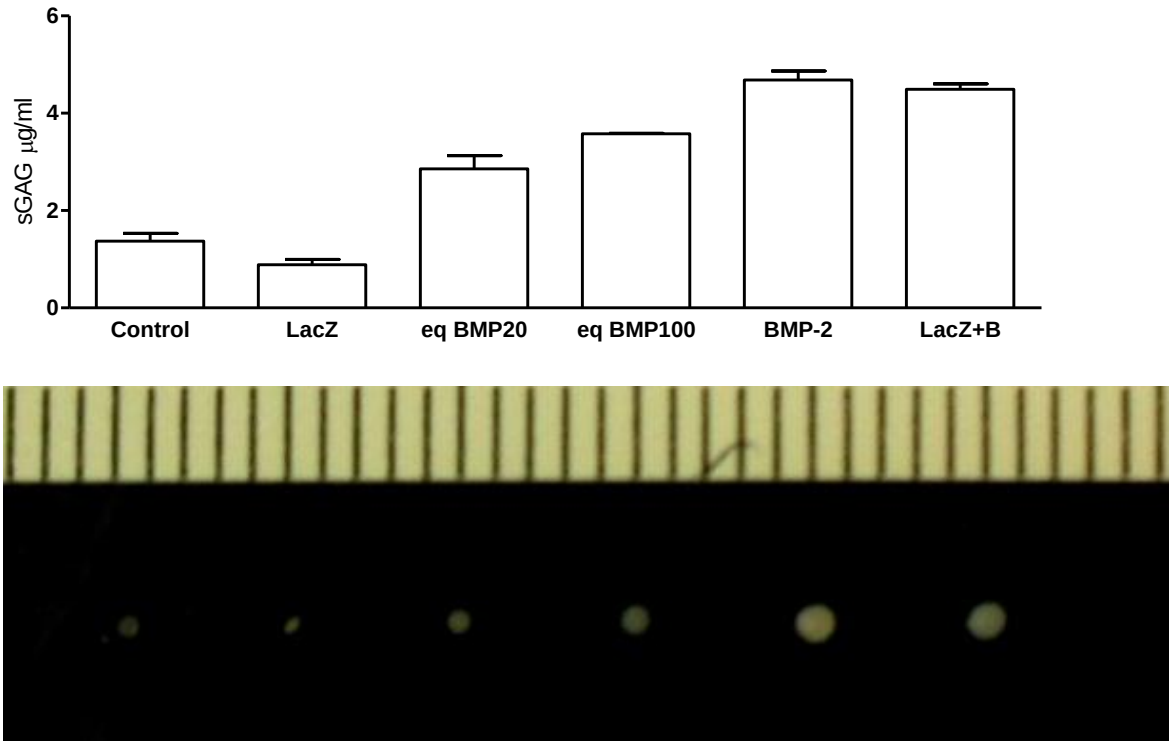

Figure 16. Ad-eqBMP2-driven chondrogenesis. The upper panel represents the deposition of sulfated glycosaminoglycans (sGAG) by SYN cell pellets, in responses to BMP-2 stimuli or controls. Immediately prior to being transferred to pellet cultures, synovial-derived progenitor cells were infected with adenoviral vectors expressing Lac-Z at MOI of 100 or equine BMP-2 at MOIs of 20 (eqBMP 20) or 100 (eqBMP 100). The Lac-Z control pellets were maintained in chondrogenic medium alone (LacZ) or were treated with 100 ng/ml of rhBMP-2 protein (LacZ +B). The BMP-2 control pellets were treated with 100 ng/ml of rhBMP-2 protein. sGAG measurements were acquired after 14 days in culture. The lower panel shows representative pellets from the experimental groups.

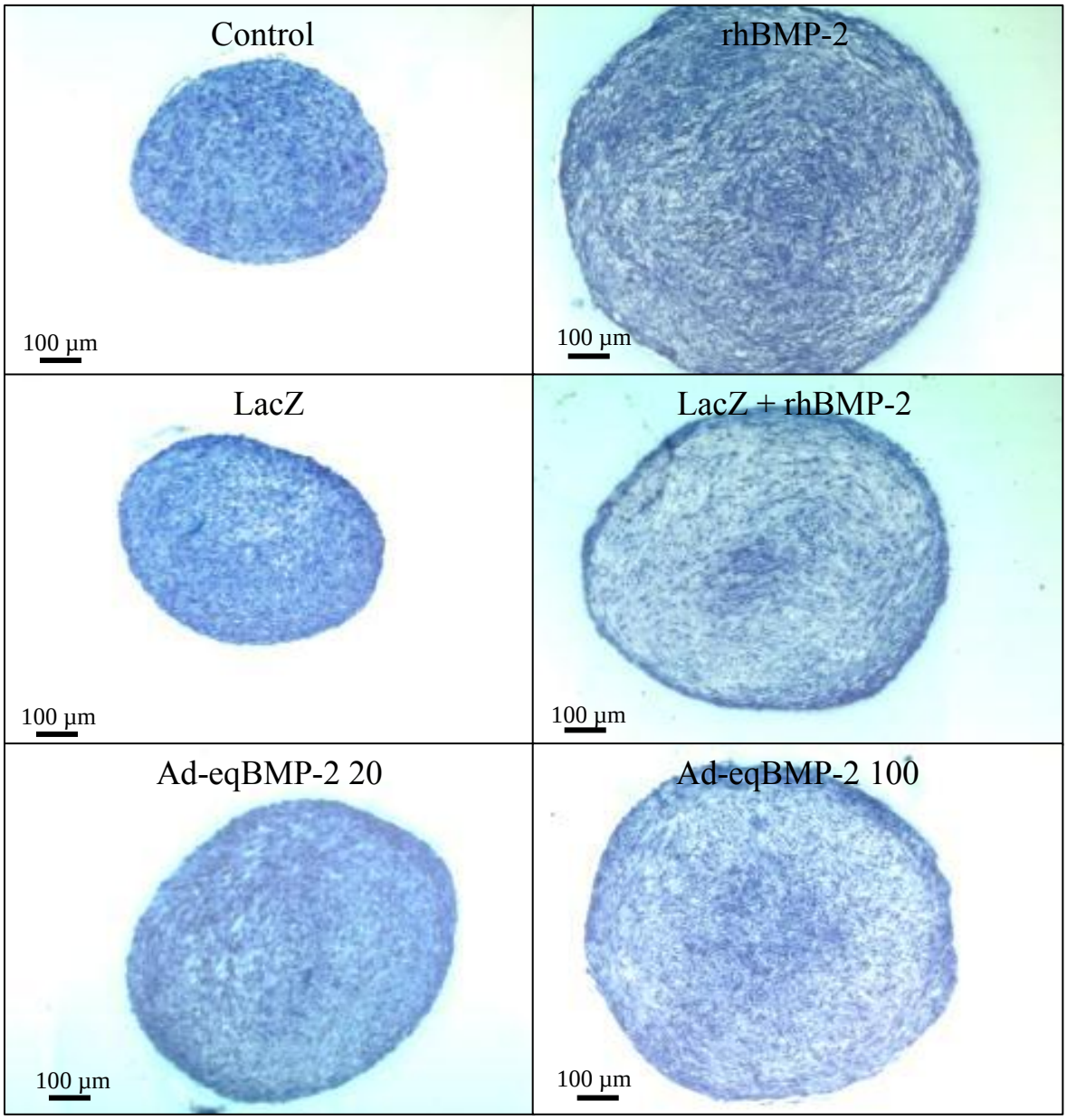

Figure 17. Representative histologic appearance of chondrogenic synovial pellets. Immediately prior to being transferred to pellet cultures, synovial-derived progenitor cells were infected with adenoviral vectors expressing Lac-Z at MOI of 100 or equine BMP-2 at MOIs of 20 (eqBMP 20) or 100 (eqBMP 100). The Lac-Z control pellets were maintained in chondrogenic medium alone (LacZ) or were treated with 100 ng/ml of rhBMP-2 protein (LacZ +B). The BMP-2 control pellets were treated with 100 ng/ml of rhBMP-2 protein. Pellets were collected after 14 days in culture, processed for histology and stained with toluidine blue.

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CHAPTER 6: CONCLUSIONS AND FUTURE DIRECTIONS

The research presented in this dissertation addressed the chondrogenic potential of equine synovial progenitor populations in two contexts. The first series of experiments focused on the chondrogenic potentials of synovial fluid- and synovium-derived cells, in Chapters 3 and 4 respectively. In addition, the chondrogenic capacity of equine synovium-derived cells was compared with that of the current 'gold standard' stem cell sources; bone marrow- and adipose-derived cells, in Chapter 4. The second series of experiments characterized equine BMP-2, and generated adenoviral expression of equine BMP-2 (Chapter 5).

The experiments addressing the chondrogenesis of synovial fluid-derived cells demonstrated that synovial fluid-derived cells possessed substantial proliferative capacity, multi-lineage potential and were capable of undergoing non-hypertrophic chondrogenesis. However, the *in vitro* expansion of primary synovial fluid cells from 1 ml aspirates required several weeks. The time required for the generation of clinically useful cell numbers could be reduced by collecting larger volumes of synovial fluid, potentially from several joints, and, in a clinical setting, collecting the fluid and expanding the requisite cell population prior to surgical intervention.

Future studies should be directed towards improving *in vitro* proliferation rates during monolayer expansion. FGF-2 accelerates *in vitro* expansion of bone marrow-derived MSC populations and further improves subsequent chondrogenic differentiation. Treating synovial fluid-derived MSC populations with FGF2 might similarly reduce the time required to produce sufficient cells for

clinical applications. Assessments of synovial fluid MSC numbers from a broader range of overtly arthritic equine joints will determine whether joint pathology in horses is associated with increased progenitor cell concentrations in synovial fluid, as has been reported in human arthritic conditions. Further, the origin(s) of synovial fluid-derived MSC populations and the techniques for recruiting these cells to cartilage defects in joints will need to be identified and validated to enable the development of in vivo strategies for articular cartilage repair.

The experiments, addressing the chondrogenic capacity of synovium-derived cells and the comparative chondrogenesis of synovium- (SYN), bone marrow- (BM), and adipose (FAT)-derived cells, demonstrated that SYN cells could be expanded in monolayer cultures with proliferation rates very similar to those of bone marrow- and adipose-derived cells and expressed the cardinal features of both osteogenic and adipogenic lineages with appropriate stimuli. In the standard chondrogenesis assay, driven by TGF-β1, BM cells expressed significantly higher levels of collagen type II and aggrecan mRNAs than SYN cells; however the relatively modest four-fold difference in collagen type II protein deposition was far less than the transcriptional disparity between BM and SYN cells. SYN cells responded more potently to BMP-2 stimulation (as opposed to TGF-β), with 2-3 fold increases in both collagen type II and sGAG secretion. Further, neither BMP-2 nor TGF-β1 induced a hypertrophic phenotype in SYN pellets. Collectively, these findings suggest that SYN cells are phenotypically more appropriate for articular chondrogenesis than MSCs from bone marrow or fat.

In future experiments, optimization of chondrogenic stimulation in SYN cells needs to be clarified, given that SYN cells might be more sensitive to BMP ligands than TGF-βs. It will be helpful to isolate pre-committed equine SYN cells by identifying appropriate differentiation-predictive markers, as has been carried out with human cells. Further, characterizing the niche of SYN cells and developing techniques for recruiting endogenous SYN cells to cartilage lesions in joints represent tissue engineering applications of these cells that could become clinically useful.

The last part of this research addressed the chondrogenic introduction of equine SYN cells by adenoviral expression of equine BMP-2. The equine coding sequence is 95.7% and 91.4% homologous to the human and murine BMP-2 sequences, respectively. The mature eqBMP-2 protein contained 114 amino acids and was completely consistent with the amino acid sequences of the mature human and murine BMP-2 proteins. The data from the reporter cell line indicated that the adenovirus, when applied to cells at an MOI of 100, generated BMP signaling activity equivalent to approximately 25 ng BMP-2/ml. The eq-BMP2-infected cells deposited significantly higher sGAG and formed larger size of pellets than did control and LacZ-infected groups, although sGAG deposition and the size of pellets did not reach the levels of pellets treated with 100 ng/ml of rhBMP-2 protein. In light of these preliminary findings, there are several potential applications of equine BMP-2 in the management of equine musculoskeletal diseases. Future studies should be directed towards optimizing delivery of BMP expression vectors to sites of injury, including technologies for regulating the BMP dose, time frame of release, and targeting vectors in cell-specific manners via cell-specific receptor interactions and enhancer-driven expression.