INVESTIGATION OF MESENCHYMAL STEM CELLS FOR CARTILAGE REPAIR

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The aim of the thesis project was to identify a growth factor that would enhance the in vitro chondrogenesis of the equine mesenchymal stem cell (MSC) and subsequently to assess the capacity of the growth factor expressing MSC to improve cartilage repair and osteoarthritis (OA) prevention in vivo.

Previous efforts on MSC enhanced cartilage repair have focused on implantation of MSCs to articular cartilage defects. This approach is only applicable to acute and focal articular cartilage injury and does not apply to the OA joint. Global joint disease, as in OA, is far more common than focal cartilage injury and no disease modifying therapies of regenerative therapies are currently available. Short term tracking studies of intra-articular injection of autologous MSCs were performed as part of this dissertation. Studies revealed that MSCs efficiently engraft the synovial membrane but not articular cartilage, whether normal or diseased.

Differing three-dimensional culture systems were tested for suitability as a system to study in vitro chondrogenesis of MSCs. In chondrogenic media, fibrin alginate culture and pellet culture (500,000 cell pellets) were superior for chondrogenic induction to agarose, alginate alone and 250,000 cells pellets. For in vitro MSC chondrogenesis, supplementation with an isoform of transforming growth factor beta (TGF-β) is required. TGF-β1, -β2 and -β3 have been used and although TGF-β1 is the most frequently reported both in vitro and in vivo, it is not clear which is superior for chondrogenic induction and prevention of hypertrophy. The chondrogenic effect of the 3 isoforms was tested in MSC pellet culture. All 3 isoforms resulted in MSC chondrogenesis, however, TGF-β3 had clear enhancement of prevention of hypertrophy.

Growth factor supplementation in vivo is difficult to maintain because of the short half-life of injected or implanted growth factors. Gene therapy techniques to induce growth factor expression by injected or implanted MSCs would allow for continuous growth factor supplementation to the joint. Adenoviral vectors for expression of TGF-β1, -β2 and -β3 were constructed and tested in MSC pellet culture to confirm transgene expression and efficacy for chondrogenic induction in long term 3-dimensional culture. Adenoviral transgene expression of TGF-β3 resulted in chondrogenic induction and reduced progression toward hypertrophy compared to Ad TGF-β1 and -β2.

Given the synovial distribution of MSCs injected to the arthritic joint, injection of growth factor enhanced and anti-catabolic MSCs to the middle carpal joint of horses in the osteochondral fragmentation model of OA was tested. This resulted in improved control of joint disease evidenced by improved effusion scores and range of motion in the week following treatment injection, reduced cartilage *MMP13* and synovial *IL1b* expression, reduced synovial fibrosis and a strong trend of increased cartilage glycosaminoglycan content.

BIOGRAPHICAL SKETCH

Ashlee Elane Watts was born on April 30, 1977 in southern California. Her family moved to Park City, Utah while she was in grade school. She graduated from Park City High School in 1995 and enrolled in college at Colorado State University in Ft. Collins, Colorado, where she majored in Animal Science. She attended veterinary school also at Colorado State University and graduated in 2003. As a veterinarian, Dr. Watts specialized in equine surgery through a 1 year internship at Pioneer Equine Hospital in Oakdale, California followed by a residency at Cornell University that was completed in 2007. To further her goal of an academic career she began pursuing a PhD in 2007 and was awarded a Cornell University Graduate Research Award, and a National Institutes of Health, Individual National Research Service Award. During this time, Dr. Watts completed that examination to be designated as a Diplomate of the American College of Veterinary Surgeons in 2008.

Dedicated to my parents, who have instilled in me the desire to excel, set high goals and achieve them and have always supported me when I needed it most; to my mentor, who is my greatest proponent and my harshest critic; and to my husband, who is my utmost supporter.

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ABSTRACT

New treatments for equine cartilage injury are being pursued because of the importance of joint disease to the athletic use of the horse, poor intrinsic healing of articular cartilage injury and limited options for ameliorating the sequelae of cartilage injury, osteoarthritis. Cell-based therapies are attractive for focal cartilage injury because they may be able to enhance chondral or osteochondral repair to that of native tissue, thereby stopping the progression to generalized osteoarthritis. Cell-based therapies, especially mesenchymal stem cells (MSCs), are attractive for the treatment of generalized osteoarthritis because they may produce trophic and mitogenic bioactive factors to stimulate endogenous repair and minimize inflammation. They may also contribute to repair directly through chondrogenic transformation and matrix production. Gene therapy might further enhance the chondrogenic, anti-inflammatory, mitogenic or trophic activities of MSCs. MSCs can be delivered by direct intra-articular injection or by surgical grafting of cells in a scaffold.

INTRODUCTION

Osteoarthritis (OA) is the most common joint malady in horses(Jeffcott et al. 1982; Rossdale et al. 1985; USDA 2000) and OA is often secondary to focal articular cartilage injury(Mankin 1982; Strauss et al. 2005). This is because articular cartilage is hypocellular, avascular and has poor intrinsic healing(Mankin 1982), especially when defects are partial thickness. In contrast to partial thickness injury, full thickness (down to subchondral bone) articular cartilage injury is repaired by MSCs that migrate to the defect largely from the exposed

subchondral bone(Campbell 1969; Shapiro et al. 1993). Despite filling of the defect with endogenous, marrow derived MSCs and production of a cartilage like matrix, the repair tissue never fully integrates to the surrounding cartilage and with continued use and shear stress, it will degenerate(Shapiro et al. 1993). Additionally, the repair tissue is biomechanically inferior because of increased collagen type I and reduced collagen type II and proteoglycan, which propagates degeneration(Howard et al. 1994). Continued degeneration of the defect tissue leads to generalized synovitis which causes dysregulation of tissue turnover in cartilage and bone, with subsequent widespread degeneration and loss of articular cartilage and subchondral bone and synovial fibrosis.

The end stage therapy for OA in humans is total joint arthroplasty and in the horse is surgical arthrodesis for selected joints. Although arthroplasty and arthrodesis can affect dramatic improvement in clinical signs, neither technique fully restores athletic potential. Even when intervention is sought early in the course of disease, athletic function is often only preserved in the short term. This is because surgical cartilage repair strategies do little to affect the long term outcome and injured joints will progress to OA(Hunziker 1999). Additionally, there are no effective disease modifying osteoarthritis drugs to stop OA progression(Hunter 2011; Qvist et al. 2008). Therefore, surgical cell based therapies(Brittberg et al. 1994) for the repair of articular cartilage and soft tissue injury are being pursued to augment articular healing and minimize development of OA(Brittberg et al. 1994; Hendrickson et al. 1994; Sams and Nixon 1995).

Similarities between horses and humans in articular cartilage thickness, heavy athletic use, importance of joint disease and reliance on arthroscopy and intra-articular medications for the treatment of joint disease, have allowed each species to benefit from discoveries made in the other. Consequently, the horse has been a useful preclinical model system for controlled studies

on articular cartilage repair and OA prevention(McIlwraith et al. 2010). This chapter will discuss cell based therapy for equine joint disease with equine evidence presented when available and human evidence presented when applicable.

CURRENT NON-CELL BASED CARTILAGE INJURY TREATMENT MODALITIES

Current non-cell based surgical treatments for articular cartilage injuries include debridement of partial thickness lesions and loosely attached cartilage and bone to expose normal subchondral bone. This minimizes the release of cartilage matrix fragments and cellular debris from diseased and loose cartilage that may propagate generalized synovitis and widespread cartilage degeneration(Thompson 1975). Debridement also exposes subchondral bone for the migration and adherence of endogenous mesenchymal stem cells from the bone marrow to the defect. Similarly, partial-thickness defects should be debrided to remove the calcified cartilage layer and any abnormal bone. In contrast, fibrillated cartilage is not manipulated and instead dictates a longer postoperative recuperation period.

Although an exogenous cell is not used in non-cell based surgical articular cartilage treatments, clearly, repair tissue is affected by local, endogenous progenitor cells that migrate to and engraft the defect. Specific local manipulative techniques, beyond debridement, have been developed to further enhance endogenous cell migration to the cartilage defect. Microfracture is the technique to make small perforations with a bone awl in the subchondral bone within the debrided area. These perforations serve to expose the defect to progenitors and growth factors present in bone marrow(Rodrigo et al. 1994; Steadman et al. 2001). Additionally, microfracture may enhance adhesion of endogenous progenitors to the subchondral bone(Lee et al. 2000). Forage(Insall 1967) and spongialization(Ficat et al. 1979) are similar but more drastic techniques

that are no longer commonly used aimed at exposing the defect to the marrow spaces to enhance progenitor and growth factor access to the defect.

Articular cartilage repair of large cartilage flaps in cases with osteochondritis dissecans (OCD) or acute traumatic joint injury by flap salvage and reattachment with bioabsorbable pins has been described.(Nixon et al. 2004) Although originally described without the use of cellular therapy, a recent paper reported the long term outcome on a broader selection of articular cartilage flap cases that were treated with flap pinning and cellular therapy (bone marrow aspirate concentrate) under the flap. (Sparks et al. 2011)

CELL BASED TECHNIQUES FOR CARTILAGE INJURY

Chondrocytes

Cell based therapy(Brittberg et al. 1994) for the repair of articular cartilage injury using autologous chondrocytes was first described in humans (Brittberg et al. 1994) and was reported in horses (allogeneic) in a fibrin matrix(Hendrickson et al. 1994) or a collagen scaffold(Sams and Nixon 1995) shortly thereafter. Due to the hypocellular nature of cartilage, harvesting of sufficient numbers of autologous chondrocytes for grafting is not possible and chondrocytes must be expanded in vitro. During expansion, chondrocytes de-differentiate to a more fibroblastic phenotype with reduced collagen type 2 and proteoglycan production. Experimentally, chondrocyte grafts have been implanted with added growth factors (IGF-1) (Fortier et al. 2002; Hidaka et al. 2003) and growth factor gene therapy (IGF-1 and BMP-7) (Goodrich et al. 2007; Hidaka et al. 2003) to direct re-differentiation of chondrocytes and enhance graft integration to native tissues and graft cell matrix production; however, the long term outcome has not been dramatically improved.

In clinical cases of medial femoral condylar cysts, repair with allogeneic chondrocyte grafts results in a positive long term outcome in horses(Ortved et al. 2011). In human articular cartilage injury, Autologous Chondrocyte Implantation (ACI) and Matrix-guided Autologous Chondrocyte Implantation (MACI), have become routine for the repair of focal articular cartilage injury. Unfortunately, ACI and MACI techniques do not consistently result in the formation of hyaline cartilage, rather than fibrocartilage, and have not been superior to other surgical interventions in systematic reviews(Hettrich et al. 2008; Wasiak et al. 2006).

It may be that de-differentiation during in vitro expansion and donor site morbidity(Matricali et al. 2010) contribute to the lack of significant long-term success of chondrocyte grafting. Allogeneic chondrocytes(Ortved et al. 2011) would negate donor site morbidity and may minimize the duration of in vitro expansion and therefore the de-differentiation but with added risk of immune rejection(Elves 1974; Hyc et al. 1997) and disease transmission. A promising new approach for cell therapy is using stem cells to engineer new cartilage.

Stem Cells

Stem cells are self-renewing, highly proliferative and capable of multi-lineage differentiation. The ultimate stem cell is made at conception. After fertilization, the zygote consists of totipotent stem cells that are able to form all 3 germ layers as well as placental tissue. After the zygote becomes a blastocyst, it consists of pluripotent stem cells (embryonic stem cells)(Thomson et al. 1998) within the inner cell mass that will give rise to the 3 germ layers; ectoderm, mesoderm and endoderm. During embryonic development, these cells will become either somatic cells that are terminally differentiated or stem cells committed to a specific

lineage. At this stage in development, the stem cells are considered multipotent and largely committed to a specific germ line. Local niches of lineage committed, multipotent stem cells remain in adult tissue throughout life for normal tissue remodeling and repair. Given their contribution to the development of musculoskeletal tissues in the body, their continued presence throughout life to generate tissue repair, and ability to expand to large cell numbers in vitro without de-differentiation like the chondrocyte, it is plausible that stem cells will be able to better affect tissue regeneration through tissue engineering techniques.

Because of ethical, political, religious and safety concerns and difficulty is isolation, embryonic stem cells are generally not considered in the treatment of non-life threatening diseases, have not been investigated extensively in the horse and will not be discussed for cartilage repair or joint therapy. In contrast, adult derived stem cells (non-embryonic) have minimal ethical, political, and religious concerns, are generally considered to be safe with little risk of tumor formation and allow the use autologous cells with little risk for disease transmission(Raghunath et al. 2005). First described for their tissue culture plastic adherence and ability to form colonies during the culture period(Friedenstein et al. 1970), adult, mesenchymal stem cells (MSCs) are considered an excellent stem cell source for autologous cartilage repair. This is because there is good proliferation and chondrogenic differentiation potential of a mesodermal lineage committed cell.(Pittenger et al. 1999; Johnstone et al. 1998; Mackay et al. 1998)

The characterization of MSCs is controversial and cell-surface markers on MSCs have broad overlap with other cell populations(Raghunath et al. 2005). Although the International Society for Cellular Therapy has a recommendation for cell-surface antigens on human MSCs, a consensus has not been reached for the horse(Dominici et al. 2006). Cell-surface marker

identification of MSCs is further complicated by variation in cell-surface marker expression that occurs during in vitro culture and expansion(Raghunath et al. 2005). Recently, one group suggested that equine bone marrow derived MSCs should express CDE29, CD44 and CD90 and lack expression of CD14, CD79 and MHC-II(De Schauwer et al. 2011); however, another group suggested that the equine MSC was a descendent of a CD14 positive cell(Hackett et al. 2011). Because of the difficulty in defining the MSC by cell surface markers, many researchers define the MSC as the tissue culture plastic adherent population of colony forming cells that have multilineage differentiation potential(Chen and Tuan 2008; Dominici et al. 2006; Jones et al. 2002; Kolf et al. 2007). In the horse, MSCs have been isolated from bone marrow(Fortier et al. 1998; Worster et al. 2000), adipose(da Silva Meirelles et al. 2009; Kisiday et al. 2008), tendon(Stewart et al. 2009), muscle(Stewart et al. 2009), umbilical cord blood and tissue(De Schauwer et al. 2011; Koch et al. 2007; Toupadakis et al. 2010), gingiva and periodontal ligament(Mensing et al. 2011), amniotic fluid(Park et al. 2011), and blood(Giovannini et al. 2007). The different tissue sources vary in the ease of harvest, expansion potential and differentiation capacity. To date, bone marrow derived MSCs from both the horse and human have been the most thoroughly studied and have the most evidence for ability to undergo chondrogenesis and contribute to cartilage repair as well as modulate inflammation within the joint.

CELL BASED TECHNIQUES FOR JOINT INJURY

MSCs - Cartilage Repair

Initial enthusiasm for the MSC's ability to impart hyaline repair to grafted cartilage defects was shown in small animal models. In 6 mm full-thickness femoral cartilage defects in rabbits, MSCs grafted in collagen type I scaffolds, resulted in hyaline-like tissue at 24 weeks

compared to controls(Wakitani et al. 1994). Subsequently, MSCs have been utilized for cartilage tissue regeneration in horses indirectly through the application of microfracture followed by intra-articular injection(McIlwraith et al. 2011) and directly with the arthroscopic application to focal cartilage defects of concentrated MSC bone marrow grafts(Fortier et al. 2010) and culture expanded MSC grafts(Kuroda et al. 2007; Matsumoto et al. 2010; Wakitani et al. 2011; Wilke et al. 2007). However, several authors consider that MSCs are inferior to chondrocytes for cartilage defect repair because they are unable to attain the chondrocyte phenotype following implantation.(De Bari et al. 2004; Wilke et al. 2007) Anecdotal reports of improved lameness resolution of return to performance following intra-articular application of MSCs to the equine joint(Ferris et al. 2009) suggest that MSCs may be capable of more than simply cartilage replacement therapy.

MSC – Immunomodulation and Anti-inflammatory Properties

There has been intense interest in the use of MSCs in regenerative medicine. The ease of isolation and expansion, ability to use autologous cells, immune tolerance of allogeneic cells and multilineage differentiation capacity of MSCs were the driving forces behind this interest. Despite tissue specific differentiation in vivo and positive outcomes after MSC therapy for several disease models(Prockop et al. 2010), MSC engraftment at the site of injury is a rare event and did not appear to result in significant long-term engraftment. However a treatment effect was documented and therefore, successful MSC engraftment is not required for a therapeutic effect(Iso et al. 2007; Caplan and Dennis 2006; Iso et al. 2007; Prockop et al. 2010). This led to the notion that mechanisms other than engraftment with tissue specific differentiation were affecting positive treatment outcomes.

Recently, the MSCs role in immunomodulation has been evolving into greater promise over tissue engineering. Numerous studies have documented that MSCs modulate the adaptive immune response through active regulation of T lymphocytes (T-cells)(Sato et al. 2007; Tse et al. 2003). Not only do MSCs regulate T-cell activation, but they secrete T-cell chemoattractants followed by T-cell suppression(Ren et al. 2008). Additionally, MSCs lack expression of MHC class II and most of the classical co-stimulatory molecules usually present on antigen presenting cells(Tse et al. 2003). The lack of expression of T-cell costimulatory molecules may contribute to the observed immune tolerance that MSCs are supposed to possess(Majumdar et al. 2003). In addition to T-cell activation, MSCs have immunomodulatory effects on dendritic cell maturation and B-cell proliferation(Yi and Song 2012). Broader immune modulating properties include inhibition of IFN-ɣ and TNF-α production and increased production of IL-10(Caplan 2009) and inhibition of natural killer cell proliferation and activity through production of PGE_2(Uccelli et al. 2008). Subsequently, there are several clinical trials investigating MSC therapy in the treatment of steroid unresponsive graft versus host disease(Lim et al. 2010; Ringden et al. 2006). Immunomodulation by MSCs may also be relevant to joint disease and OA progression.

MSCs - Joint Disease

Mesenchymal stem cells may be useful where generalized joint treatment rather than direct implantation to focal articular cartilage defects is required(Chen and Tuan 2008). In OA, all tissues within the joint are affected and inflammation and tissue catabolism is up-regulated while tissue anabolism is down-regulated. Given their trophic, mitotic, immunomodulatory(Nauta and Fibbe 2007), and anti-inflammatory(Nemeth et al. 2009)(Nauta and Fibbe 2007) effects, chondrogenic potential(Mackay et al. 1998), and ability to engraft to the site of injury(Sordi 2009; Yagi et al. 2010), intra-articular injection of MSCs may be useful for

OA therapy. In the inflamed joint, MSCs inhibit activation of T lymphocytes and secretion of inflammatory cytokines while concurrently stimulating secretion of anti-inflammatory interleukins (Gonzalez-Rey et al. 2010; Zheng et al. 2008). MSCs also secrete tissue inhibitors of metalloproteinases, the natural inhibitors of matrix metalloproteases(Lozito and Tuan 2011) that are largely responsible for increased cartilage matrix degradation. Indeed, MSCs are exquisitely responsive to their micro-environment via cell to cell contacts(Prockop 2009) and contact-independent mechanisms(Groh et al. 2005) and are expected to behave in accordance to the environment in which they are placed. In this manner, MSCs would respond appropriately to the degree of OA and synovitis and modulate the local articular environment in favor of reduced inflammation, reduced apoptosis and enhanced matrix synthesis of endogenous progenitors and tissue specific cells.

Intra-articular injection of MSCs has been investigated in animal models of OA with promising results across several different research groups where injected MSCs reduced OA progression and improved healing of soft tissue articular injury.(Agung et al. 2006; Frisbie et al. 2009; Horie et al. 2009; Lee et al. 2007; McIlwraith et al. 2011; Mokbel et al. 2011; Murphy et al. 2003; Sato et al. 2012; Toghraie et al. 2011) In a rabbit joint destabilization model, $1x10^6$ allogeneic adipose derived MSCs (infrapatellar fat pad) were injected 12 weeks after OA induction. Eight weeks later, there was reduced cartilage degeneration, osteophyte formation, and subchondral bone sclerosis in the MSC treated joints. In a model of spontaneous OA in the guinea pig, intra-articular injection of $7x10^6$ commercially available human MSCs resulted in improved cartilage repair with increased collagen type II content(Sato et al. 2012). Positive clinical results in veterinary (Black et al. 2007; Black et al. 2008; Ferris et al. 2009) and human patients(Centeno et al. 2008; Centeno et al. 2011; Davatchi et al. 2011) provide additional

evidence for the value of direct, intra-articular injection of MSCs for the treatment of OA. This has led to phase II clinical trials utilizing allogenic cultured MSCs for the treatment of human knee OA (NCT01448434).

Despite the evidence for reduced articular injury in OA models(Agung et al. 2006; Frisbie et al. 2009; Horie et al. 2009; Lee et al. 2007; McIlwraith et al. 2011; Mokbel et al. 2011; Murphy et al. 2003; Toghraie et al. 2011) and reduced clinical symptoms in veterinary(Black et al. 2008; Black et al. 2007; Ferris et al. 2009) and human patients, (Centeno et al. 2008; Centeno et al. 2011; Davatchi et al. 2011) there is conflicting information regarding the ability of the MSC to engraft to abnormal cartilage surfaces. Two studies suggest MSCs can populate injured articular cartilage,(Agung et al. 2006; Mokbel et al. 2011) while other studies indicate MSCs do not embed to injured articular cartilage.(Jing et al. 2008; Murphy et al. 2003) However, there is good evidence to suggest that MSCs injected to the joint engraft efficiently to and improve healing of intra-articular soft tissues including meniscus(Agung et al. 2006; Izuta et al. 2005; Murphy et al. 2003). Full characterization of tissue specific engraftment and long term survival of MSCs following direct intra-articular injection to the joint are indicated.

Equine Bone Marrow Collection and MSC Isolation

Bone marrow aspirates were originally used directly for equine musculoskeletal applications(Herthel 2001). However, only 0.001–0.01% of the total nucleated cells in bone marrow aspirates are MSCs(Jones et al. 2002), making expansion in culture necessary to get numbers suitable for clinical applications. This has led to interest in creating a bone marrow aspirate concentrate (BMAC) for stall side enhanced MSC concentration above that of raw marrow(Fortier et al. 2010; Owens et al. 2011). Similarly, several research groups have

investigated cell surface marker and sorting techniques to enrich for the stem cell portion of the nucleated cell population of raw bone marrow.

The sternum is an often used and reliable site for bone marrow aspiration in the horse(Russell et al. 1994) and for the collection of mesenchymal stem cells(Fortier et al. 1998; Taylor and Clegg 2011). Although thoracic puncture leading to pneumopericardium in a 10 year old Warmblood(Berggren 1981; Durando et al. 2006) and acute death due to laceration of the left ventricle leading to cardiac tamponade in a 6-year-old Quarter Horse gelding(Jacobs et al. 1983) has been reported following BM aspiration, sternal bone marrow aspiration is safe and easy to perform. The fifth sternebra is the most often used and likely is the safest as it has the greatest dorsoventral depth and is cranial to the heart apex. The tuber coxae of the ilium is also a commonly used site for bone marrow collection from the horse. On investigative group compared bone marrow collection from tuber coxae of the ilium and sternum, finding no differences in the concentration of stem cells nor in their growth potential from either site(Adams et al. 2011).

Although bone marrow collection can be performed while the horse is anesthetized, local anesthesia and light sedation are most often used for bone marrow collection. Bone marrow biopsy needles (Jamshidi, VWR Scientific, Bridgeport, NJ) are used to aspirate bone marrow into 60-mL syringes containing heparin for a final concentration of 1,000 units/ml (APP Pharmaceuticals, LLC; Schaumburg, IL 60173). Each 60 ml is collected from a separate site with advancement of the Jamshidi needle after each 15 ml of marrow had been drawn. It has recently been described that the highest concentration of MSCs reside within the first 5 ml of bone marrow aspirate(Kasashima et al. 2011). Therefore, periodic advancement of the bone marrow biopsy needle to a new trabecular region during bone marrow aspiration is important to

maximize the concentration of MSCs per ml of marrow drawn. Because the density and expansion potential of MSCs decreases with age(Singer and Caplan 2011) it would be especially important to advance the needle often to maximize the concentration of collected cells in older horses. Similarly, when utilizing bone marrow for BMAC, one would want to optimize the concentration of stem cells during bone marrow collection. Bone marrow aspirate can then be processed by centrifugal gradient purification to enhance nucleated cell portion of the bone marrow aspirate or plated directly to tissue culture flasks.

For centrifugal gradient purification, bone marrow aspirate is diluted 1:3 using phosphate buffered saline with 0.5% bovine serum albumin and layered 2:1 onto Ficoll-Paque Plus (Amersham Biosciences, Pistcataway, NJ) for enrichment of the nucleated cells by centrifugation for 30 minutes. Bone marrow or the nucleated cells from centrifugation are then isolated through selective tissue culture plastic adherence. Briefly, 30 ml of is plated to 175 cm^2 flasks, 1:1 in growth medium (Dulbecco's modified Eagles' media; 1,000 mg/L glucose; 2 mM L-glutamine; 100 units/ml penicillin-streptomycin; 1 ng/ml bFGF; 10% fetal calf serum). Addition of bFGF to the isolation and growth medium has become a routine part of MSC isolation because of maintained differentiation capability and increased divisions prior to cellular senescence(Stewart et al. 2007). Tissue culture flasks are maintained at 37°C, 5% CO_2, and 95% humidity in room air. Non-adherent cells are removed through daily feeding. Once colony formation is evident, adherent cells are passaged and replated at 10-12,000 cells/cm^2, and fed every other day.

GROWTH FACTORS

Addition of growth factors to cell cultures and application to joints has been used to enhance chondrogenic differentiation or re-differentiation. Since methods for in vitro chondrogenesis of MSCs were first described(Johnstone et al. 1998) TGF-β has been the most

commonly utilized growth factor for MSC chondrogenic induction(Puetzer et al. 2010)(Johnstone et al. 1998; Puetzer et al. 2010). Several in vitro studies have demonstrated that transduction with TGF-β significantly increases the MSC proliferation and synthesis of hyaline cartilage (proteoglycans and type II collagen) in species other than the horse(Caplan 2000; Gafni et al. 2004; Guo et al. 2006a; Mason et al. 2000; Trippel et al. 2004). In vivo and clinical studies have also demonstrated the utility of TGF-β gene transduction for chondrogenic induction and chondrogenic maintenance of MSCs and chondrocytes(Ha et al. 2012). In full thickness articular cartilage defects in rabbits, MSCs (Guo et al. 2006b) or chondrocytes(Song et al. 2005) with TGF-β1 transgene expression resulted in improved synthesis of hyaline cartilage, improved reconstitution of the subchondral bone and inhibited inflammatory immune responses. In a human safety study, osteoarthritic knees were injected with allogeneic chondrocytes expressing a TGF-β1 transgene over a 2 week period. No serious adverse events were noted, patients had improved symptoms and evidence of increased cartilage matrix thickness at cell higher doses(Ha et al. 2012).

In addition to being chondrogenic, intra-articular TGF-β has anti-inflammatory properties(Harvey et al. 1991; Hui et al. 2003; Redini et al. 1993; Roberts and Sporn 1993), immune modulating properties(Blumenfeld and Livne 1999), stimulates chondrocyte matrix production, inhibits chondrocyte terminal differentiation and mineralization(van Beuningen et al. 1994a; van Beuningen et al. 1994b; van Osch et al. 1998) and reduces the height of the cartilage hypertrophic zone(Itayem et al. 1997). These additional properties make TGF-β a favorable growth factor to augment the MSC autocrine and paracrine functions within the joint.

Recently, the intra-articular application of TGF-β has been criticized for intra-articular use(Fortier et al. 2011) because of its role in wound healing as a fibrotic agent(Branton and Kopp

1999). Additionally, intra-articular injection of TGF-β1 induced synovial fibrosis and osteophyte formation in a mouse OA model(van Beuningen et al. 1994b). It is important to note that there are marked isoform specific differences among the TGF-β peptides. TGF-β1 and -β2 are associated with fibrosis and scarring whereas TGF-β3 results in reduced scarring(Shah et al. 1995).

In addition to TGF-β, other growth factors have been investigated for articular therapy including IGF-1, BMPs, FGFs, and EGF.(Steinert et al. 2007; Steinert et al. 2008) Recombinant growth factor therapy in bone repair has become routine for human clinical patients with fracture delayed-unions, non-unions, segmental bone defects and vertebral fusions. Despite the beneficial effects of articular growth factor therapy, widespread clinical application of TGF-β or other growth factors to the joint for cartilage repair and osteoarthritis therapy has been limited by the short half-life and short term effects of recombinant proteins(Ha et al. 2012) and need for repeated injections(Hardingham et al. 1992). This is in contrast to bone healing, where transient growth factor influence may be sufficient. In vivo, gene therapy would allow MSCs would continue to express the transgene, increasing the duration of growth factor (TGF-β) exposure compared to injection of recombinant protein. In the joint, the transgene growth factor (TGF-β) would have both autocrine and paracrine functions. Through autocrine action, TGF-β would induce chondrogenesis of implanted MSCs. Through paracrine action, TGF-β would affect endogenous progenitors, synovial membrane and chondrocytes. Gene therapy (direct and cell mediated) for growth factor delivery in bone healing has been extensively studied in animals including the horse(Ishihara and Bertone 2012) demonstrating efficacy and short term safety. Similar studies should be performed for intra-articular growth factor therapy in the horse.

GENE THERAPY

Use of a corrective gene(s) may improve upon unsatisfactory current treatment modalities for many diseases. Unlike many other areas in medicine where transfer of therapeutic genes may be useful, gene transfer techniques to facilitate musculoskeletal tissue repair may only require transient, localized expression of a specific transgene product. Success of gene therapy techniques has already been achieved by gene transfer to augment bone healing(Baltzer and Lieberman 2004; Ishihara and Bertone 2012). The application of gene transfer to articular tissues was pioneered by Evans and co-workers, as a means to treat rheumatoid arthritis(Evans et al. 1996; Evans et al. 2005). Initial retroviral gene delivery in animal models led to a human clinical trial to evaluate the safety and feasibility of using gene therapy for rheumatoid arthritis(Evans et al. 1996; Evans et al. 2005; Ghivizzani et al. 1998). The study was completed without incident; the procedure was well tolerated, and intra-articular gene transfer and expression was observed in all joints(Evans et al. 1996; Evans et al. 2005). Despite the progress in gene therapy, the repair of focal articular cartilage defects and control of OA progression with gene therapy techniques has not yet been achieved.

Gene therapy techniques are preferable to peptide depots or repeated injection for growth factor delivery as endogenous growth factor production by gene therapy improves efficiency. This is because the peptide will undergo appropriate post-translational modification by the producing host cell and will be produced continuously for a given duration(Evans et al. 1999). Many methods for gene transfer in orthopedics are available. For satisfactory transduction efficiency and effective protein expression, the best-described gene therapy procedures involve viral vectors such as retrovirus, adeno-associated virus, adenovirus, herpes simplex virus, simian virus 40 (SV40), and papilloma virus(Evans and Robbins 1994). Non-viral methods, such as electroporation, microinjection, protoplast fusion, or the application of liposomes, Polybrene, or

calcium phosphate, have also been described in other tissues, although none have achieved significant success in joint therapy(Evans and Robbins 1994). Of the virally mediated gene therapy techniques, there is great variability in the genomic integration ability and subsequently the duration of gene over-expression. It is unknown whether a long duration of transgene expression or permanent transgene expression would be required to enhance and maintain MSC chondrogenesis and control inflammation in joint disease.

Continued exposure to growth factors post-implantation through gene therapy induced (Ha et al. 2012)expression has been successful for cartilage defect repair with improved hyaline-like tissue in chondrocyte grafted defects(Goodrich et al. 2007; Hidaka et al. 2003). For chondrogenic induction of MSCs, transforming growth factor beta (TGF-β1, -β2 or -β3) supplementation is used routinely in vitro(Mackay et al. 1998; Tuan 2004) and it has been suggested that continued supplementation of TGF-β may improve the quality of repair tissue following MSC implantation to cartilage defects(Ha et al. 2012).

CONCLUSION

Cell-based therapies are attractive for focal cartilage injury and osteoarthritis prevention. The autologous bone marrow derived MSC is of mesodermal lineage and has trophic (inhibits scar formation, inhibits apoptosis, increases angiogenesis and stimulates endogenous progenitors), mitogenic, anti-inflammatory and immunomodulatory properties. Thus the MSC serves as an ideal cell source for regenerative therapy and disease modifying osteoarthritis therapy. Because the MSC is so responsive to its environment, direct intra-articular injection may be all that is needed to direct their action for OA therapy. If additional manipulation or augmentation of intrinsic properties is required, the MSC might be an excellent vehicle for gene

therapy. The MSC can be delivered by direct intra-articular injection which is preferable to surgical grafting of cells due to reduced invasiveness, cost and complications.

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Distribution of stem cells after intra-articular injection to normal and osteoarthritic joints

Abstract/Summary

Objective: To provide tissue distribution of autologous bone marrow derived mesenchymal stem cells (MSC) after intra-articular injection to normal joints and joints that had developed spontaneous osteoarthritis (OA).

Methods: Twenty nine metacarpophalangeal or femoropatellar joints from 10 horses were characterized as normal or osteoarthritic based upon clinical and radiographic examination. Autologous bone marrow derived MSCs were isolated through tissue culture plastic adherence. Passage 2 MSCs were labeled for tracking using fluorescent quantum dots and injected into the joints. Tissues were retrieved 1 week after MSC injection and the synovial membrane and cartilage sectioned and examined under fluorescent microscopy.

Results: Twenty-nine joints were characterized, with 17 having OA and 12 normal. Tissue sections of synovial membrane were positive for labeled MSCs significantly more often than cartilage sections. When sections were positive, there was a greater number of labeled cells present per field on synovial membrane versus cartilage sections. The proportion of positive sections from synovium and cartilage was no different between OA and normal joints.

Conclusions: Injected MSCs engrafted the synovial membrane more efficiently than cartilage. Moreover, MSCs did not engraft areas of cartilage injury more often than normal cartilage. These data indicate intra-articular injection of MSCs may not be an effective direct treatment for resurfacing articular cartilage injury, and would rely on other indirect benefits to improve joint function.

Introduction

Osteoarthritis (OA) is the most common musculoskeletal disease in man and is expected to be the fourth leading cause of disability by the year 2020.(Woolf and Pfleger 2003) Current treatment is symptomatic, until end-stage joint disease necessitates total joint arthroplasty.(Wieland et al. 2005) In contrast, successful regenerative techniqueshave been developed to improve the repair of focal articular cartilage injury and prevent OA, including mosaicplasty(Matsusue et al. 1993) and autologous chondrocyte implantation.(Brittberg et al. 1994) Unlike acute articular cartilage injury, OA often affects cartilage throughout the joint, and extends to include the synovial membrane and subchondral bone. Generalized joint disease generally precludes focal autologous therapy options(Knutsen et al. 2004)and most currently available regenerative therapies. A significant unmet need exists to develop a regenerative method applicable to generalized OA in man. Stem cell therapy may provide some of the elements to assist in OA control.

Friedenstein(Friedenstein et al. 1970) was the first to describe colony producing fibroblastic cells derived from bone marrow that are now most commonly referred to as mesenchymal (stromal) stem cells (MSCs). Mesenchymal stem cells may be an ideal cell choice for treating joint disease, as the cells are appropriately lineage committed to tissues of the joint. Differentiation studies have confirmed the ability of MSCs to undergo chondrogenesis in vitro(Johnstone et al. 1998; Mackay et al. 1998; Pittenger et al. 1999), and animal studies have confirmed their chondrogenic potential in vivo.(Wakitani et al. 1994; Yan and Yu 2007)Subsequently, MSCs have been utilized for joint tissue regeneration indirectly through the application of microfracture,(Rodrigo et al. 1994; Steadman et al. 2001) and directly with the

arthroscopic application of culture expanded MSC grafts to focal chondral defects.(Kuroda et al. 2007; Matsumoto et al. 2010; Wakitani et al. 2011; Wilke et al. 2007)

Mesenchymal stem cells may also be useful in the treatment of OA, where generalized joint treatment rather than direct implantation to focal articular cartilage defects is required(Chen and Tuan 2008). Given their trophic, mitotic, immunomodulatory(Nauta and Fibbe 2007), and anti-inflammatory(Nemeth et al. 2009)(Nauta and Fibbe 2007) effects, chondrogenic potential(Mackay et al. 1998), and ability to engraft to the site of injury(Sordi 2009; Yagi et al. 2010), intra-articular injection of MSCs has been investigated in several animal models of OA. In these models, intra-articular injection of MSCs reduced OA progression and improved healing of soft tissue articular injury.(Agung et al. 2006; Frisbie et al. 2009; Horie et al. 2009; Lee et al. 2007; McIlwraith et al. 2011; Mokbel et al. 2011; Murphy et al. 2003; Toghraie et al. 2011) Positive clinical results in veterinary (Black et al. 2007; Black et al. 2008; Ferris et al. 2009) and human patients(Centeno et al. 2008; Centeno et al. 2011; Davatchi et al. 2011) provide additional evidence for the value of direct intra-articular injection of MSCs for the treatment of OA. This has led to phase II clinical trials utilizing allogenic cultured MSCs for the treatment of knee OA (NCT01448434). Despite the evidence for reduced articular injury in OA models(Agung et al. 2006; Frisbie et al. 2009; Horie et al. 2009; Lee et al. 2007; McIlwraith et al. 2011; Mokbel et al. 2011; Murphy et al. 2003; Toghraie et al. 2011) and reduced clinical symptoms in veterinary(Black et al. 2008; Black et al. 2007; Ferris et al. 2009) and human patients, (Centeno et al. 2008; Centeno et al. 2011; Davatchi et al. 2011) there is little information regarding the ability of the MSC to engraft to abnormal cartilage surfaces. Two studies suggest MSCs can populate injured articular cartilage,(Agung et al. 2006; Mokbel et al.

2011) while other studies indicate MSCs do not embed to injured articular cartilage.(Jing et al. 2008; Murphy et al. 2003)

The purpose of this study was to investigate the distribution of autologous bone marrow derived culture expanded MSCs following intra-articular injection of normal joints and joints with naturally developed osteoarthritis. The horse was selected as a model because spontaneous and traumatic OA in the equine athlete are similar to human OA.(McIlwraith et al. 2010) Additionally, there is published evidence for the efficacy of intra-articular MSC therapy in clinical equine joint disease,(Frisbie et al. 2007) similar to that reported for human OA patients.(Centeno et al. 2008; Centeno et al. 2011; Davatchi et al. 2011)(Centeno et al. 2008; Centeno et al. 2008; Centeno et al. 2011)Tracking studies utilized OA and normal joints from 10 equine athletes and 6 horses with normal joints were used for control injections to characterize synovial reaction to the vehicle for cell injection. We hypothesized that MSCs would engraft to abnormal articular cartilage surfaces in OA joints to a greater extent than to the articular cartilage of normal joints.

Materials & Methods

Study Outline

Bone marrow was harvested from the sternebrae and mesenchymal stem cells (MSCs) were isolated and expanded from 16 skeletally mature horses that had been retired from athletic performance and donated for lameness due to osteoarthritis of a metacarpophalangeal and/or femoropatellar joint. For 10 horses, autologous passage 2 adherent MSCs were labeled with fluorescent quantum dots (Qtracker®, Invitrogen), and injected to OA or normal metacarpophalangeal and/or femoropatellar joints (MSC injection). When OA was present in the

metacarpophalangeal and femoropatellar joint of the horse,both joints were utilized. Metacarpophalangeal and femoropatellar joints that were identified on radiographs to be normal in many of the horses, were included as non OA joints. Metacarpophalangeal and femoropatellar joints from these 10 horses not used for Qdot labeling were injected with unlabeled MSCs to compare the synovial reaction to Qdot-laden and naïve MSCs. One week later, joints were collected for synovial fluid cytology, characterization of MSC distribution via fluorescent microscopy, and characterization of articular cartilage injury via routine microscopy.

The remaining 6 horses (control injections) were injected with cell suspension medium (MEM) only, without cells, to a metacarpophalangeal and a femoropatellar joint, and synovial fluid assessed at 1 week after joint injection. This study was approved by the university's animal care and use committee.

OA Characterization

The presence and severity of metacarpophalangeal and femoropatellar osteoarthritis was characterized as mild, moderate, severe, or no joint disease (normal) in each horse via lameness examination and radiographic assessment. For lameness examination, horses were observed at the walk and trot before and after joint flexion and given a lameness score.(American Association of Equine Practitioners 1991) For radiographic examination, orthogonal radiographic projections of affected joints were assessed for the presence and severity of osteophytes, joint space narrowing and subchondral lysis or sclerosis. If the joint was radiographically abnormal (OA) and osteophytosis was the sole radiographic finding, the OA score was modified based upon lameness score where a lameness of 0-1/5 was considered mild OA and a lameness score of 2/5 was considered moderate OA. If the joint was radiographically

abnormal with findings in addition to osteophytosis, such as joint space narrowing and subchondral lysis, the joint automatically received an OA score of severe, regardless of lameness score.

During post mortem examination, appropriate ante mortem OA characterization was confirmed with gross examination of articular structures and histologic examination of osteochondral sections. Joints with full thickness cartilage loss and exposed subchondral bone were assigned to severe OA, if not already in this category.

Stem Cell Isolation and Labeling

Bone marrow aspirates were obtained from the sternum of each horse.(Fortier et al. 1998) Local anesthesia and light sedation was used for bone marrow collection. Bone marrow biopsy needles (Jamshidi, VWR Scientific, Bridgeport, NJ) were used to aspirate bone marrow into four 60-mL syringes containing heparin (10,000 units/ml; APP Pharmaceuticals, LLC; Schaumburg, IL 60173), for a final concentration of 1,000 units/ml. Each 60 ml was collected from a separate site with advancement of the Jamshidi needle after each 15 ml of marrow had been drawn. Bone marrow aspirate was diluted 1:1 in growth medium (Dulbecco's modified Eagles' media; 1,000 mg/L glucose; 2 mM L-glutamine; 100 units/ml penicillin-streptomycin; 1 ng/ml bFGF; 10% stem cell tested fetal calf serum) and 60 ml was plated to T-175 tissue culture flasks each. Non-adherent cells were removed through daily feeding. Once colony formation was evident, adherent cells were passaged using trypsin and replated at 20,000 cells/cm^2, and fed every other day. Monolayer cultures were passaged a second time when plates were 80-90% confluent. When passage 2 cultures were 80-90% confluent, adherent MSCs were labeled and prepared for joint injection.

Cell labeling with fluorescent quantum dots was performed as per manufacturer instructions (Qtracker®, Invitrogen). Quantum dots fluorescing at 625 mm (red) and 525 mm (green) were used. Based upon preliminary studies in our laboratory (data not shown), 10 nM labeling solution was used for 1 hour at 37°C. Briefly, Reagent A and Reagent B (Qtracker kit) were mixed in a 50 ml conical tube and incubated at room temperature for 5 minutes. Growth medium was used to dilute the Reagent A & B mix at 100:1 followed by a 30 second vortex to complete the labeling solution. Medium was aspirated from the monolayer culture and 3 ml of the 10 nM labeling solution was added to culture flasks containing approximately $7.5x10^6$ adherent MSCs for 1 hour at 37°C. Labeling solution was aspirated and labeled adherent cells were washed twice with growth medium and then prepared for joint injection.

Joint Injection

Labeled cells were collected by trypsinization, washed once in phosphate buffered saline and washed twice in Modified Eagles' Medium (MEM). A portion of unlabeled MSCs were also collected for control cell injections (MSC injected). Unlabeled MSC injections were injected into the contralateral joint to the labeled MSC injected articulation. Cells were prepared as $3x10^6$ and $5x10^6$ labeled MSC aliquots for the metacarpophalangeal and femoropatellar joint, respectively. Cells were diluted in MEM ($1x10^6$ cells/ml) containing 40 µg/ml gentamicin immediately prior to injection. When horses were injected bilaterally with labeled MSCs, different wavelength quantum dots were used in each joint (i.e. right metacarpophalangeal joint received 625 nm quantum dots and left metacarpophalangeal joint received 525 nm quantum dots). Horses in the control group (not receiving MSCs in any joint) were injected in the metacarpophalangeal and femoropatellar joints with a similar volume of cell free MEM and gentamicin.

Horses were sedated for joint injection. Injection to the metacarpophalangeal joint was made through the lateral collateral sesamoidean ligament and to the femoropatellar joint between the middle and medial patellar ligaments. Following injection, the limb was taken through passive range of motion for 20 joint flexion and extensions to distribute cells. Horses were administered a non-steroidal anti-inflammatory drug at the time of joint injection (phenylbutazone; 4.4 mg/kg, IV) and once daily for two days after joint injection (phenylbutazone; 2.2 mg/kg, PO). Metacarpophalangeal joints were maintained under a clean bandage for 3 days after joint injection. Femoropatellar joints were not bandaged. Horses were housed individually in box stalls without forced exercise until euthanasia with an overdose of pentobarbital, 1 week after joint injection. Control cases were not euthanized and had synovial fluid collected 1 week after joint injection.

Reaction to Injection

Effusion after injection and lameness in the injected limbs were re-assessed on day 1 and 7. Effusion was rated as similar or increased. Lameness was scaled using the same 5-level lameness descriptor applied pre-injection.

Joint Tissue Processing & Analysis

Synovial fluid was collected from injected joints and examined by a board certified veterinary pathologist. Cell counts and cell differentials were obtained. Synovial fluid cytospins were examined under fluorescent miscroscopy for the presence of fluorescent labeled cells (y/n) using filter sets for visualization of both 525 nm and 625 nm quantum dots. Joints were opened, gross articular cartilage lesions were mapped and articular surfaces were digitally photographed. Samples of articular cartilage and synovial membrane from different regions of the joint (Table

2.1) were collected and prepared for frozen sectioning by embedding in OCT medium. Osteochondral samples were collected (Table 2.1), fixed in 4% paraformaldehyde, decalcified in 10% EDTA, and embedded in paraffin. Presence (y/n) of a gross lesion on collected cartilage and osteochondral sections was recorded. If a gross cartilage lesion was not collected, given the sampling areas outlined in Table 1, it was collected in addition to the planned collection sites. Frozen sections were cover slipped with a nuclear stain (propidium iodide for 525 cell label or Hoescht for 625 cell label) and examined for the presence of fluorescent labeled cells (y/n) using both a 525 filter set and a 625 filter set. Osteochondral sections were stained with Hematoxylin and Eosin, examined and digitally photographed (50 x magnification).

Table 2.1. Joint tissues and site collected for fluorescent microscopy (cartilage and synovial membrane) and osteoarthritis characterization (osteochondral).

Table 2.1. Joint tissues collected for fluorescent microscopy and osteoarthritis characterization.			
	Cartilage	Synovial Membrane	Osteochondral
Femoropatellar joint	Lateral trochlear ridge Medial trochlear ridge Distal patella	Lateral cul-de-sac Medial cul-de-sac Proximal pouch	Lateral trochlear ridge Medial trochlear ridge
Metacarpo-phalangeal joint	Sagittal ridge of distal metacarpus Dorsomedial proximal phalanx Palmaromedial distal metacarpal condyle Medial sesamoid	Palmarolateral Palmaromedial Dorsomedial Dorsolateral	Palmaromedial distal metacarpal condyle Dorsomedial proximal phalanx

Statistical Analyses

Comparisons in cell distribution and synovial cytology were made between joints injected with labeled MSCs and unlabeled MSCs, and between OA joints and normal joints. Comparison between MSC injected joints and MEM injected joints was only made for synovial fluid cytology. Synovial fluid cytology parameters were reported as a median and a 95% confidence interval. Synovial fluid cytology parameters were tested for differences with Wilcoxon's Rank Sum test. The number of sections positive for labeled cells was reported as a percentage of all sections evaluated. Synovial membrane and cartilage sections were tested for differences in the proportion of tissue sections that were positive for labeled MSCs with Fisher's Exact test. Statistical analysis was performed using commercially available software (Statistix 9). For all tests a $p \leq 0.05$ was considered significant.

Results

Animals

Horses ranged in age from 3-7 years of age. There were 11 females and 6 males. Horses were Thoroughbreds (14) or Thoroughbred crosses (2).

Joint Characterization

Twenty-two joints (11 normal, 2 mild, 4 moderate, 5 severe OA) were injected with labeled MSCs and 7 joints (6 normal and 1 severe OA) were injected with unlabeled MSCs (Fig 1). Twelve joints were injected with MEM only (6 normal, 3 mild, 2 moderate, 1 severe OA). No joints were reassigned to a different disease category following post mortem examination and gross and histological OA grades were appropriate (Fig 2.1).

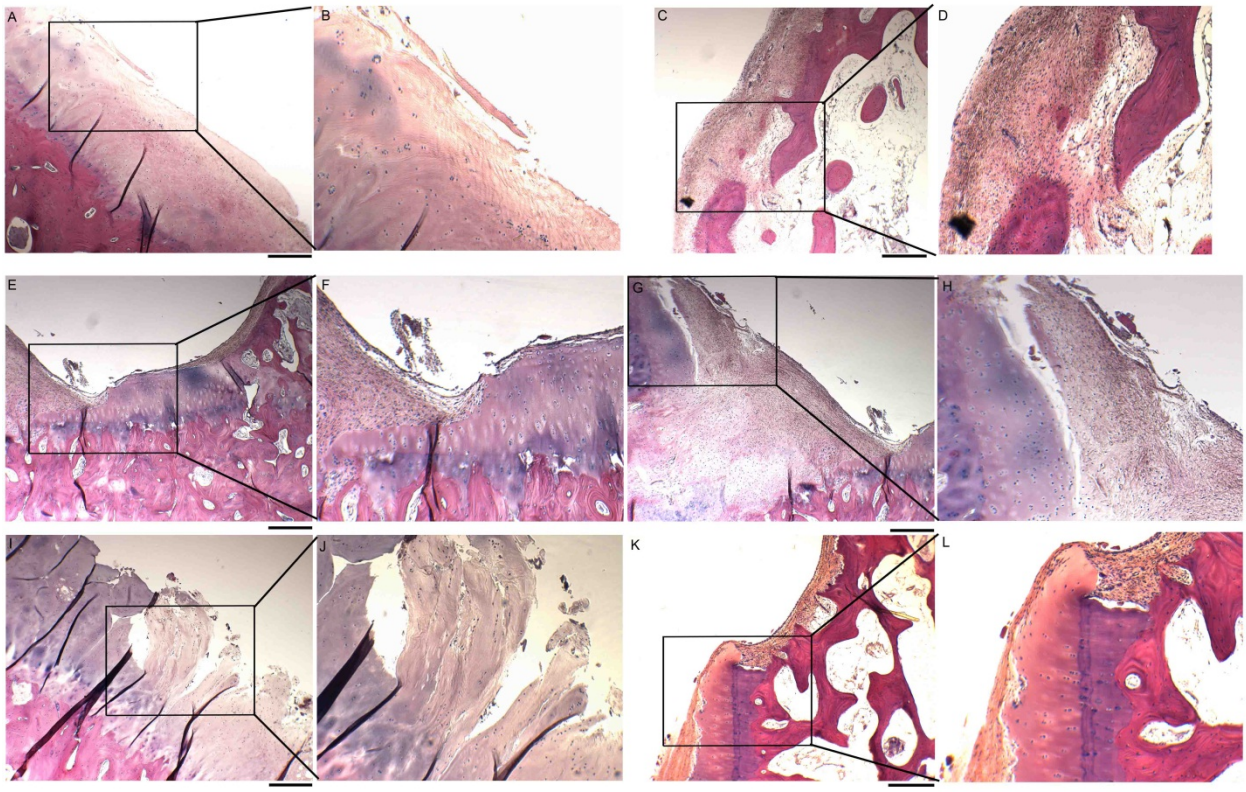

Figure 2.1. Hematoxylin and Eosin stained osteochondral sections from (A-D) mild OA metacarpophalangeal joint showing surface fibrillation (B) and transitional zone osteophyte (D), (E-H) severe OA metacarpophalangeal joint with full thickness cartilage erosions, (I-L) severe OA femoropatellar joint with deep fibrillation (J) and erosion extending into the subchondral bone (L). Images were taken at 50x magnification. Expanded images denoted by the black box. Black bar = 200 µm for original images.

Clinical Reaction to Injection

Two horses had increased lameness in the injected limb on day 1, and 19 of 29 injected joints had an increase in joint fluid effusion.

Synovial Cytology

Cell injected joints had significantly increased total nucleated cell counts and total protein compared to MEM injected controls (Table 2.2). The cell differential had a significantly increased percentage of macrophages in cell injected joints but no difference in the percentage of lymphocytes or neutrophils. There were no statistically significant differences ($p>0.1$) in synovial cytology parameters between labeled MSC and unlabeled MSC injected joints. In the comparison between cell injected OA joints versus cell injected normal joints, only the total nucleated cell count was significantly different (OA=2,300 cells/µl; 1,350-4,320 cells/µl versus normal=3,400 cells/µl; 2,870-4,830 cells/µl; $p=0.04$).

Cytospins of synovial fluid collected from injected joints were positive for labeled cells from all of the labeled MSC injected joints and none were positive from the unlabeled MSC injected joints or MEM injected joints. When the contralateral joint was also injected with labeled cells, no quantum dots from the contralateral joint (different fluorescence quantum dot) were identified in any joints.

Table 2.2. Synovial fluid analysis including total nucleated cell count, differential cell count based on smears, and protein quantification.

Table 2.2. Synovial cytology total nucleated cell count and differential cell count.					
	MSC injected joints n=29		MEM injected joints n=12		P value
	Median	95% confidence interval	Median	95% confidence interval	
TNCC $x10^3$ cells/µl	2.8	2.6-4.2	.85	0.5-1.4	**<0.001**
Macrophages %	64	51-60	42	30-60	**0.05**
Lymphocytes %	32	27-41	34	28-48	0.5
Neutrophils %	.05	-1.3-13	7	5-29	0.12
Total Protein g/dl	2.8	<2.5-2.9	<2.5	<2.5-<2.5	**0.01**

Tissue Sections

Tissue sections from labeled MSC injected joints were positive for labeled MSCs significantly more often ($p<0.0001$) on synovial membrane sections (54/70; 70%) than on cartilage sections (17/97;18%). When sections were positive, there was a greater number of cells present per field on synovial membrane versus cartilage sections (Fig 2.2). When comparing OA joints to normal joints, the proportion of positive sections from synovium and cartilage was not different (synovium $p=0.72$; cartilage $p=0.79$) between OA and normal joints (Fig 2.3). Tissue sections from unlabeled MSC injected joints were not positive for labeled MSCs.

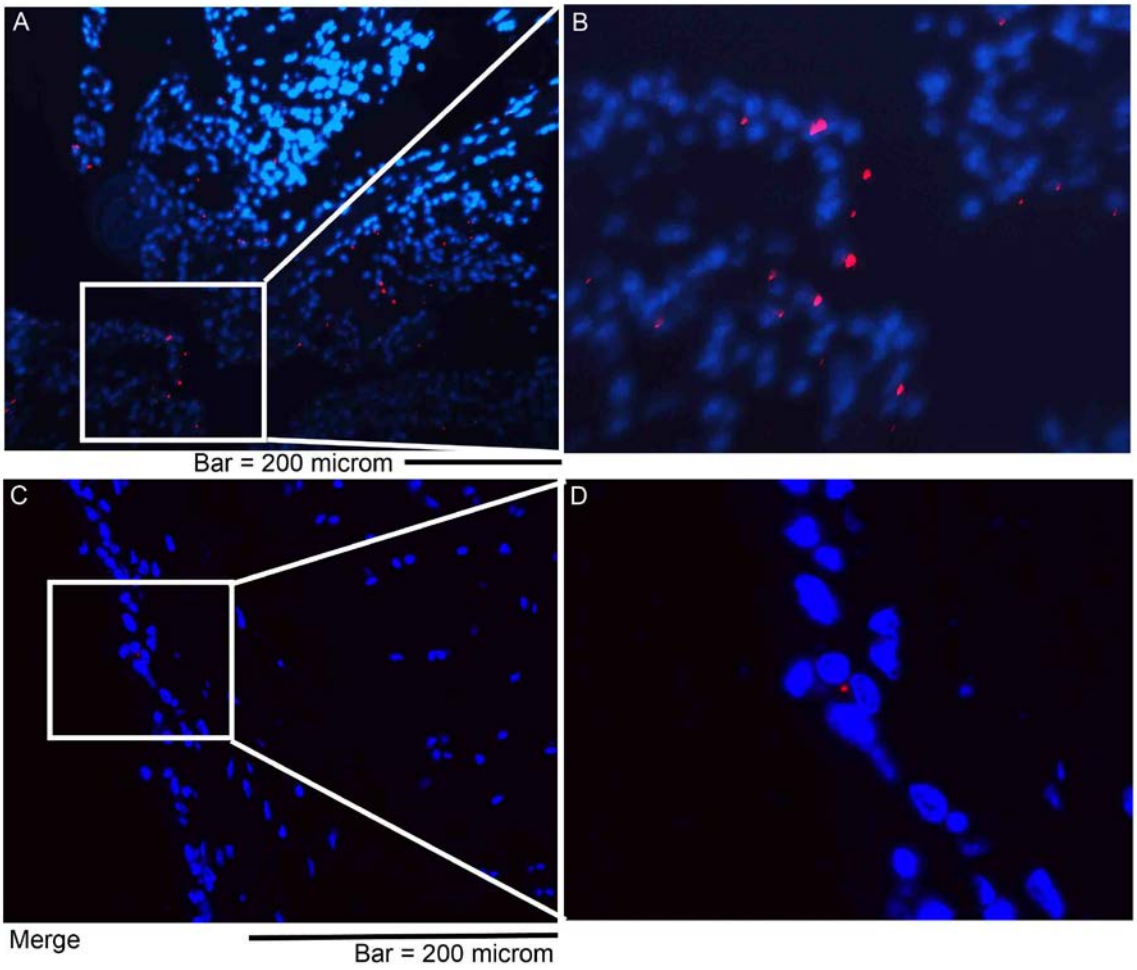

Figure 2.2. Distribution of 625 nm quantum dot labeled MSCs (red) to synovial membrane (A, B) and articular cartilage (C, D) with a nuclear counter-stain (Hoescht). Sections are from a severe OA metacarpophalangeal joint. Odot labeled cells are abundantly present in the intimal layers of the synovial membrane villi, and a single labeled cell is evident in the fibrotic surface of the OA cartilage. Images were taken at (A) 200x and (C) 400x magnification. (B, D) Expanded images from A and C denoted by the white box. Bar = 200 µm in original images.

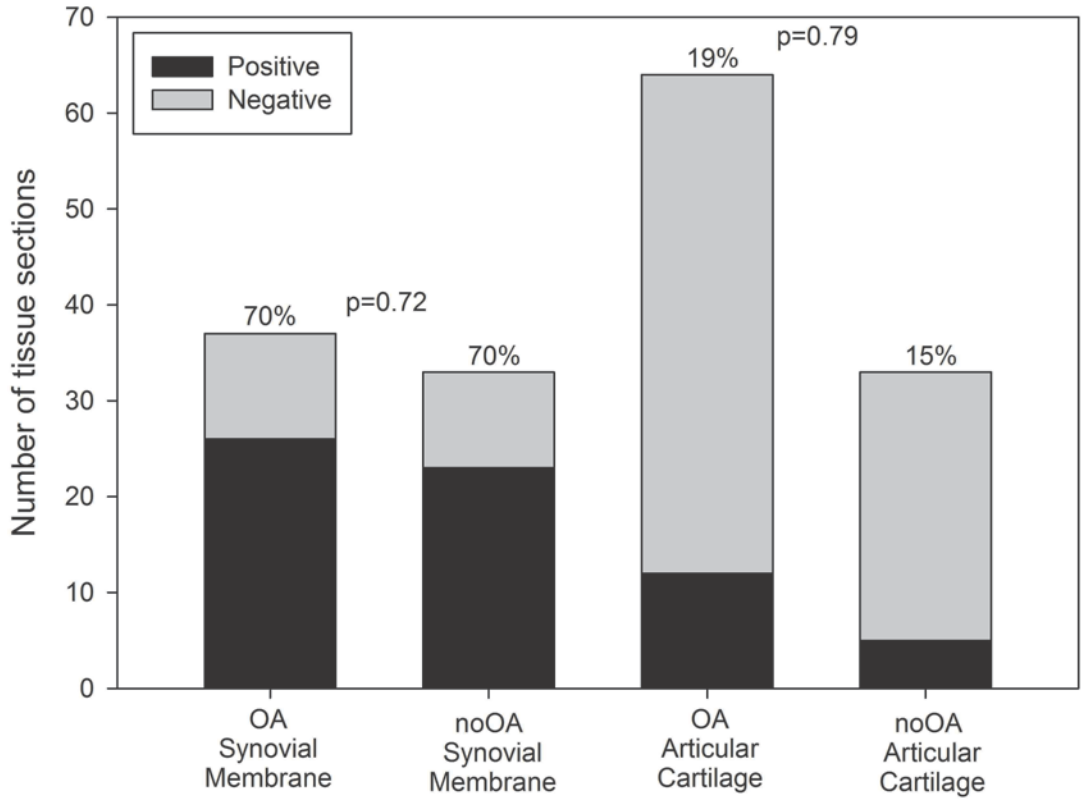

Figure 2.3. Number of tissue sections from synovial membrane and articular cartilage that were positive (black bar) and negative (grey bar) for the presence of labeled MSCs. Listed p values for a comparison between OA and normal joints.

Discussion

Our results confirm reports from a rabbit and goat OA model(Jing et al. 2008; Murphy et al. 2003) where the majority of injected MSCs engrafted the synovial membrane rather than the articular cartilage. When we separated joints by the presence of OA versus normal, there was no difference in the proportion of labeled synovial membrane and cartilage sections. This was disappointing as many investigators have speculated that the uneven fibrillated and ulcerated cartilage surface in OA joints would help capture and retain stem cells that might then contribute to local repair. Thus we rejected our hypothesis that OA joints would have greater MSC engraftment to cartilage than normal joints.

Given the low rate of MSC adherence to articular cartilage, it seems unlikely that these cells, if they persisted, would contribute directly to significant cartilage repair. Rather, the enhanced repair of cartilage due to MSC injection seen in animal models(Lee et al. 2007; McIlwraith et al. 2011; Murphy et al. 2003; Toghraie et al. 2011) and clinical patients,(Black et al. 2007; Black et al. 2008; Centeno et al. 2008; Centeno et al. 2011; Davatchi et al. 2011; Frisbie et al. 2007) may be the result of a paracrine interaction between injected MSCs that have populated the synovial membrane and endogenous repair mechanisms from the synovium, subchondral bone, and progenitors within the joint, as has been suggested by others.(Jing et al. 2008; Murphy et al. 2003) This paracrine interaction may occur through one of several mechanisms known to occur with MSCs: modulation of the immune response; stimulation of growth of host cells; recruitment of endogenous progenitors; or prevention of an inappropriate fibrotic response.(Caplan 2009)

Further evidence for the ability of MSCs to modulate the articular environment has been shown when cells were confined to the articular defect as well. When implanted allograft MSCs were investigated alongside chondrocytes, fibroblasts, umbilical cord blood stem cells, and empty defects in the rabbit knee, MSCs and chondrocytes both resulted in hyaline like tissue within the defect compared to the other treatments. Interestingly, MSCs were superior to chondrocytes with better cell arrangement, improved subchondral remodeling, and better integration with surrounding tissue at 6 and 12 weeks.(Yan and Yu 2007) The authors suggested the differences may have been due to bioactive factors released from MSCs or greater adaptability or proliferative capacity of the MSC compared to the less active chondrocytes.

We elected to use fluorescent quantum dot labels because of the narrow emission spectrum, good stability, long luminescence time, minimal overlap with auto-fluorescence, and no effect on cell behavior of labeled cells.(Shah and Mao 2011) Although use in equine MSCs has not been reported, quantum dots do not change the differentiation or growth potential of human or mouse bone marrow derived MSCs.(Muccioli et al. 2011; Ohyabu et al. 2009; Shah and Mao 2011) Prior to this study, we tested differing concentrations and labeling durations for quantum dot labeling of equine MSCs, leading to our use of 10 nM for 1 hour, where there was 100% labeling efficiency without obvious toxicity (data not shown). We also confirmed maintenance of a cytoplasmic fluorescent signal after 1 week of three-dimensional culture (data not shown). In this study,quantum dot labeling of equine bone marrow derived MSCs labeled all the MSCs for injection, and allowed us to characterize MSC distribution 1 week after intra-articular injection without any reaction in the joint due to the quantum dots.

Clinically, we utilize hyaluronic acid as a carrier for intra-articular injection of MSCs as was performed in a caprine OA study.(Murphy et al. 2003) However, in this study, we used

MEM as the MSC carrier. This obviated the possible reaction to HA, which has been described in the horse,(Kuemmerle et al. 2006) and in humans,(Goldberg and Coutts 2004; Magilavy et al. 2004; Roos et al. 2004) where intra-articular injection of HA alone can occasionally induce severe inflammatory joint reaction. We wanted to assess the synovial fluid for reaction to quantum dot labeled cells and to MSCs applied intra-articularly, as severe post-injection reaction to MSCs has been reported in the horse.(Carrade et al. 2011)

Synovial fluid cytologic abnormalities were noted in all cell injected joints with elevated total protein and elevated total nucleated cell counts, consisting primarily of large mononuclear cells (histiocytic) and small lymphocytes. This indicates mild non-septic inflammation and antigenic stimulation. It is possible that the mildly elevated nucleated cell count simply reflects free floating MSCs from injection 1 week prior. Unfortunately, fluorescent examination was not performed concurrent with cytologic exam on all synovial fluid samples and we cannot make this conclusion. Regardless, given the elevated total protein, it seems likely that there was mild non-septic inflammation and elevated cell counts weren't simply free-floating MSCs. Additionally, horses had detectable lameness (2 horses) and increased joint effusion (19/29 joints) following MSC injection, confirming mild joint reaction. Control joints that were injected with media alone did not have perturbations in synovial cytology, lameness, or joint effusion suggesting that reaction was not to the cell carrier. There were no differences between differential cell counts from labeled MSC injected joints and unlabeled MSC injected joints, indicating the mild joint reaction was not due to the quantum dot label. Given our experience of MSC injection to the joints of clinical equine patients, we suspect that reaction was due to foreign proteins present in growth medium during MSC culture.

The significant difference in total nucleated cell counts from MSC injected OA joints versus normal joints is intriguing. This may reflect a greater degree of engraftment (to synovial membrane particularly) and thus fewer MSCs free-floating in the synovial fluid in OA joints. Alternatively, it could be that MSCs are reacting according to the environment in which they are placed (OA or normal) and have a greater anti-inflammatory effect within the inflamed OA joint.(English et al. 2007; Shi et al. 2010)

There were limitations to this study. The distribution of labeled MSCs to the synovium and cartilage, compared to MSCs remaining free floating in the synovial fluid were not quantified. Methodology to allow for quantification, such as MRI of superparamagnetic iron oxide (SPIO) labeled cells may allow more thorough characterization of cell distribution.(Heymer et al. 2008; Jing et al. 2008) Second, because we used naturally occurring disease, OA severity was not uniform among OA joints. A spectrum of different degrees of OA severity was considered important to assess the receptivity of degenerate cartilage, particularly the depth of fibrillation, to MSC seeding. However, it is possible that a narrower spectrum of OA joints may have better allowed a difference to be detected if one existed.

Intra-articular MSC injection offers a simple regenerative therapy for modifying the structural progression of OA and reducing clinical symptoms by treating all tissues within the joint.(Chen and Tuan 2008) We utilized 29 joints from 10 horses to characterize the articular tissue distribution of MSCs 1 week after intra-articular injection. In our study, intra-articular injection did not appear to be an efficient method for delivery of MSCs to articular cartilage of unaffected joints or those with OA. The efficiency of engraftment to articular cartilage was low and was no different between unaffected and OA joints. To achieve efficient MSC engraftment

to articular cartilage, more invasive arthroscopic or open joint approaches would be required for direct MSC application to cartilage.

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A comparison of three-dimensional culture systems to evaluate in vitro chondrogenesis of equine mesenchymal stem cells

ABSTRACT

Objective: To compare chondrogenesis of bone marrow derived mesenchymal stem cells (MSCs) in three-dimensional culture systems: fibrin 0.3% alginate, 1.2% alginate, $2.5x10^6$ cell pellets, $5x10^6$ cell pellets and 2% agarose. **Methods**: MSCs from 5 horses 2-3 years of age were consolidated to the differing culture sytstems and maintained in chondrogenic medium with supplemental TGF-β1 for 4 weeks. Pellets and media were tested at days 1, 14 and 28 for selected gene expression (*ACAN*, *COL2B*, *COL10*, *SOX9*, *18S*), and histology (H&E, toluidine blue). **Results**: Fibrin, fibrin alginate and both pellet culture systems resulted in chondrogenic transformation. Adequate RNA quality or quantity was not isolated from agarose cultures at any time point. There was increased *COL2B*, *ACN* and *SOX9* expression on day 14 from both pellet culture systems. On day 28, increased expression of *COL2B* was maintained in $5x10^5$ cell pellets and there was no difference in *ACN* and *SOX9* between fibrin alginate and both pellet cultures. *COL10* expression was significantly lower from fibrin alignate cultures on day 28. **Conclusion**: Equine MSCs respond to three-dimensional culture in fibrin alginate and both pellet culture systems with chondrogenic induction. For prevention of terminal differentiation and hypertrophy, fibrin alginate culture may be superior.

INTRODUCTION

Osteoarthritis is the most common joint malady in horses(Jeffcott et al. 1982) and is often a sequelae to focal articular cartilage injury.(Mankin 1982; Strauss et al. 2005) Cell based therapy(Brittberg et al. 1994) for the repair of articular cartilage injury(Brittberg et al. 1994; Hendrickson et al. 1994; Sams and Nixon 1995)has been pursued due to the poor intrinsic healing of injured cartilage,(Mankin 1982) the poor long term response to surgical therapies(Hunziker 1999) and the lack of effective disease modifying osteoarthritis drugs.(Hunter 2011; Qvist et al. 2008) Repair with chondrocyte grafts improves long term outcome(Ortved et al. 2011) but must be either allogeneic, with risk of immune rejection,(Elves 1974; Hyc et al. 1997) or autogenous, with donor site morbidity.(Matricali et al. 2010) Adult, bone-marrow derived mesenchymal stem cells (MSCs) are a stem cell source for autologous cell transplantation to musculoskeletal tissues with minimal donor site morbidity and good proliferation and chondrogenic differentiation potential.(Pittenger et al. 1999)(Johnstone et al. 1998; Mackay et al. 1998) MSCs have been utilized for joint tissue regeneration in horses indirectly through intra-articular injection, after microfracture(McIlwraith et al. 2011), and directly with the arthroscopic application to focal cartilage defects of concentrated bone marrow grafts(Fortier et al. 2010) and culture-expanded MSC grafts.(Kuroda et al. 2007; Matsumoto et al. 2010; Wakitani et al. 2011; Wilke et al. 2007) However, several authors consider that MSCs are inferior to chondrocytes for cartilage defect repair because they are unable to attain the chondrocyte phenotype following implantation.(De Bari et al. 2004; Wilke et al. 2007) Many studies have evaluated culture additives and conditions that might drive chondrogenesis of MSCs in vivo, however, long-term outcome data suggests persisting fibrous tissue throughout

the repaired defect. Additional work using culture models that more completely mimic the in-vivo conditions experienced by implanted MSCs is clearly indicated.

For chondrogenic induction of MSCs, transforming growth factor beta supplementation of a defined serum free medium in a three-dimensional pellet culture is used routinely in vitro and has been well characterized for several species(Mackay et al. 1998; Tuan 2004). Pellet culture is a stable, biomaterial free culture system and is the gold standard for both chondrocyte re-differentiation studies as well as MSC differentiation studies in vitro. However, many investigators report that equine bone marrow derived MSCs have poor long term survival in pellet culture, with apoptosis and necrosis of central cells, poor RNA quality and quantity, and reduced total chondrogenesis(Rickey and Nixon 2007). In order to fully evaluate in vitro methods to optimize MSC chondrogenesis, a three-dimensional system that will allow cell survival, growth, differentiation, and matrix production for at least 1 month is required.

The purpose of this study was to investigate the effect of differing three-dimensional culture systems on MSC chondrogenesis in long term in vitro culture. We used three-dimensional systems that have been characterized to induce MSC chondrogenesis for species other than the horse; pellet culture, (Buschmann et al. 1992)agarose, alginate, and fibrin-alginate composites(Buschmann et al. 1992; Diduch et al. 2000; Ho et al. 2010; Johnstone et al. 1998; Yang et al. 2004). Identification and use of the best system will allow better characterization of MSC chondrogenesis and methods to improve it. Further, the best system may also elucidate methods to enhance in vivo MSC survival and engraftment.

MATERIALS AND METHODS

Study Design

Bone marrow derived MSCs from 5 horses were isolated, expanded and dispensed to each culture condition and maintained in chondrogenic medium. Pellets and supernatant media were collected on days 1, 14 and 28 for gene expression of selected marker genes and routine histology.

Bone Marrow Collection and MSC Isolation

Bone marrow aspirates were obtained from the sternum of 5 horses, 2-3 years of age(Fortier et al. 1998). Local anesthesia and light sedation was used for bone marrow collection. Bone marrow biopsy needles (Jamshidi, VWR Scientific, Bridgeport, NJ) were used to aspirate bone marrow into four 60-mL syringes containing heparin (APP Pharmaceuticals, LLC; Schaumburg, IL 60173) for a final concentration of 1,000 units/ml. Each 60 ml was collected from a separate site with advancement of the Jamshidi needle after each 15 ml of marrow had been drawn. Bone marrow aspirate was diluted 1:1 in growth medium (Dulbecco's modified Eagles' media; 1,000 mg/L glucose; 2 mM L-glutamine; 100 units/ml penicillin-streptomycin; 1 ng/ml bFGF; 10% fetal calf serum) and 60 ml was plated to T-175 tissue culture flasks, and maintained at 37°C, 5% CO_2, and 95% humidity in room air. Non-adherent cells were removed through daily feeding. Once colony formation was evident, adherent cells were passaged using trypsin and replated at 20,000 cells/cm^2, and fed every other day. Monolayer cultures were passaged a second time when plates were 80-90% confluent. When passage 2 cultures were 80-90% confluent, adherent MSCs from each horse were cryopreserved.

At the start of the in vitro experiment, cryopreserved MSCs were plated in monolayer culture at 20,000 cells/cm^2. When cultures were 85% confluent, cells from each horse were trypsinized, counted, and aliquoted to each group.

Fibrin Alginate

For culture in a fibrin alginate (ultra-pure low viscosity 67% guluronate, UPLVG, NovaMatrix, FMC Corporation, PA, USA) scaffold (FA), cells were resuspended to $10x10^6$ cells/ml in 30 mg/ml fibrinogen in Dulbecco's Phosphate Buffered Saline (dPBS) and mixed 1:1 with 0.6% alginate for a final concentration of 0.3% alginate. Allogeneic fibrinogen had been cryoprecipitated from plasma collected from horses using a technique previously described(Dresdale et al. 1985). For scaffold polymerization, the cell suspension was dropped via a 19 gauge 1.5 inch needle into 102 mM $CaCl_2$ with 5 units/ml bovine thrombin, for approximately 150,000 cells per bead. After 10 minutes, $CaCl_2$ solution was aspirated and FA beads were rinsed 3x in dPBS.

Alginate Culture

For culture in an alginate scaffold, cells were resuspended to $10x10^6$ cells/ml in dPBS and mixed 1:1 with 2.4% alginate (ultra-pure low viscosity 67% guluronate, UPLVG, NovaMatrix, FMC Corporation, PA, USA) in dPBS for a final concentration of 1.2% alginate. For scaffold polymerization, the cell suspension was dropped via a 19 gauge 1.5 inch needle into 102 mM $CaCl_2$ for approximately 125,000 cells per alginate bead. After 5 minutes, $CaCl_2$ was aspirated and beads were rinsed 3x with dPBS.

Agarose

For culture in an agarose (Ultra-Pure LMP Agarose, Invitrogen, Carlsbad, CA) scaffold, cells were resuspended to $60x10^6$ cells/ml in dPBS and mixed 1:1.5 with 3% agarose in dPBS for a final concentration of 2% agarose. Low melting temperature agarose was maintained at 38°C during cell preparation. The agarose MSC suspension was then dispensed to a casting frame (10cm petri dish) to a thickness of 1.6 mm and allowed to polymerize at room temperature for 10

minutes. Once polymerized, a 6 mm biopsy punch was used to cut discs containing approximately $1.8x10^6$ cells per disk.

Pellet Culture

MSCs were resuspended to $2x10^6$ cells/ml ($5x10^5$ pellets) and $1x10^6$ cells/ml ($2.5x10^5$ pellets) in chondrogenic medium. Aliquots of 250 µl were dispensed to wells of 96 well, 'v' bottom, polypropylene plates (PHENIX Research Products, 73 Ridgeway Road Candler, NC 28715). Plates were spun in a swinging bucket rotor at 400g for 10 minutes for pellet formation of $2.5x10^5$ and $5x10^5$ cells per pellet.

Three-dimensional Culture

Each group was maintained in chondrogenic medium (CM; high glucose DMEM; 12.5 ml/500 ml HEPES buffer; 100 nM dexamethasone; 50 µg/ml ascorbate 2 phosphate; 100 µg/ml sodium pyruvate; 40 µg/ml proline; 1x ITS+; 100 IU/ml penicillin-streptomycin) supplemented with 10 ng/ml TGF-β1 (Recombinant Human TGF- β1; Gibco Invitrogen 542 Flynn Road, Camarillo, CA 93012). Two hundred µl of CM was exchanged daily and pellets were maintained at 37°C, 5% CO_2, and 95% humidity in room air until the harvest time point at day 1, 14 or 28. All culture systems were maintained in wells of 12 well polypropylene plates except pellet cultures, which were maintained in polypropylene 96 well plates from pellet initiation.

For scaffold dissolution and RNA isolation at the end of the culture period, FA beads were rinsed once in dPBS, incubated 120 minutes at 37°C in 0.25% bacterial collagenase (Sigma, St. Louis, MO, USA) in dPBS, vortexed and then incubated until dissolved (5-15 minutes) at 37°C in reconstruction buffer (50 mM EDTA, 10 mM HEPES at pH 7.4), vortexed and centrifuged at 470 g for 5 minutes. The resulting cell pellet was washed three times in dPBS and frozen at -80°C in RNA lysis solution. Alginate beads were rinsed once in dPBS, incubated

until dissolved (5-15 minutes) at 37°C in reconstruction buffer, vortexed and centrifuged at 470 g for 5 minutes. The resulting cell pellet was washed three times in dPBS and frozen at -80°C in RNA lysis solution. Alginate beads at day 14 and 21 required pretreatment with 0.25% bacterial collagenase in addition to reconstruction buffer, similar to dissolution for FA beads. Pellets and agarose cultures were pulverized with a mortar and pestle while immersed in Lysis Buffer and kept on ice. All samples were collected and frozen (-80°C) in lysis buffer for later RNA isolation at a later time. Each condition was homogenized with a commericial homogenizer solution (Qiashredder, Qiagen) followed by RNA extraction with a commericial kit (RNeasy® Plus Mini Kit, Qiagen). Genomic DNA was removed from RNA samples prior to PCR, by selective filter centrifugation.

Gene Expression

Six pellets, beads or discs from each group and horse were isolated at each time point (days 1, 14, or 28) with combination of 2 per sample, for an n=3 for each group, horse and time point. For quantitative PCR, the primers and dual-labeled fluorescent probe [6-FAM as the 5' label (reporter dye) and TAMRA as the 3' label (quenching dye)] were designed using Primer Express Software version 2.0b8a (Applied Biosystems) using equine specific sequences published in Genbank:

18S-Fwd CGGCTTTGGTGACTCTAGATAACC *18S*-Rev CCATGGTAGGCACAGCGACTA; *COL2b*-Fwd CGCTGTCCTTCGGTGTCA, *COL2b*-Rev CTTGATGTCTCCAGGTTCTCCTT; *COL10a*-Fwd GAGAACATGCTGCCACAAACA, *COL10a*-Rev TCAGCATAAAACTCGCCATGAA; *AGC*-Fwd GATGCCACTGCCACAAAACA, *AGC*-Rev GGGTTTCACTGTGAGGATCACA; *SOX9*-Fwd CAGGTGCTCAAGGGCTACGA, *SOX9*-Rev GACGTGAGGCTTGTTCTTGCT.

Total RNA was reverse transcribed and amplified using the One-Step RTPCR technique and the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Life Technologies, Carlsbad, CA). Samples for each molecule for each time point were assessed on the same qPCR plate to minimize variation. The qPCR program included reverse transcription at 48°C for 30 minutes and denaturing at 95°C for 10 minutes, followed by 40 cycles of 90°C for 15 seconds and 60°C for 1 minute. Each well of the qPCR plate was loaded with 10 ng of RNA in 20 µl. Other than *18S*, a standard curve was generated from equine specific plasmid DNA for each gene at known concentrations to allow copy number estimation. All samples were run in duplicate on the qPCR plate and total copy number per 10 ng of RNA of each gene was obtained from a standard curve and normalized to *18S* gene expression.

Histology

Two pellets, beads or discs per group, horse and time point, were fixed in 4% paraformaldehyde for 12 hours. Cultures were then confined to 2% agarose gel, processed and embedded in paraffin, sectioned (5µm) and stained using standard procedures for hematoxylin and eosin (H&E) and toluidine blue.

Statistical Analysis

As data were not normally distributed, non-parametric tests were used. Differences between groups were detected by Kruskal-Wallis 1-way ANOVA and Dunn multiple comparison test. Data were reported as a median and inter-quartile range. Statistical analyses were performed with a commercially available software (Statistix 9; Tallahassee, FL, 32317) and the level of significance was set at $P<0.05$.

RESULTS

MSC Harvest and Culture

At least $100x10^6$ passage 2 cells per horse were cryopreserved after primary cell harvest. All horses had good viability (>85%) post freeze. Cell suspension in each scaffold or pellet culture was successful for all groups. By 1 week, FA and alginate cultures had become opaque and remained this way until harvest. Pellets (opaque and white) and agarose (translucent without color) did not change in color or translucency during the culture period (Fig 3.1)

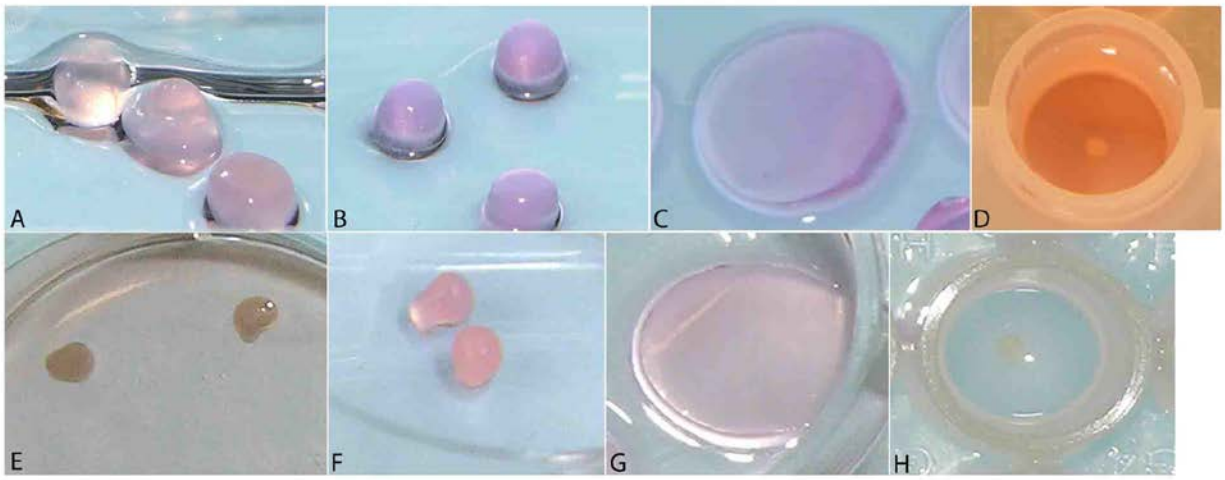

Figure 3.1. Photographs of three-dimensional constructs on day1 (A, Fibrin Alginate; B, Alginate; C, Agarose; D, pellet) and day 28 (E, Fibrin Alginate; F, Alginate; G, Agarose; H, pellet) of culture.

Histology

Each group had matrix accumulation evident starting on day 14 and increasing on day 28 (Fig 3.2). There was increased matrix staining in $2.5x10^5$ pellets and $5x10^5$ pellets compared to alginate and FA and very little matrix accumulation in agarose on day 14 and 28. Within both pellet cultures, there was reduced matrix staining and obvious stratification with elongated cells and lacunae-like structures in the outer third of the pellets at day 28. The development of lacunae-like structures within the central region did not appear to be different between the different groups within each time point. Cellular migration within the scaffold, with subsequent clustering of cells was greatest at day 28 in alginate and FA cultures, and was occasionally present in agarose cultures but was not noted in either $2.5x10^5$ pellets or $5x10^5$ pellets.

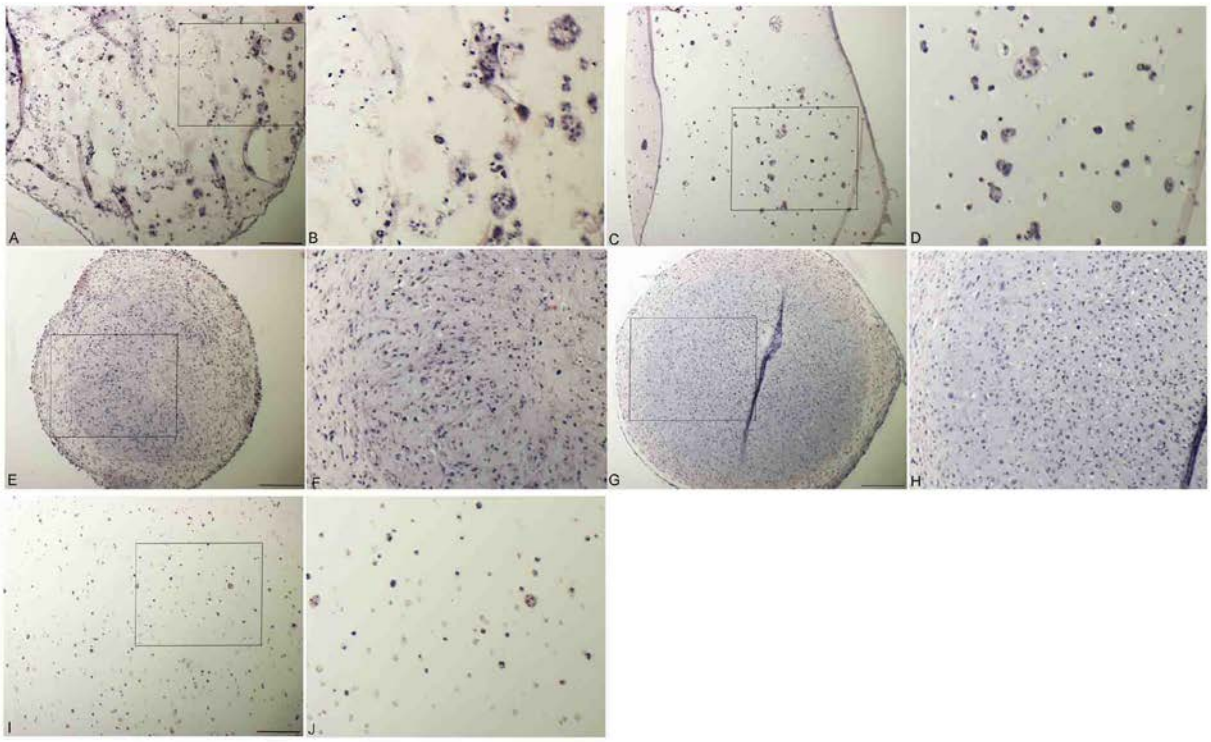

Figure 3.2. Photomicrographs of mesenchymal stem cell three-dimensional cultures collected at 28 days and stained with hematoxylin and eosin. (A, B) Fibrin Alginate, (C, D) Alginate, (E, F) $2.5x10^5$ Pellet, (G, H) $5x10^5$ Pellet, and (I, J) Agarose. 200x magnification. Images were taken at 100 x magnification. Black box represents area for magnified image adjacent to original. Scale bar = 200 µm

Gene expression

Good quality RNA was isolated from 3-d cultures at all time points from all groups, other than agarose, with significant differences between groups in the total RNA isolated within each time point (Table 3.1). Sufficient quantities of RNA were not generated from agarose at any time point and therefore rtPCR was not performed on agarose samples. On day 14 and day 28, but not day 1, there were significant differences between the groups in *ACN* and *COL2b* expression. On day 1, 14 and 28 there were significant differences between the groups in *COL10* and *SOX9* expression. On day 28 *ACN*, *COL2B* and *SOX9* were significantly lower from alginate cultures and *COL10* was significantly lower from fibrin alginate cultures (Fig 3.3).

Table 3.1 Good quality RNA was isolated from 3-d cultures at all time points from all groups, other than agarose, with significant differences between groups in the total RNA isolated within each time point.

Table 3.1. Total RNA (µg/ml) in 35 µl from MSC 3-dimensional culture systems. Superscript letters demonstrate statistically significant differences within each time point.

	Day 1		Day 14		Day 28	
	Median	IQR	Median	IQR	Median	IQR
FA	94^{AB}	54-168	113^{A}	44-248	96^{A}	35-190
Alginate	48^{B}	41-55	10^{B}	9-17	30^{AB}	5-60
Pellet 2.5	49^{AB}	38-70	18^{B}	14-24	10^{B}	5-15
Pellet 5	121^{A}	91-144	20^{B}	13-23	12^{B}	8-18
Agarose	1^{C}	0-2	1^{C}	1-2	3^{C}	2-4

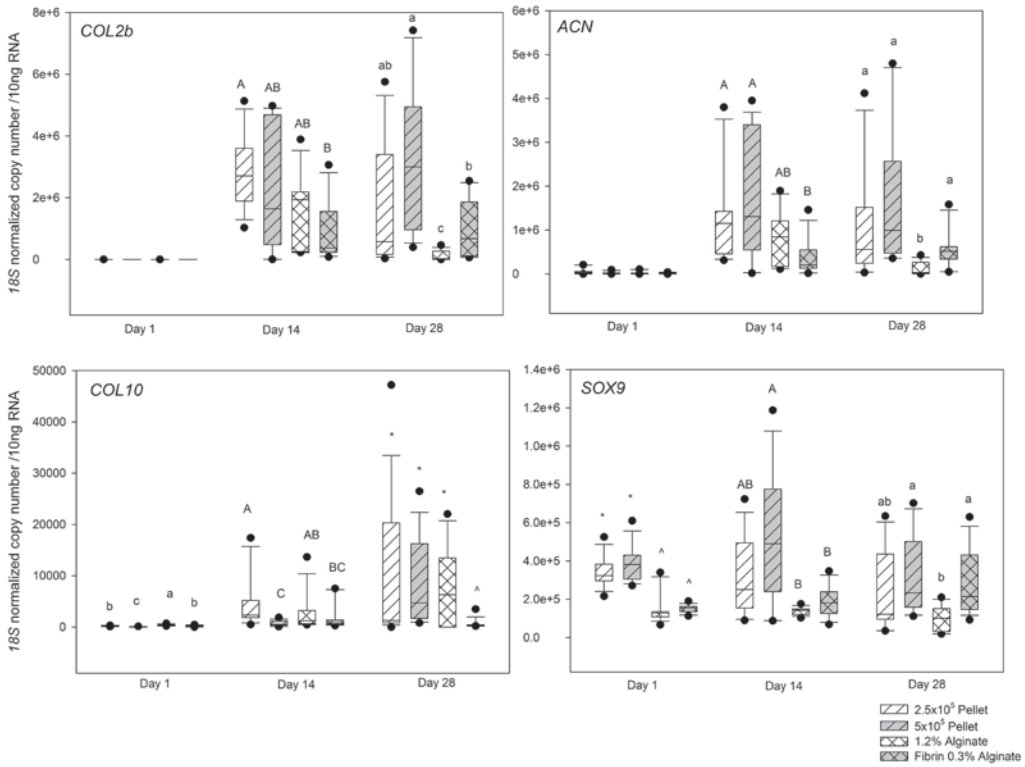

Figure 3.3. Box plots of gene expression for *COL2b*, *ACN*, *COL10* and *SOX9* from three-dimensional cultures of mesenchymal stem cells. Differing superscript letters and symbols indicate statistical differences between groups within each time point.

DISCUSSION

This study confirmed the utility of FA, alginate and pellet culture but not agarose, for long term three-dimensional culture of equine MSCs for studying chondrogenesis. All culture conditions induced some degree of MSC chondrogenesis, confirmed by rounding of cells and formation of lacunae-like structures. However, isolation of adequate RNA with good quality at all time points, and confirmed matrix accumulation only developed from FA, alginate and pellet ($2.5x10^5$ pellet and $5x10^5$ pellet) culture systems. FA, $2.5x10^5$ pellet and $5x10^5$ pellet systems were superior to alginate in chondrogenic induction with increased *ACN* and *SOX9* expression. The biomaterial vehicle free pellet culture systems were superior for chondrogenic induction on the basis of increased *COL2b* expression, and therefore may be best for the study of chondrogenesis.

The fibrin-alginate culture provides several compelling features. Although the FA scaffold had lower *Col2b* expression at 4 weeks compared to pellet culture, *ACN* or *SOX9* were no different, and *COL10* was dramatically lower compared to all other systems. In vivo, collagen type 10 is produced by pre-hypertrophic and hypertrophic chondrocytes during chondrogenesis,(Lefebvre et al. 1998; Lefebvre and Smits 2005) and its expression is used as a marker of chondrocyte hypertrophy.(Ichinose et al. 2005; Winter et al. 2003) It is not surprising that pellet culture induced *COL10* expression, as the cell to cell interactions of pellet culture closely mimic those that occur in pre-cartilage condensation of the physis during development. The reduced *COL10* message at day 28 in FA beads suggests that in longer term culture, $2.5x10^5$ pellet and $5x10^5$ pellet MSCs are progressing on to terminal differentiation and hypertrophy while FA MSCs are remaining in a more hyaline-like differentiated state. This would be

desirable in cartilage repair applications and may support the value of fibrin glue for MSC grafting to cartilage defects.

In addition to reduced cell to cell contact in FA cultures compared to pellets, that may influence the reduced COL10 expression, FA may contain growth factors that are enhancing a hyaline-like state of chondrogenesis. Cryopreciopitate fibrinogen has platelet derived growth factors such as TGF-β1 and VEGF(Rock et al. 2006) and the combination of recombinant TGF-β3 exposure from chondrogenic medium and TGF-β1 exposure from the fibrin, may have influenced the progression toward hypertrophy.

Pellet culture of equine MSCs had not been an ideal model in our lab, given the central necrosis and lamination that frequently developed, and it was surprising that the pellet culture system was successful, allowing adequate RNA isolation and the highest degree of MSC chondrogenesis. Many investigators, including ourselves, utilize a mixed pool of MSCs from several different donors for in vitro studies. This is done to increase the number of early passage MSCs available for the experiment and to minimize the effect of inter-animal heterogeneity of MSC cultures and subsequent variability in growth characteristics, and response to experimental conditions. However, MSCs are exquisitely responsive to their micro-environment and cell to cell contacts,(Prockop 2009) and mixing of MSCs from different donors may confound MSC studies. The experiment we report here was performed with 5 individual horses rather than a mixed population of donors and this may explain the success of both pellet culture systems in this experiment in contrast to our laboratory's previous experience with pellet culture. Subsequent use of the pellet culture system in our laboratory using individual horses has had similar success and when we have used a mixed population there has been reduced longevity and

chondrogenesis of pellet cultures. Further investigation into the response of MSCs to the combination of donors versus individual donors is indicated.

One drawback of using pellet culture compared to biomaterial scaffolds such as alginate, fibrin, or agarose, is that a very large number of cells are required to generate sufficiently sized constructs. This is especially important when trying to avoid mixing of different donors within 3-dimensional constructs. It has recently been shown that the yield of MSCs from raw marrow is significantly greater in the first 5 ml collected, compared to subsequent aliquots in equine sternal bone marrow collection (Kasashima et al. 2011). Therefore, to facilitate expansion of high numbers of early passage MSCs from individual donors, we maximized the MSC concentration from each bone marrow collection by aspirating only 15 ml per sternum site and collected a total of 180 ml of marrow per donor. By advancing the biopsy needle after each 15 ml of bone marrow had been drawn, we collected a larger proportion of MSCs to marrow and this allowed expansion of MSCs to at least $100x10^6$ passage 2 cells.

Our results demonstrate the utility of FA, alginate or pellet culture at both $2.5x10^5$ and $5x10^5$ cells per pellet, but not agarose, for long term three-dimensional culture of equine MSCs when gene expression is the study end point. Adequate RNA quantity and quality was isolated from the successful culture systems that would allow assessment of treatments for the induction of chondrogenesis. Both $2.5x10^5$ pellet and $5x10^5$ pellet systems appear to be superior for chondrogenic induction studies, based upon *COL2b* gene expression and matrix accumulation within the pellets.

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Transforming growth factor beta-3 improves chondrogenesis without inducing hypertrophic differentiation of mesenchymal stem cell compared to transforming growth factor beta -1 and -2

ABSTRACT

Objective: To compare chondrogenesis of bone marrow derived mesenchymal stem cells (MSCs) in pellet culture supplemented with transforming growth factor beta-1, -2 or -3 (TGF-β1, -β2 or -β3). **Methods**: MSCs from 9 young adult horses were consolidated individually to pellets and maintained in chondrogenic medium with TGF-β1, -β2 or -β3 for 4 weeks. Pellets and media were tested at days 1, 14 and 28 for selected gene expression (*ACAN*, *COL2B*, *COL10*, *SOX9*, *TGFB1*, *TGFB2*, *TGFB3*, *18S*), total glycosaminoglycan (GAG) and DNA content, media alkaline phosphatase (ALP) activity and GAG content, and histology (H&E, toluidine blue, collagen II and X immunohistochemistry). **Results**: All 3 peptides resulted in chondrogenic transformation without differences between the isoforms in *ACAN* or *COL2B* gene expression at 14 and 28 days. TGF-β3 supplementation resulted in reduced *COL10* and increased *SOX9* gene expression, and reduced ALP activity in the media at 28 days. TGF-β3 supplemented pellets also had increased GAG accumulation in the supernatant media at all 3 time points. **Conclusion**: Chondrogenic transformation was successful with all three TGF-β isoforms; however, TGF-β3 resulted in a reduction in the degree of hypertrophic differentiation and may better maintain the hyaline-like tissue phenotype of chondrogenically induced MSCs compared to the other TGF-β isoforms.

INTRODUCTION

Cell based therapies for the repair of articular cartilage injuries are being pursued due to poor intrinsic healing and the limited efficacy of pharmacologic therapies. Adult, bone-marrow derived mesenchymal stem cells (MSCs) are a stem cell source for autologous cell transplantation to musculoskeletal tissues with good proliferation and chondrogenic differentiation potential.(Pittenger et al. 1999)(Johnstone et al. 1998; Mackay et al. 1998)

Unlike chondrocytes that maintain their hyaline phenotype following implant, chondrogenic MSCs often undergo hypertrophy toward terminal chondrocyte differentiation(Ichinose et al. 2005; Pelttari et al. 2008; Winter et al. 2003) with vascular invasion and calcification after subcutaneous transplantation to SCID mice.(Cui et al. 2007; Liu et al. 2008; Pelttari et al. 2006) The tendency for bone marrow derived MSCs undergoing chondrogenesis to continue toward hypertrophy has been documented in vitro(Johnstone et al. 1998; Mueller and Tuan 2008) by increased *COL10* expression,(Ichinose et al. 2005; Winter et al. 2003) similar to expression of *COL10* in growth plate cartilage,(Schmid and Linsenmayer 1985) and increased alkaline phosphatase activity,(Hennig et al. 2007; Pelttari et al. 2006) which is an enzyme marker of matrix mineralization. Continued chondrocytic differentiation of MSCs toward a hypertrophic phenotype post-implantation is a concern in cartilage tissue engineering. Failure to prevent hypertrophic differentiation would lead to apoptosis, matrix calcification, vascular ingrowth and advancement of the subchondral bone.(Goldring et al. 2006) Therefore, identification of methods to prevent hypertrophic differentiation of chondrifying MSCs is a major concern for the orthopedic surgeon prior to application of MSCs to chondral defects for regenerative therapy.(Fischer et al. 2010; Steinert et al. 2007)

Supplementation of TGF-β to chondrocytes prevents their hypertrophic differentiation,(Ballock et al. 1993; Ballock et al. 1993; Bohme et al. 1995; Mello and Tuan 2006) and may also have similar effects when applied to MSCs for prevention of hypertrophy and terminal differentiation. TGF-β1, -β2 or -β3 supplementation is used routinely for chondrogenic induction of MSCs in vitro and TGF-β is withdrawn from medium when inducing MSC hypertrophy in vitro.(Mueller and Tuan 2008) Although an early report directly compared TGF-β1, -β2 or -β3, for differences in chondrogenesis, finding that TGF-β2 and -β3 were superior for MSC chondrogenesis,(Barry et al. 2001) TGF-β1 remains the most commonly reported isoform used for in vitro cultures (Mueller et al. 2010; Solchaga et al. 2011) as well as in vivo MSC cartilage repair studies.(Chang et al. 2011) Therefore, it is not clear from the literature which TGF-β isoform is the most efficient at MSC chondrogenic induction.

For prevention of hypertrophy, the literature is even less clear. It has been reported that TGF-β1 prevents hypertrophy of embryonic chick MSCs;(Mello and Tuan 2006) however, another study found no difference between TGF-β1 and TGF-β3 supplementation for chondrogenesis and prevention of hypertrophic induction of MSCs in pellet culture.(Mueller et al. 2010) A recent report compared all three TGF-β isoforms in MSC culture for chondrogenesis and prevention of hypertrophy and found no differences in chondrogenesis or hypertrophy, other than reduced mineralization of TGF-β1 supplemented cultures after addition of β-glyerophosphate.(Cals et al. 2011) In summary, progression toward chondrocytic hypertrophy in chondrifying MSCs due to TGF-β supplementation has not been thoroughly investigated.

The purpose of this study was to investigate the effect of TGF-β1, -β2 or -β3 supplementation on MSC chondrogenesis and hypertrophic differentiation. Given the successful use of the 3 isoforms in MSC chondrogenesis studies, we hypothesized that the three TGF-β

isoforms would induce MSC chondrogenesis without differences, but that there would be differences in the degree of hypertrophic differentiation.

MATERIALS AND METHODS

Bone Marrow Collection, MSC Isolation and Pellet Culture

Bone marrow aspirates were obtained from the sternum of 9 healthy horses ranging in age from 2–5 years.(Fortier et al. 1998) Horses were sedated and bone marrow biopsy needles (Jamshidi, VWR Scientific, Bridgeport, NJ) were inserted into one or several sternebra under local anesthesia, to aspirate bone marrow into four 60-mL syringes containing heparin (APP Pharmaceuticals, LLC; Schaumburg, IL 60173) for a final concentration of 1,000 units/ml. Each 60 ml syringe was withdrawn from a separate site with redirection of the Jamshidi needle after the first 15 ml of marrow had been drawn. Bone marrow aspirate was diluted 1:3 using phosphate buffered saline with 0.5% bovine serum albumin and layered 2:1 onto Ficoll-Paque Plus (Amersham Biosciences, Pistcataway, NJ) for enrichment of the nucleated cells by centrifugation. MSCs were then isolated through selective tissue culture plastic adherence. Briefly, 30 ml of centrifuged bone marrow was plated to T-175 plates, 1:1 in growth medium (Dulbecco's modified Eagles' media; 1,000 mg/L glucose; 2 mM L-glutamine; 100 units/ml penicillin-streptomycin; 1 ng/ml bFGF; 10% fetal calf serum). Non-adherent cells were removed through daily feeding. Once colony formation was evident, adherent cells were passaged and replated at 10-12,000 cells/cm^2, and fed every other day.

Chondrogenic differentiation was performed via pellet culture.(Johnstone et al. 1998; Penick et al. 2005) Expanded passage 2 cells for each horse were collected with trypsinization and resuspended to $2x10^6$ cells/ml of chondrogenic medium (CM; high glucose DMEM; 12.5

ml/500 ml HEPES buffer; 100 nM dexamethasone; 50 µg/ml ascorbate 2 phosphate; 100 µg/ml sodium pyruvate; 40 µg/ml proline; 1x ITS+; 100 IU/ml penicillin-streptomycin; 10 ng/ml of either TGF-β1, -β2 or -β3 peptide [Recombinant Human TGF-β1, -β2 or -β3; Gibco Invitrogen, 542 Flynn Road, Camarillo, CA 93012]). Aliquots of 250 µl were dispensed to wells of 96 well, 'v' bottom, polypropylene plates (PHENIX Research Products, 73 Ridgeway Road Candler, NC 28715) for each horse, so that there were 16 pellets for each horse, under each peptide condition for each time point. Plates were spun by centrifugation in a swinging bucket rotor at 400g for 10 minutes to form pellets containing $5x10^5$ cells per pellet.

Two hundred µl of CM was exchanged daily and pellets were maintained at 37°C, 5% CO_2, and 5% humidity in room air until the harvest time point at day 1, 14 or 28.

Gene Expression

Samples of monolayer cells from each horse prior to pellet formation were collected and frozen in lysis buffer for later RNA isolation. Six pellets from each horse (horse 1-9) for each culture condition (TGF-β1, -β2 or -β3) were isolated at each time point (days 1, 14, or 28) with combination of 2 pellets per sample, for an n=3 for each horse, condition, and time point. Pellets were pulverized with a mortar and pestle while immersed in Lysis Buffer and kept on ice followed by homogenization with a commercially available homogenizer (Qiashredder, Qiagen) and RNA extraction kit (RNeasy® Plus Mini Kit, Qiagen). For quatitative PCR, the primers and dual-labeled fluorescent probe [6-FAM as the 5' label (reporter dye) and TAMRA as the 3' label (quenching dye)] were designed using Primer Express Software version 2.0b8a (Applied Biosystems) using equine specific sequences published in Genbank (Table 4.1)

Table 4.1 Forward and reverse primers for rt-PCR.

Table 4.1

18S-Fwd CGGCTTTGGTGACTCTAGATAACC *18S*-Rev CCATGGTAGGCACAGCGACTA;

COL2b-Fwd CGCTGTCCTTCGGTGTCA, *COL2b*-Rev CTTGATGTCTCCAGGTTCTCCTT;

COLXa-Fwd GAGAACATGCTGCCACAAACA, *COLXa*-Rev TCAGCATAAAACTCGCCATGAA;

AGC-Fwd GATGCCACTGCCACAAAACA, *AGC*-Rev GGGTTTCACTGTGAGGATCACA;

SOX9-Fwd CAGGTGCTCAAGGGCTACGA, *SOX9*-Rev GACGTGAGGCTTGTTCTTGCT;

TGFB1-Fwd TCCTGGCGCTACCTCAGTAAC, *TGFB1*-Rev TGACATCAAAGGACAGCCATTC;

TGFB2-Fwd CGCTCGATATGGACCAGTTCA, *TGFB2*-Rev CTGGTGCTGTTGTAGATGGAAATC;

TGFB3-Fwd GCCTGGCGGAGCACAAT, *TGFB3*-Rev CGGAATTCTGCTCGGAACA.

Genomic DNA was removed from RNA samples prior to PCR, by selective filter centrifugation (RNeasy® Plus Mini Kit, Qiagen). Total RNA was reverse transcribed and amplified using the One-Step RT-PCR technique and the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Life Technologies, Carlsbad, CA). Samples for each molecule for each time point were assessed on the same qPCR plate to minimize variation. The qPCR program included reverse transcription at 48°C for 30 minutes and denaturing at 95°C for 10 minutes, followed by 40 cycles of 90°C for 15 seconds and 60°C for 1 minute. Each well of the qPCR plate was loaded with 10 ng of RNA in 20 µl. Other than *18S*, a standard curve was generated from equine specific plasmid DNA for each gene at known concentrations to allow copy number estimation. All samples were run in duplicate on the qPCR plate and total copy number per ng of RNA of each gene was obtained from a standard curve and normalized to *18S* gene expression. The fold difference from the mean expression of each gene from monolayers for all 9 horses prior to pellet culture was calculated.

Biochemical Analysis of Pellets

Six pellets for each horse, condition,or time point were combined and lyophilized for biochemical assays. For total glycosaminoglycan and total DNA assay, samples were digested in papain (1 mL papain [0.5mg/ml]/pellet) at 65°C for 4 and 24 hours, respectively. The samples were mixed with dimethylmethylene blue dye for glycosaminoglycan quantification by colorimetric assay(Farndale et al. 1986) and bisbenzimide compound for DNA quantification by fluorometric assay (Kim et al 1988) in triplicate aliquots.

Biochemical Media Analysis

Three aliquots of culture supernatants were collected for each horse and condition at days 1, 14 and 28. For total glycosaminoglycan, samples were digested in papain (1 mL papain

[0.5mg/ml]/ ml conditioned media) at 65°C for 4 hours. The samples were mixed with dimethylmethylene blue dye for glycosaminoglycan quantification by colorimetric assay(Farndale et al. 1986) in triplicate aliquots. Total alkaline phosphatase activity was determined in triplicate aliquots using a commercially available fluorometric assay (SensoLyte®, Freemont, CA, USA) according to the manufacturer's directions, and measured spectrophotometrically in a microplate reader with results calculated based on manufacturer supplied standards to develop a standard curve.

Histology

Four pellets per horse, condition, and time point, were fixed in 4% paraformaldehyde-phosphate buffered saline for 12 hours. Pellets were then confined by 2% agarose gel, processed and embedded in paraffin, sectioned (5µm) and stained using standard procedures for hematoxylin and eosin (H&E), toluidine blue, and alcian blue. Immunohistochemical reaction was performed against collagen type 2 and 10 using mouse anti-human (Col2) and mouse anti-pig (Col10) antibody followed by anti-mouse secondary antibody,and horseradish peroxidase and DAB substrate for color detection.

Statistical Analysis

Prior to statistical testing, gene expression data was log transformed to minimize variation. Except for biochemical pellet assays, where there was only 1 replicate per horse, condition, and time point, samples were compared for differences due to condition (TGF-β1, -β2 or -β3) within each time point by a randomized ANOVA blocked by horse. For biochemical pellet assay, samples within each time point were compared by 1-way ANOVA. Statistical analyses were performed with a commercially available software (Statistix 9; Tallahassee, FL, 32317) and the level of significance was set at $P<0.05$.

RESULTS

Bone marrow collection and pellet formation

Bone marrow collection was performed successfully without modification in technique and MSC isolation resulted in cell numbers greater than the minimum requirement for the experiment ($>72x10^6$) by passage 2 for each of the 9 horses. At the time of initiation of pellet culture, all cultures appeared to be largely a homogenous population of cells with fibroblastic morphology consistent with bone marrow derived MSCs. Pellet formation was successful for all horses. Other than horse 9, for which the pellets lost shape and fragmented during the third week of culture, pellets were stable, maintained shape through the 28 day time point, and were increasingly difficult to break apart for RNA isolation due to matrix accumulation, which was confirmed histologically. Subjectively, there were no differences between the peptide groups in the pellet size, either grossly or on histologic sections (Fig 1), suggesting matrix accumulation was similar for TGF-β1, -β2 or -β3 supplementation. Finally, the development of lacunae-like structures within the central region of the pellets did not appear to be different between the different peptide groups.

Histology

Collagen type 2 and collagen type 10 reaction was apparent at day 14 and to a greater extent on day 28, without differences between the peptide supplemented groups. Metachromatic staining on toluidine blue and alcian blue histochemistry demonstrated accumulation of GAG at day 14, with a greater extent on day 28 (Fig 4.1). There were no differences in matrix GAG staining between peptide supplemented groups for most horses; however when a difference existed, there was reduced GAG accumulation in TGF-β1 supplemented pellets.

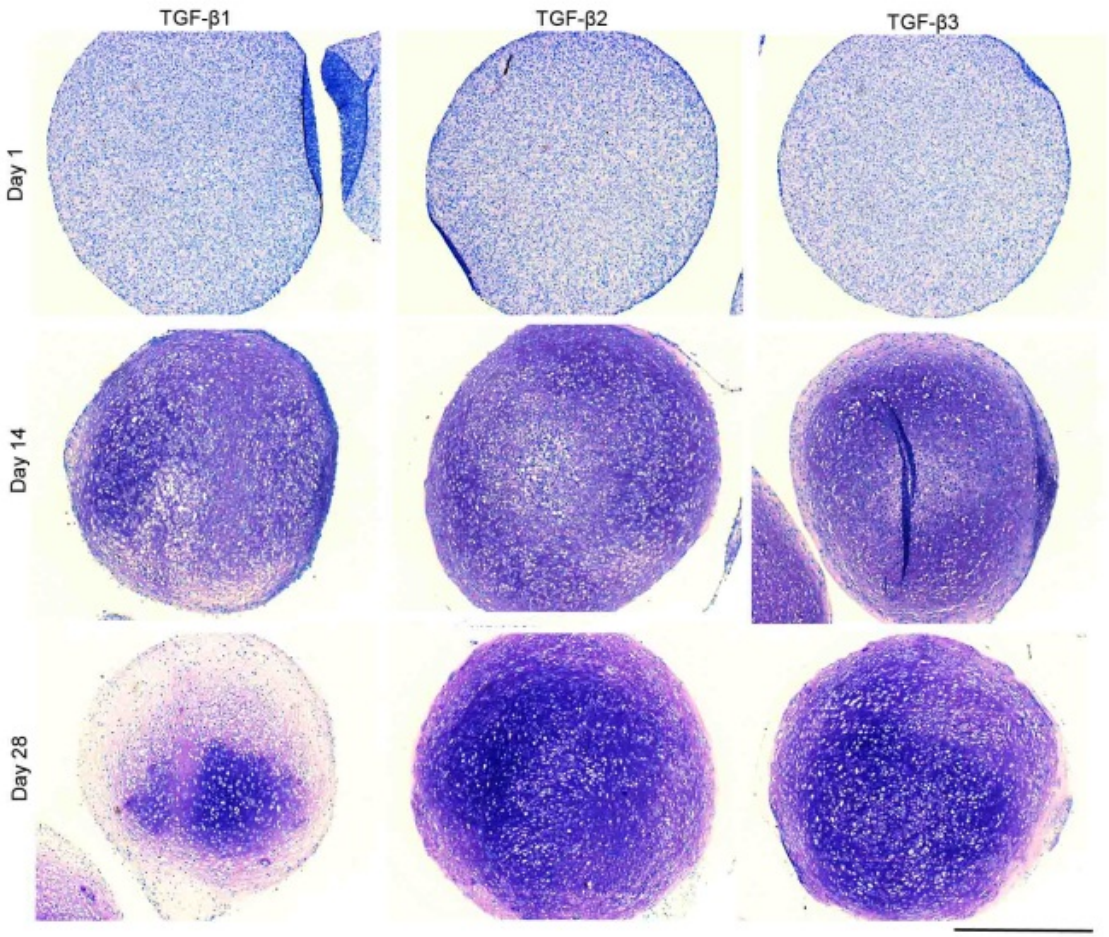

Figure 4.1. Photomicrographs of pellets collected at days 1, 14 and 28 of pellet culture and stained with toluidine blue for glycosaminoglycan accumulation under the three culture conditions of supplementation with recombinant TGF-β1, - β2 or - β3. Matrix GAG staining increased from day 1 to day 14 or 28 samples. For several but not all horses, TGF-β1 at day 28 had reduced matrix staining compared to TFG-β2 or -β3. Scale bar = 200 μm

Gene expression

On day 1 of culture there was increased *COL2b* message in both TGF-β2 and -β3 supplemented pellets compared to TGF-β1, although this difference did not persist at day 14 and 28, where no differences were detected (Fig 4.2). At day 28, *SOX9* gene expression was significantly higher and *COL10* was significantly lower in TGF-β3 treated pellets compared to TGF-β1 and -β2 (Fig 3.2). TGF-β peptide supplementation with all 3 isoforms induced up-regulation of *TGFB1*, *TGFB2* and *TGFB3* message compared to the message from un-supplemented monolayer cultures (Fig 4.3). For day 1 cultures, there were significant differences in the degree of up-regulation: TGF-β1 peptide significantly induced gene expression of all three TGF-β isoforms, with significantly higher expression of *TGFB1* and *TGFB2* compared to *TGFB3*; TGF-β2 peptide resulted in significantly greater auto-induction compared to induction of *TGFB1* and *TGFB3*; and TGF-β3 peptide resulted in significantly greater induction of *TGFB1* and *TGFB2* compared to auto-induction of *TGFB3*.

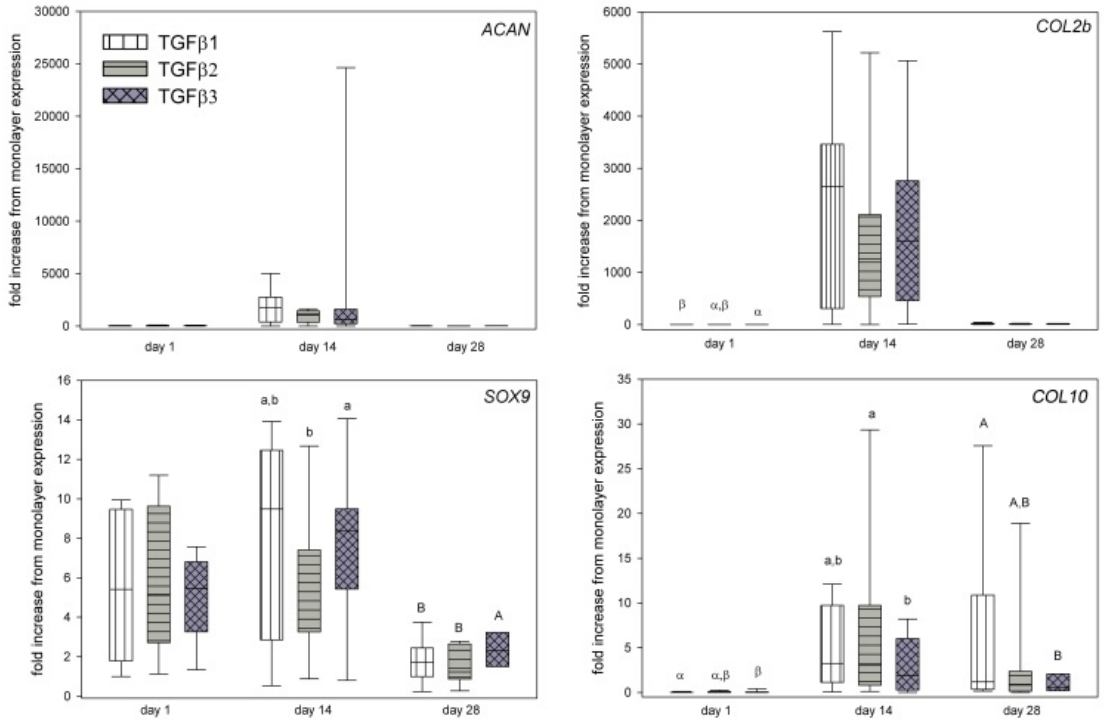

Figure 4.2. Box plots of gene expression for *aggrecan*, *collagen type IIb*, *SOX9*, and *collagen type 10* from pellets collected at days 1, 14 and 28 of pellet culture under the three culture conditions of supplementation with recombinant TGF-β1, -β2 or - β3. Differing symbols or letters demonstrate significant differences when they were detected between peptide supplemented groups within each timepoint.

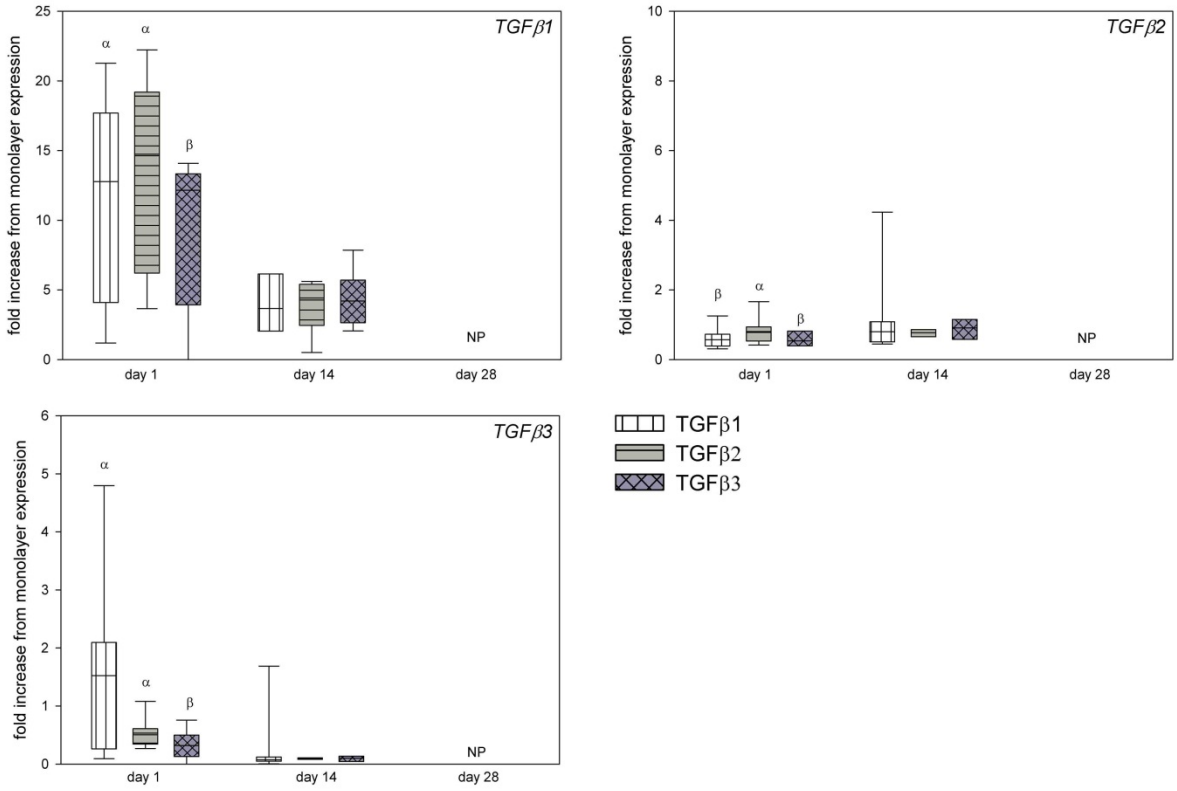

Figure 4.3. Box plots of gene expression for *TGFB1*, *2* and *3* from pellets collected at days 1, 14 and 28 of pellet culture under the three culture conditions of supplementation with recombinant TGF-β1, - β2 or - β3. Differing symbols or letters demonstrate significant differences when they were detected between peptide supplemented groups within each timepoint.

Supernatant media ALP activity and GAG content

At days 1 and 14, alkaline phosphatase activity of the supernatant media was not significantly different between TGF-β1, -β2 or -β3 supplementation. However at day 28, the TGF-β2 pellets had significantly higher ALP activity compared to TGF-β1, but not different compared to TGF-β3 (Fig 4.4). There was increased total GAG content in supernatant medium for the TGF-β3 supplemented pellets collected as early as day 1 of culture that persisted through the 28 day culture period (Fig 4.4).

Biochemical assays

There were no differences in the total GAG content or DNA content of the pellets within each timepoint (Fig 4.4).

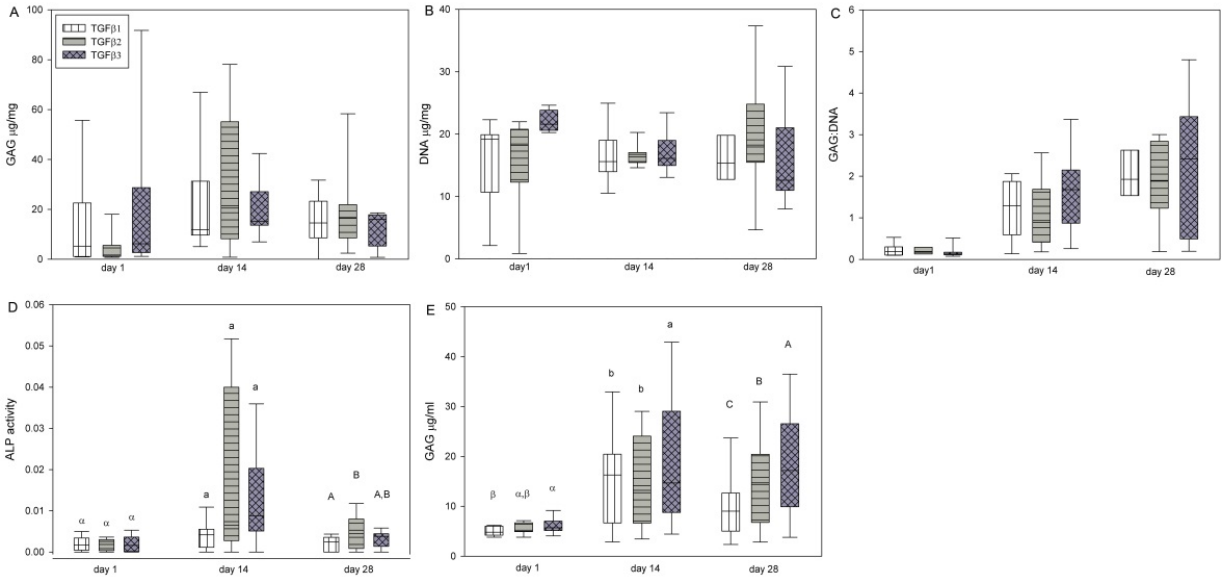

Figure 4.4. Box plots of results for biochemical assays; A) total glycosaminoglycan content (GAG), B) total DNA content, and C) GAG:DNA ratio of the pellets, D) total alkaline phosphatase activity (ALP) and E) total GAG content from the supernatant media from pellet cultures at days 1, 14, and 28 of pellet culture supplemented with recombinant TGF-β1, -β2 or -β3. Differing superscript symbols or numbers indicate statistical differences between TGF-β treated groups.

DISCUSSION

Novel to this study, we showed that there are differences in the degree of chondrogenesis and hypertrophy following three-dimensional pellet culture, using established methodology(Johnstone et al. 1998; Yoo et al. 1998) of MSCs in chondrogenic medium supplemented with 10 ng/ml of either TGF-β1, -β2 or -β3. In our study, TGF-β3 improved chondrogenesis, through maintenance of greater *SOX9* expression, and reduced indices of hypertrophy with reduced *COL10* expression and supernatant media ALP activity, and increased supernatant media GAG content. Our findings are in contrast to recent publications where there were minimal differences due to TGF-β isoform supplementation. Cals et al. found no difference in chondrogenesis and hypertrophy and minimal differences in amount of mineral deposition due to TGF-β1, -β2 or -β3 and β-glycerophosphate supplementation.(Cals et al. 2011) Similarly, Mueller et al. found no difference in chondrogenesis and terminal differentiation due to TGF-β1 versus -β3 peptide supplementation.(Mueller et al. 2010)

Our study demonstrated minimal differences in MSC chondrogenic transformation to a hyaline like phenotype between the three TGF-β isoforms. Other than increased *COL2b* message on day 1 in TGFβ1 and 2 supplemented pellets, there were no significant differences in the chondrogenic markers, *ACAN* and *COL2b*, at all other time points, nor were there differences in the amount of total GAG accumulation within the pellets assessed biochemically and histologically. There was, however, a significantly higher amount of GAG accumulation within the supernatant media of TGF-β3 supplemented pellets, throughout the experiment, suggesting that TGF-β3 supplementation might be superior for chondrogenic induction.

During development and endochondral ossification, Sox9 is required for the induction of chondrogenesis,(Bi et al. 1999) and during the transition to chondrocyte hypertrophy, *SOX9* expression must be turned off.(Bi et al. 1999; Bi et al. 2001; Lefebvre et al. 1998) Conversely, it is known that maintenance of *SOX9* expression will prevent chondrocyte hypertrophy and terminal differentiation.(Akiyama et al. 2004) Therefore, the increased *SOX9* message, starting at day 14 and persisting through day 28, in the TGF-β3 treated pellets, suggests that there is improved signaling for the prevention of chondrocyte hypertrophy with TGF-β3 supplementation versus TGF-β1 and -β2.

Collagen type X is produced exclusively by pre-hypertrophic and hypertrophic chondrocytes during chondrogenesis,(Lefebvre et al. 1998; Lefebvre and Smits 2005) and its expression is used as a marker of chondrocyte hypertrophy.(Ichinose et al. 2005; Winter et al. 2003) The increased *COL10* message in TGF-β1 and -β2 supplemented pellet cultures compared to TGF-β3, further supports the notion that TGF-β3 supplementation improved prevention of chondrocyte-like MSC hypertrophy. Additional tests to assess the progression toward terminal differentiation, such as Von Kossa staining for the presence of mineralized matrix, was not performed as hypertrophic medium without TGF-β and dexamethasone but with β-glycerophosphate,(Mueller and Tuan 2008) was not utilized and cultures were only continued to day 28; therefore, mineralization was not expected. Staining for ALP activity may have also provided additional evidence for differences in hypertrophy, but was not performed due to paraformaldehyde fixation rather than cryo-preservation of pellets.

It is well known that MSCs from bone marrow origin are a morphologically and functionally heterogeneous population of cells with both between-donor and within-donor variability.(Kucia et al. 2005; Phinney et al. 1999) This variability can have an effect on the

chondrogenic potential of MSCs during pellet culture.(Cals et al. 2011; Mueller and Tuan 2008; Mwale et al. 2006) Many investigators utilize a mixed pool of MSCs from several different donors, presumably in an effort to minimize the effect of heterogeneity of MSC cultures. However, we elected to perform the experiment with pellets from 9 individual donors rather than a mixed population of donors. This was done because MSCs are exquisitely responsive to their micro-environment and cell to cell contacts,(Prockop 2009) and mixing of MSCs from different donors may confound chondrogenesis studies. This resulted in wide variability between horses in molecular and biochemical data. However, despite the variability, we were able to detect differences due to differing peptide supplementation because there was minimal variability within the replicates for each horse. Additionally, to lessen culture induced variability between horses, we used early passage cells to minimize the inadvertent selection of different populations of cells from each donor. Finally, by using a large group of donors (n=9), we hoped to account for the variability of bone marrow derived MSC populations.

Although we found differences in chondrogenesis and hypertrophy of MSC pellet cultures, we did not directly assess progression of chondrogenesis to terminal differentiation. Although it seems likely that a change in the degree of hypertrophy would affect the degree of mineralization, we have no direct evidence for this and therefore, we cannot make this conclusion. Changes in terminal differentiation due to pre-differentiation with the TGF-β isoforms could be investigated through continuation of pellet culture beyond day 28 in medium containing a phosphate donor to assess for mineralization as was performed by Mueller and Tuan.(Mueller and Tuan 2008)

It is clear that although TGF-β3 supplementation was able to reduce indices of chondrocyte hypertrophy, it was not complete. Despite maintenance of the increased *SOX9* and

reduced *COL10* expression, and continued high GAG production to the supernatant media, of TGF-β3 treated MSC pellets at day 28, there was a marked reduction, although still above that of the monolayer cultures, in *collagen type II* and *aggrecan* gene expression. Identification of other factors that could be used in concert with TGF-β3 supplementation may further reduce progression toward terminal differentiation.

In summary, our observations have shown that equine bone marrow derived MSCs in pellet culture have clear differences in the degree of chondrogenesis and chondrocyte hypertrophy due to differing TGF-β peptide supplementation of the chondrogenic medium, favoring TGF-β3 versus , -β1 or -β2. This was demonstrated on the basis of reduced *COL10* expression and media ALP activity (compared to TGF-β2) and increased *SOX9* expression and GAG production to supernatant media. Therefore, it is important that future MSC chondrogenesis studies report the isoform used during chondrogenic studies. Because the pre-differentiated state at the time of implantation determines MSC fate in vivo,(Dickhut et al. 2009; Liu et al. 2008; Pelttari et al. 2006) the results of this experiment should now be tested for effects of the TGF-β isoforms during pre-differentiation prior to three-dimensional culture and cartilage defect implantation.

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Overexpression of transforming growth factor beta-3 improves chondrogenesis of equine mesenchymal stem cells compared to transforming growth factor beta -1 and -2

ABSTRACT

Objective: To compare chondrogenesis of bone marrow derived mesenchymal stem cells (MSCs) in pellet culture over-expressing transforming growth factor beta-1, -2 or -3 (TGF-β1, -β2 or -β3). **Methods**: MSCs from 5 young adult horses were mixed, transduced with adenovirus, consolidated to pellets and maintained in chondrogenic medium without supplemental TGF-β for 3 weeks. Pellets and media were tested at days 1, 7, 14 and 21 for selected gene expression (*ACAN, COL2B, TGFB1, TGFB2, TGFB3, 18S*), total glycosaminoglycan (GAG) and DNA content, supernatant media GAG content, and histology (H&E, toluidine blue). **Results**: All three AdTGF-β groups resulted in significantly increased transgene expression at all time points and subsequent chondrogenic transformation. By day 7, AdTGF-β3 had resulted in significantly higher *ACAN* and *COL2B* gene expression compared to AdTGF-β1 or -β2. There was higher GAG accumulation in AdTGF-β3 pellets at day 7 and 14 based upon dimethylmethylene blue assay. Histologically, there was minimal GAG accumulation in pellet matrices from any group at any time point. There were no differences in GAG accumulation in the supernatant media at any time point. **Conclusion**: Viral delivery and TGF-β3 gene overexpression was more effective at chondrogenic lineage patterning of equine MSCs in three-dimensional culture than TGF-β1 or β2. Ad-TGF-β3 transgene MSCs may be useful in the treatment of cartilage injury for cell replacement and for short term TGF-β3 delivery to the joint. In addition to the induction of chondrogenesis of implanted MSCs, transgene TGF-β3 may influence endogenous progenitors, surrounding chondrocytes, and the synovial membrane.

INTRODUCTION

Osteoarthritis is the most common joint malady in horses(Jeffcott et al. 1982) and is often a sequelae to focal articular cartilage injury.(Mankin 1982; Strauss et al. 2005) Cell based therapy(Brittberg et al. 1994) for the repair of articular cartilage injury(Brittberg et al. 1994; Hendrickson et al. 1994; Sams and Nixon 1995)is being pursued due to poor intrinsic healing of injured cartilage,(Mankin 1982) poor long term response to surgical therapies(Hunziker 1999) and lack of effective disease modifying osteoarthritis drugs.(Hunter 2011; Qvist et al. 2008) Repair with chondrocyte grafts improves long term outcome(Ortved et al. 2011) but must be either allogeneic, with risk of immune rejection,(Elves 1974; Hyc et al. 1997) or autogenous, with donor site morbidity.(Matricali et al. 2010) Adult, bone-marrow derived mesenchymal stem cells (MSCs) are a stem cell source for autologous cell transplantation to musculoskeletal tissues with minimal donor site morbidity and good proliferation and chondrogenic differentiation potential.(Pittenger et al. 1999; Johnstone et al. 1998; Mackay et al. 1998) MSCs have been utilized for joint tissue regeneration in horses indirectly through the application of microfracture followed by intra-articular injection(McIlwraith et al. 2011) and directly with the arthroscopic application to focal cartilage defects of concentrated bone marrow MSCs grafts(Fortier et al. 2010) and culture expanded MSC grafts.(Kuroda et al. 2007; Matsumoto et al. 2010; Wakitani et al. 2011; Wilke et al. 2007) However, it has been noted that MSCs are no better than chondrocytes in cartilage defect repair because they are unable to maintain the chondrocyte phenotype following implantation.(De Bari et al. 2004) When implanted subcutaneously to the nude mouse, MSCs form bone(Pelttari et al. 2006) unless they are influenced by exogenous TGF-β, in which case they form stable hyaline cartilage.(Re'em et al. 2012) Therefore, in order to achieve chondrogenesis of superior quality from MSCs in vivo, continued exposure of chondrogenic growth factors after implantation may be required.

Gene therapy approaches provide long-term exposure of implanted cells in cartilage defects to mitogenic and anabolic growth factors, and both IGF-1 and BMP-7 overexpression in transplanted chondrocytes has facilitated early cartilage healing(Ha et al. 2012).(Goodrich et al. 2007; Hidaka et al. 2003) For chondrogenic induction of MSCs in vitro, transforming growth factor beta (TGF-β1, -β2 or -β3) supplementation has been used routinely,(Mackay et al. 1998; Tuan 2004) and it has been suggested that continued supplementation of TGF-β may improve the quality of repair tissue following MSC implantation to cartilage defects.(Ha et al. 2012) To achieve continued TGF-β supplementation without recombinant peptide, an adenoviral vector was constructed for transgene over-expression of TGF-β1, -β2 or -β3.

Gene induced chondrogenesis of MSCs has been evaluated using single TGF-β isoforms(Guo et al. 2006; Pagnotto et al 2007, or combinations of TGF-β1 and SOX transcription factors or BMPs.(Steinert et al 2007 &2009) Several studies suggest SOX9 or the SOX trio (SOX5,6, and 9) genes can also drive MSC chondrogenesis.(Park et al. 2011) However, the focus of most experiments assessing MSC differentiation down the cartilage lineage continues to involve TGF-β isoforms. A direct comparison of the potency of TGF-β1, -β2 or -β3 gene transduction for MSC chondrogenesis has not been described.

The purpose of this study was to compare the differing efficiencies of the TGF-β isoforms for chondrogenic induction ofMSCs in pellet culture, by utilizing TGF-β1, -β2 or -β3 gene transduction. High efficiency adenoviral vectors over-expressing the TGF-β genes were used in a 3D micropellet culture model. Ultimately, adenoviral delivery of a TGF-β transgene to MSCs being prepared for cartilage defect repair could be useful to improve immediate and later chondrogenesis of MSCs as they populate cartilage defects in vivo. Previously, we have demonstrated improved chondrogenesis of equine MSCs exposed to supplemental recombinant

TGF-β3 compared to TGF-β1 and -β2.(Watts et al. 2011) Given the improved MSC chondrogenesis from TGF-β3 peptide supplementation, we hypothesized that the transgenes would be effective for chondrogenic induction and that AdTGF-β3 would induce MSC chondrogenesis more thoroughly compared to AdTGF-β1 and -β2.

MATERIALS AND METHODS

Study Design

Bone marrow derived MSCs were transduced with AdTGF-β1, AdTGF-β2, AdTGF-β3, AdGFP, or no transduction control. Twenty four hours after transduction or no transduction control, MSCs were consolidated to cell pellets (500,000 cells/pellet) and maintained in chondrogenic medium without supplemental TGF-β (for AdTGF-β1, AdTGF-β2, AdTGF-β3, AdGFP, no transduction control negative cultures), or chondrogenic medium with supplemental TGF-β3 (no transduction control positive). Pellets and supernatant media were collected on days 1, 7, 14 and 21 for gene expression of selected genes, biochemical assays for total glycosaminoglycan (GAG) and DNA content, and routine histology.

Adenovirus Generation

A recombinant, replication deficient (E1 and partial E3 deleted from the Ad serotype 5 genome) adenovirus was constructed via Cre-lox recombination with cDNA encoding equine TGF-β1, -β2 or -β3 and GFP in the human embryonic kidney cell line (HEK 293 cells).(Hardy et al. 1997) The construct contained the cytomegalovirus early promoter enhancer, the transgene of interest and the SV 40 polyA tail. The construct for AdTGF-β3 was made in combination with a GFP reporter. Virus was purified by centrifugal separation in a cesium chloride gradient and salts were removed by overnight dialysis in a sucrose buffer. The viral titer was estimated

spectrophotometrically, and infectious titer carrying the transgene estimated to be 1% of this total.

Bone Marrow Collection and MSC Isolation

Bone marrow aspirates were obtained from the sternum of 5 young adult horses.(Fortier et al. 1998) Local anesthesia and light sedation was used for bone marrow collection. Bone marrow biopsy needles (Jamshidi, VWR Scientific, Bridgeport, NJ) were used to aspirate bone marrow into four 60-mL syringes containing heparin (APP Pharmaceuticals, LLC; Schaumburg, IL 60173) for a final concentration of 1,000 units/ml. Each 60 ml was collected from a separate site with advancement of the Jamshidi needle after each 15 ml of marrow had been drawn. Bone marrow aspirate was diluted 1:1 in growth medium (Dulbecco's modified Eagles' media; 1,000 mg/L glucose; 2 mM L-glutamine; 100 units/ml penicillin-streptomycin; 1 ng/ml bFGF; 10% fetal calf serum) and 60 ml was plated to T-175 tissue culture flasks each and maintained at 37°C, 5% CO_2, and 95% humidity in room air. Non-adherent cells were removed through daily feeding. Once colony formation was evident, adherent cells were passaged using trypsin and replated at 20,000 cells/cm^2, and fed every other day. Monolayer cultures were passaged a second time when plates were 80-90% confluent. When passage 2 cultures were 80-90% confluent, adherent MSCs from each horse were combined and plated at 20,000 cells/cm^2. The following day cultures were prepared for transduction.

Transduction

Monolayers were washed twice with serum free balanced salt solution. Three ml of high glucose DMEM with 10,000 infectious viral particles per cell (100 MOI), or control with no virus, was applied in T175 tissue culture flasks. Cells were incubated at 37°C for 90 minutes without agitation. After 90 minutes, 30 ml of chondrogenic medium (CM; high glucose DMEM;

12.5 ml/500 ml HEPES buffer; 100 nM dexamethasone; 50 µg/ml ascorbate 2 phosphate; 100 µg/ml sodium pyruvate; 40 µg/ml proline; 1x ITS+; 100 IU/ml penicillin-streptomycin) without supplemental TGF-β was added to monolayers, and flasks were returned to the incubator for an additional 24 hours.

Pellet Culture

Chondrogenic differentiation was performed via pellet culture.(Johnstone et al. 1998; Penick et al. 2005) Transduced cells were collected via trypsinization and resuspended to $2x10^6$ cells/ml of chondrogenic medium. Aliquots of 250 µl were dispensed to wells of 96 well, 'v' bottom, polypropylene plates (PHENIX Research Products, 73 Ridgeway Road Candler, NC 28715) for each group, so that there were 28 pellets for each group and each time point. Plates were spun in a swinging bucket rotor at 400g for 10 minutes for pellet formation of $5x10^5$ cells per pellet. For the no transduction control groups, CM with 10 ng/ml supplemental TGF-β3 was used (Recombinant Human TGF- β3; Gibco Invitrogen 542 Flynn Road, Camarillo, CA 93012) for CM+ group, and CM without added TGF-β3 for CM- group.

Two hundred µl of CM was exchanged daily and pellets were maintained at 37°C, 5% CO_2, and 95% humidity in room air until the harvest time point at day 1, 7, 14 or 21.

Gene Expression

Samples of monolayer cells from each group prior to pellet formation were collected and frozen (-80°C) in lysis buffer for later RNA isolation. Twelve pellets from each group were isolated at each time point (days 1, 14, or 28) with combination of 2 pellets per sample, for an n=6 for each group and time point. Pellets were pulverized with a mortar and pestle while immersed in Lysis Buffer and kept on ice followed by homogenization with a commercially available homogenizer (Qiashredder, Qiagen) and RNA extraction kit (RNeasy® Plus Mini Kit,

Qiagen). For quantitative PCR, the primers and dual-labeled fluorescent probe [6-FAM as the 5' label (reporter dye) and TAMRA as the 3' label (quenching dye)] were designed using Primer Express Software version 2.0b8a (Applied Biosystems) using equine specific sequences published in Genbank (Table 5.1).

Table 5.1 Forward and reverse primers used for rt-PCR.

Table 5.1

18S-Fwd CGGCTTTGGTGACTCTAGATAACC *18S*-Rev CCATGGTAGGCACAGCGACTA;

COL2b-Fwd CGCTGTCCTTCGGTGTCA, *COL2b*-Rev CTTGATGTCTCCAGGTTCTCCTT;

ACN-Fwd GATGCCACTGCCACAAAACA, *ACN*-Rev GGGTTTCACTGTGAGGATCACA;

TGFB1-Fwd TCCTGGCGCTACCTCAGTAAC, *TGFB1*-Rev TGACATCAAAGGACAGCCATTC;

TGFB2-Fwd CGCTCGATATGGACCAGTTCA, *TGFB2*-Rev CTGGTGCTGTTGTAGATGGAAATC;

TGFB3-Fwd GCCTGGCGGAGCACAAT, *TGFB3*-Rev CGGAATTCTGCTCGGAACA.

Genomic DNA was removed from RNA samples prior to PCR, by selective filter centrifugation. Total RNA was reverse transcribed and amplified using the One-Step RT-PCR technique and the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Life Technologies, Carlsbad, CA). Samples for each molecule for each time point were assessed on the same qPCR plate to minimize variation. The qPCR program included reverse transcription at 48°C for 30 minutes and denaturing at 95°C for 10 minutes, followed by 40 cycles of 90°C for 15 seconds and 60°C for 1 minute. Each well of the qPCR plate was loaded with 10 ng of RNA in 20 µl. Other than *18S*, a standard curve was generated from equine specific plasmid DNA for each gene at known concentrations to allow copy number estimation. All samples were run in duplicate on the qPCR plate and total copy number per 10 ng of RNA of each gene was obtained from a standard curve and normalized to *18S* gene expression.

Biochemical Analysis of Pellets

Twelve pellets for each group and time point were collected and combined for an n=6 for biochemical assays. For total glycosaminoglycan and total DNA assay, samples were digested in papain (1 mL papain [0.5mg/ml]/pellet) at 65°C for 4 and 24 hours, respectively. The samples were mixed with dimethylmethylene blue dye for glycosaminoglycan quantification by colorimetric assay(Farndale et al. 1986) and bisbenzimide compound for DNA quantification by fluorometric assay (Kim et al. 1988) in triplicate aliquots.

Biochemical Media Analysis

Four aliquots of culture supernatants were collected for each group, at days 1, 7, 14 and 21. For total glycosaminoglycan, samples were digested in papain (1 mL papain [0.5mg/ml]/ ml conditioned media) at 65°C for 4 hours. The samples were mixed with dimethylmethylene blue

dye for glycosaminoglycan quantification by colorimetric assay(Farndale et al. 1986) in triplicate aliquots.

Histology

Four pellets per group and time point, were fixed in 4% paraformaldehyde-phosphate buffered saline for 12 hours. Pellets were then stabilized in 2% agarose gel, processed and embedded in paraffin, sectioned (5µm) and stained using standard procedures for hematoxylin and eosin (H&E) and toluidine blue.

Statistical Analysis

As data were not normally distributed, non-parametric tests were used. Differences between groups were detected by Kruskal-Wallis 1-way ANOVA with follow up multiple comparisons. Statistical analyses were performed with a commercially available software (Statistix 9; Tallahassee, FL, 32317) and the level of significance was set at $P<0.05$.

RESULTS

Adenoviral Transduction & Pellet Culture

Transduction with all adenovirus constructs was successful with transgene expression and translation. The efficiency of transduction based upon GFP positive cells for GFP containing constructs was estimated to be 90-95% (data not shown). Subjectively, there were no differences between groups in pellet size, either grossly or on histologic sections (Fig 1), suggesting matrix accumulation was not different between groups.

Histology

Pellets from each groups had matrix accumulation evident between days 1, 7, and 14. Pellets on day 14 and 21 had increasingly dark staining nuclei in the pellet centers and further

matrix accumulation was not different between day 14 and 21. The development of lacunae-like structures within the central region of the pellets did not appear to be different between the different groups and did not appear to progress between days 14 and 21. Slight metachromatic staining on toluidine blue histochemistry demonstrated slight GAG accumulation at day 7 (Fig 5.1) without additional accumulation at day 14 or 21. Subjectively, there were no differences between the groups in GAG accumulation.

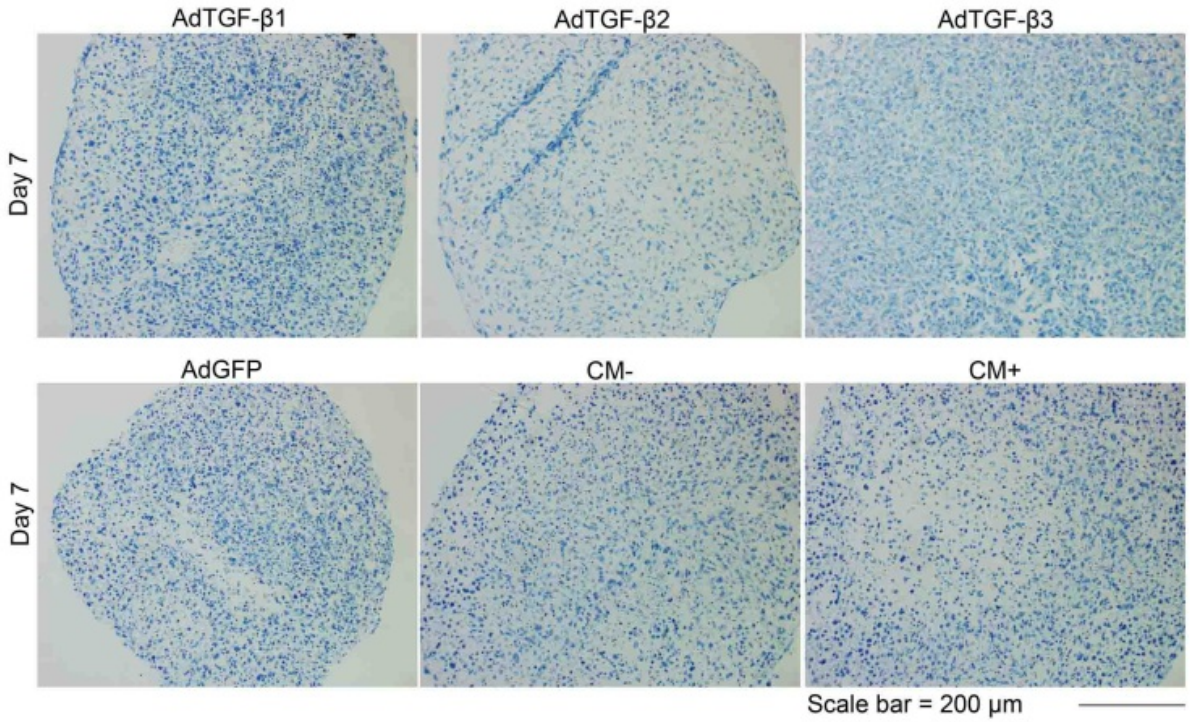

Figure 5.1. Photomicrographs of pellets collected on day 7 of pellet culture and stained with toluidine blue for glycosaminoglycan accumulation during chondrogenesis of mesenchymal stem cells transduced with AdTGF-β1 –β2 or –β3, AdGFP or no transduction controls with (CM+) and without (CM-) supplemental TGF-β3 peptide. 200x magnification. Scale bar = 200 μm

Gene expression

Good quality RNA was isolated from monolayers and pellets at day 1, 7 and 14 from all groups. Day 21 pellets did not yield sufficient RNA for gene expression analysis from any group. After transduction, there was significantly greater TGF-β1, -β2 and -β3 from the respective AdTGF-β1, -β2 and -β3 groups without differences between the other groups at all time points tested (monolayer, days 1, 7, 14). In monolayer cultures, 24 hours after transduction, there were no significant differences in *ACN* expression. On day 1 and day 7, there were significant differences in *ACN* expression (Table 4.2), with AdTGF-β1 and -β3 resulting in prominent increases in ACN expression compared to AdGFP. These differences were evident in day 7 samples, however, only AdTGF-β3 stimulated *ACN* expression beyond that evident in AdGFP cultures. There were no differences in *ACN* expression between groups on day 14 (Table 4.2). There was no gene expression of *COL2b* from monolayer or day 1 pellet cultures. On day 7, there were significant differences in *COL2b* gene expression between treatment groups with significant increases in AdTGF-β3 transduced cultures. AdTGF-β1, -β2, and AdGFP culture had no *COL2b* expression. On day 14, very low copy numbers of *COL2b* was expressed sporadically by all groups without significant differences (Table 5.2).

Table 5.2. Aggrecan gene expression in MSC cultures after transduction with AdTGF-β1, -β2 and -β3, AdGFP, and chondrogenic medium (CM+), or basal medium (CM-). 18S normalized copies/10ng. Data presented as median and interquartile range. Differing superscript letters indicate statistical differences between groups within each time point.

Table 5.2. Aggrecan gene expression. 18S normalized copies/10ng.				
	mono	day 1	day 7	day 14
AdTGF-B1	135(108-195)^{A}	2,152(1,936-2376)^{A}	259(167-369)^{BC}	0(0-310)^{A}
AdTGF-B2	228(179-253)^{A}	317(243-380)^{C}	120(70-350)^{C}	96(0-220)^{A}
AdTGF-B3	309(288-347)^{A}	1,361(1,238-1,503)^{AB}	4,787(3,603-11,244)^{AB}	95(0-260)^{A}
CM-	316(273-380^{A}	358(300-416)^{BC}	8,306(3,1157-16,770)^{A}	60(20-130)^{A}
CM+	224(193-266)^{A}	528(398-710)^{ABC}	3,554(2,380-4,630)^{AB}	45(0-120)^{A}
AdGFP	276(272-385)^{A}	328(232-346)^{C}	280(215-355)^{ABC}	130(0-400)^{A}

Table 5.3. Collagen type 2b gene expression in MSC cultures after transduction with AdTGF-β1, -β2 and -β3, AdGFP, and chondrogenic medium (CM+), or basal medium (CM-). 18S normalized copies/10ng. Data presented as median and interquartile range. Differing superscript letters indicate statistical differences between groups within each time point. ND = No expression detected.

Table 5.3. Collagen type 2b gene expression 18S normalized copies/10ng. Median and IQR.				
	mono	day 1	day 7	day 14
AdTGF-B1	ND	ND	0^{B}	ND
AdTGF-B2	ND	ND	0^{B}	ND
AdTGF-B3	ND	ND	$90(30\text{-}170)^{A}$	ND
CM-	ND	ND	$80(15\text{-}165)^{AB}$	ND
CM+	ND	ND	$80(50\text{-}105)^{AB}$	ND
AdGFP	ND	ND	0^{B}	ND

Supernatant media GAG content

There were no differences in accumulation of GAG to the supernatant media from any group within each time point. There were no differences between time points except media from day 21, which was significantly lower for all treatment groups.

Biochemical assays

There were significant differences in total GAG content in pellets on day 7 and day 14. MSC cultures transduced with TGF-β3 had increased GAG content compared to TGF-β1 and –β2 overexpressing cells. None of the gene transduced cultures developed GAG levels as high as the positive control culture pellets, exposed to steady state levels of TGF-β1 recombinant protein (Fig 5.2). There were no differences in the total GAG content of pellets on day 1 or day 21.

DNA content of pellets was similar on Day 1. On days 7, 14 and 21, there were significant differences in the total DNA content of pellets (Fig 5.3). TGF-β3 over-expression induced DNA increases compared to TGFβ1 and –β2 on all days assessed with a significant difference on day 14. DNA content in TGF-β3 transduced pellets was similar to cultures containing rhTGF-β1 at all time points.

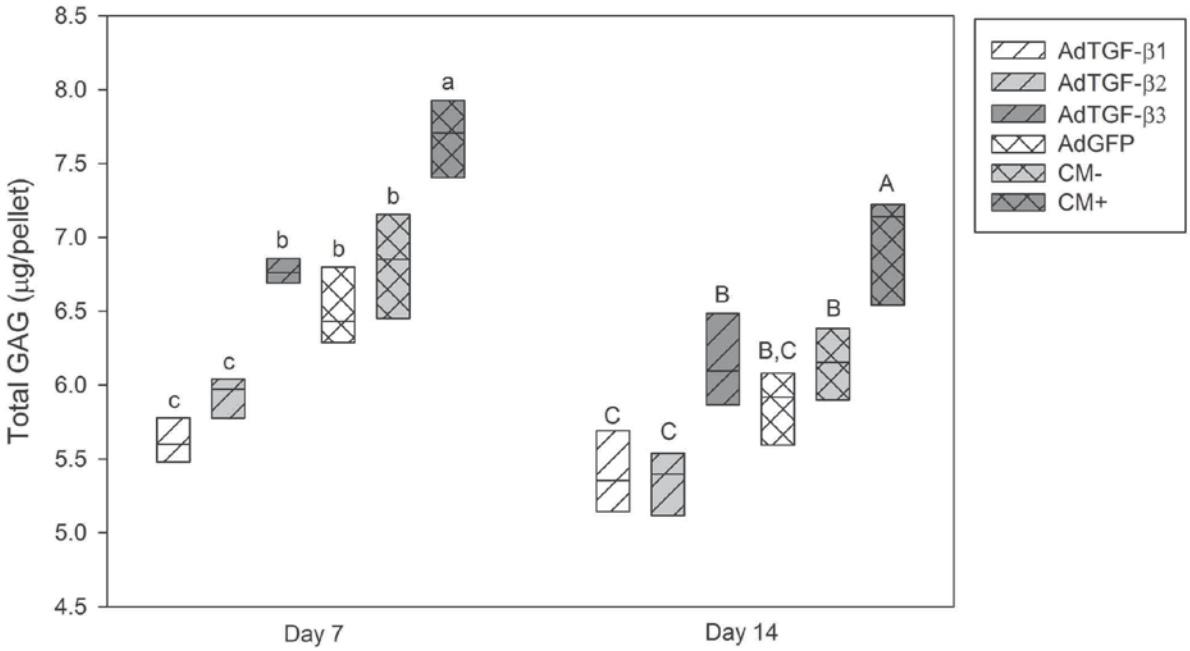

Figure 5.2**.** Box plots of total glycosaminoglycan per pellet at day 7 and 14 during chondrogenesis of mesenchymal stem cells transduced with AdTGF-β1 –β2 or –β3, AdGFP or no transduction controls with (CM+) and without (CM-) supplemental TGF-β3 peptide. Differing superscript letters indicate statistical differences between groups within each time point.

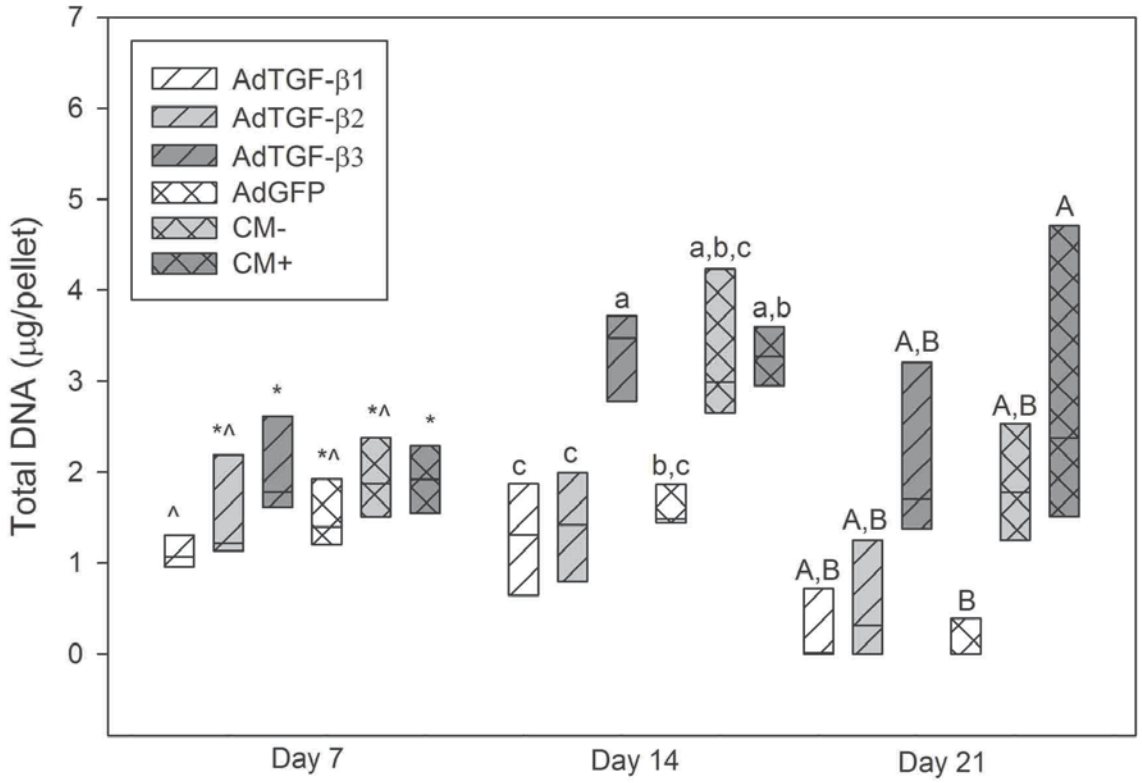

Figure 5.3**.** Box plots of total DNA per pellet at days 7, 14, and 21 during chondrogenesis of mesenchymal stem cells transduced with AdTGF-β1 –β2 or –β3, AdGFP or no transduction controls with (CM+) and without (CM-) supplemental TGF-β3 peptide. Differing superscript symbols or letters indicate statistical differences between groups within each time point.

DISCUSSION

Our results show the efficacy of adenoviral transduction of cultured mesenchymal stem cells with TGF-β transgenes to enhance chondrogenesis. Gene induced chondrogenesis with AdTGF-β3 most closely resembled the response in TGF-β1 recombinant peptide supplemented cultures (CM+),compared to AdTGF-β1 and -β2. The superiority of AdTGF-β3 was predominantly evident in increased *ACN* and *COL2b* expression and GAG accumulation, all markers of significant chondrogenesis.

Gene therapy techniques are preferable to peptide depots or repeated injection for growth factor delivery, as sustained endogenous growth factor production by gene therapy improves efficiency. Indeed, the design of these experiments may not provide a true reflection of *in vivo* responses of MSC to rhTGF-B which may only facilitate stem cell function for several hours before eluting from the graft. Conversely, in these long-term cultures, rhTGF-β1 is supplemented daily (CM+), which does not mimic the in vivo circumstance. Gene transduction of MSCs prior to implantation unquestionably extends TGF-β exposure. The synthesized peptide will undergo appropriate post-translational modification by the producing host cell, and will be produced continuously for a given duration depending on the viral vector and gene integration.(Evans et al. 1999)

Many methods for gene transfer in orthopedics are available. For satisfactory transduction efficiency and effective protein expression, the best-described gene therapy procedures involve viral vectors such as retrovirus, adeno-associated virus, adenovirus, herpes simplex virus, simian virus 40 (SV40), and papilloma virus.(Evans and Robbins 1994) Non-viral methods, such as electroporation, microinjection, protoplast fusion, or the application of liposomes, polybrene, or calcium phosphate, have also been described in other tissues, although

none have achieved significant success in joint therapy.(Evans and Robbins 1994) Of the virally mediated gene therapy techniques, there is major variability in genomic integration and subsequently the duration of gene over-expression. Data from this experiment suggest that the adenoviral vectors produced at least 2 weeks of transgene expression in equine MSCs. It is unknown whether a longer duration of transgene expression or permanent transgene expression would be required to enhance and maintain MSC chondrogenesis. Given the limitations of in vitro culture systems (day 21 cultures had variable nuclei pyknosis), it is quite likely this question will only be answered by in vivo experimentation.

Since methods for in vitro chondrogenesis of MSCs were first described,(Johnstone et al. 1998) TGF-β has been the most commonly utilized growth factor for MSC chondrogenic induction.(Puetzer et al. 2010)(Johnstone et al. 1998; Puetzer et al. 2010) Several in vitro studies have demonstrated that transduction with TGF-β significantly increases MSC proliferation and synthesis of hyaline cartilage (high molecular weight proteoglycans and type II collagen) in species other than the horse.(Caplan 2000; Gafni et al. 2004; Guo et al. 2006a; Mason et al. 2000; Trippel et al. 2004) In vivo and clinical studies have also demonstrated the utility of TGF-β gene transduction for chondrogenic induction and chondrogenic maintenance of MSCs and chondrocytes.(Ha et al. 2012) In full thickness articular cartilage defects in rabbits, MSCs (Guo et al. 2006b) or chondrocytes(Song et al. 2005) overexpressing the TGF-β1 transgene resulted in improved synthesis of hyaline cartilage, improved reconstitution of the subchondral bone and suppressed inflammatory immune responses. In a human safety study, osteoarthritic knees were injected with allogeneic chondrocytes expressing a TGF-β1 transgene over a 2 week period. No serious adverse events were noted; patients had improved symptoms and evidence of increased cartilage matrix thickness at higher cell doses.(Ha et al. 2012) While it seems intuitively obvious

that TGF-β exposure of MSCs will benefit chondrogenesis in situ, the benefits of TGF-β for enhancing matrix synthesis by chondrocytes are often found inferior to other growth factors such as IGF-1, BMP-2, and BMP-7. Given the extensive literature that supports the differing roles of TGF-β compared to other more anabolic growth factors, the aim of using TGF-β seems more appropriately targeted at cell proliferation and differentiation.

Despite studies elucidating the negative aspects of rhTGF-β use in joints, which include fibrosis and osteophyte formation, there have been reports of significant attributes to TGF-β application. Intra-articular TGF-β has anti-inflammatory properties,(Harvey et al. 1991; Hui et al. 2003; Redini et al. 1993; Roberts and Sporn 1993) immune modulating properties,(Blumenfeld and Livne 1999) inhibits chondrocyte terminal differentiation and mineralization(van Beuningen et al. 1994a; van Beuningen et al. 1994b; van Osch et al. 1998) and reduces the height of the cartilage hypertrophic zone.(Itayem et al. 1997) Despite the beneficial effects, widespread clinical application of TGF-β to the joint for cartilage repair and osteoarthritis therapy has been limited by the short half-life and short term effects of recombinant proteins(Ha et al. 2012) and need for repeated injections.(Hardingham et al. 1992) In vivo, AdTGF-β MSCs would continue to express the transgene, increasing the duration of TGF-β exposure compared to injection of recombinant protein. In the joint, TGF-β would also have autocrine and paracrine functions. Through autocrine action, TGF-β would induce chondrogenesis of implanted MSCs. Through paracrine action, TGF-β would affect endogenous progenitors in the subchondral bone and synovium, and potentially aid local chondrocyte function.

Recently, the intra-articular application of TGF-β has been criticized for intra-articular use(Fortier et al. 2011) because of its role in wound healing as a fibrotic agent.(Branton and

Kopp 1999) Additionally, intra-articular injection of TGF-β1 induced synovial fibrosis and osteophyte formation in a mouse OA model.(van Beuningen et al. 1994b) Worse still, in a study of AdTGF-β1 injection to rabbit knees, TGF-β1 overproduction resulted in massive fibrosis and mortality.(Mi et al. 2003) It is important to note that there are marked isoform specific differences among the TGF-β peptides; TGF-β1 and -β2 are associated with fibrosis and scarring whereas TGF-β3 results in reduced scarring.(Shah et al. 1995)

We propose that the adenoviral vector could be used to deliver a chondrogenic stimulus to MSCs in vitro, during isolation and expansion of bone marrow derived cells. One to 2 days after transduction, transgenic MSCs could be delivered to joints by direct injection or by arthroscopic implantation to cartilage defects. In this protocol, there would be minimal host response to the adenoviral vector because the viral capsid will deliver the transgene to the MSC nucleus and then disassemble while MSCs are still in vitro.(Trotman et al. 2001) Loss of the capsid(Molinier-Frenkel et al. 2000) prior to intra-articular implantation will minimize inflammatory host response to the adenoviral vector.{{1068 Molinier-Frenkel,V. 2000}}

A limitation of the MSC pellet culture system in the present study was the trend toward cellular senescence and apoptosis beyond day 14 of culture. Evidence for apoptosis included reduced total DNA content at day 21 for several groups, and pyknotic nuclei on histologic sections at days 14 and 21. Cellular senescence may have resulted in the lack of continued GAG accumulation in the pellets and media beyond day 14, and poor RNA quality and quantity on day 21. This is in contrast to our previous experience with equine MSC pellet culture where cultures can be continued to at least day 28. The reduced longevity of MSC pellet cultures was not a result of the adenoviral vectors, as the un-transduced controls (both CM- and CM+) did not have better longevity compared to non-transduced groups. Lack of long term survival may have

resulted from the combination of MSCs from different horses. MSCs are exquisitely responsive to their micro-environment and cell to cell contact,(Prockop 2009) and mixing of MSCs from different donors may confound pellet culture studies. Although this limited our assessments at the later time point, it did not hinder our ability to test the chondrogenic response of MSCs to AdTGF-β1, -β2 and -β3.

Our results demonstrate the efficacy of adenoviral transduction of cultured mesenchymal stem cells with engineered TGF-β genes to enhance chondrogenesis by delivering a biologically effective concentration of TGF-β over a 2 week duration. In agreement with work using TGF-β peptide supplementation during pellet culture in our lab, AdTGF-β3 was superior for the induction of MSC chondrogenesis compared to AdTGF-β1 or -β2 based on the increased *ACN* expression and GAG accumulation. This suggests the value of adenoviral-TGF-β3 gene therapy to MSCs for improved cartilage healing.

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Dual axis gene-therapy using stem cells overexpressing TGF-β3 in combination with IL-1β and TNF-α RNA silencing for osteoarthritis control in a large animal osteochondral chip fracture model.

ABSTRACT

Objective: To investigate the potential of a combinatorial gene therapy approach to osteoarthritis (OA) control using stem cells targeted to the chondrocyte lineage by TGF-β3 overexpression, while co-expressing a long hair-pin RNA silencing motif for IL-1 and TNF-α knockdown in an equine model.

Methods: Early OA was induced in a middle carpal joint of 13 horses by osteochondral fragmentation and treadmill exercise. The contralateral joint was sham operated. Treatments with either MSCs overexpressing TGF-β3 and the long hairpin silencing construct (n=6) or placebo (n=7) were injected to OA joints 14 days after OA induction. Sham joints were injected with placebo. Clinical exams were performed weekly. Tissues were retrieved 70 days after OA induction for molecular, biochemical and histology evaluation.

Results: No adverse treatment effects were observed. There were significant improvements in range of motion and effusion in the week following MSC treatment injection, joint effusion on day 49, and selected gene expression, otherwise, there no differences due to treatment. There were significant deteriorations due to OA induction in several clinical parameters, synovial GAG concentrations, synovial cytology parameters and molecular assays. Subjectively, there was increased synovial thickening and fibrosis in OA placebo injected compared to OA MSC injected groups.

Conclusions: These data indicate intra-articular injection of MSCs with transgene overexpression of TGF-β3 and knockdown of IL-1β and TNF-α may be an effective treatment for OA.

INTRODUCTION

Osteoarthritis (OA) is the most common musculoskeletal disease in man and is expected to be the fourth leading cause of disability by the year 2020.(Woolf and Pfleger 2003) Current treatment is symptomatic, until end-stage joint disease necessitates total joint arthroplasty.(Wieland et al. 2005) In contrast, successful regenerative techniques have been developed to improve repair of focal articular cartilage injury and prevent OA, including mosaicplasty(Matsusue et al. 1993) and autologous chondrocyte implantation.(Brittberg et al. 1994) Unlike acute articular cartilage injury, OA often affects cartilage throughout the joint, and extends to include the synovial membrane and subchondral bone. Generalized joint disease generally precludes focal autologous therapy options(Knutsen et al. 2004). A significant unmet need exists to develop a regenerative method applicable to generalized OA. Stem cell delivery of therapeutic genes may provide some of the elements to assist in OA control.

Friedenstein(Friedenstein et al. 1970) was the first to describe colony producing fibroblastic cells derived from bone marrow that are now most commonly referred to as mesenchymal (stromal) stem cells (MSCs). Mesenchymal stem cells may be an ideal cell choice for treating joint disease, as the cells can be manipulated in pre-implantation culture down appropriate articular tissue lineages. Differentiation studies have confirmed that MSCs can undergo chondrogenesis in vitro(Johnstone et al. 1998; Mackay et al. 1998; Pittenger et al. 1999), and implantation in animal studies have confirmed their chondrogenic potential in vivo.(Wakitani et al. 1994; Yan and Yu 2007)Subsequently, MSCs have been utilized for cartilage regeneration indirectly through intra-articular injection(McIlwraith et al. 2011), and directly by arthroscopic application of culture expanded MSCs in adherent vehicles to focal chondral defects.(Kuroda et al. 2007; Matsumoto et al. 2010; Wakitani et al. 2011; Wilke et al.

2007) These experiments in various animal models suggested expanded clinical trials were indicated. However, efficacy in preliminary clinical studies in man was not overwhelmingly positive. Issues of cell persistence, incomplete lineage penetration, and poor control of joint homeostasis all limit more widespread application.

Mesenchymal stem cells may also be useful in the treatment of more generalized joint diseases such as OA, where MSCs can impact synovial and capsular tissues, as well as seed fibrillated surfaces, rather than just directly seed focal articular cartilage defects.(Chen and Tuan 2008) Given their trophic, mitotic, immunomodulatory(Nauta and Fibbe 2007), and anti-inflammatory(Nemeth et al. 2009)(Nauta and Fibbe 2007) effects, coupled with chondrogenic potential(Mackay et al. 1998) and ability to engraft to the site of injury(Sordi 2009; Yagi et al. 2010), intra-articular injection of MSCs has been investigated in several animal models of OA. In these models, joint injection of MSCs reduced OA progression and improved healing of soft tissue articular injury(Nauta and Fibbe 2007) (Agung et al. 2006; Frisbie et al. 2009; Horie et al. 2009; Lee et al. 2007; McIlwraith et al. 2011; Mokbel et al. 2011; Murphy et al. 2003; Toghraie et al. 2011) Positive clinical results in veterinary (Black et al. 2007; Black et al. 2008; Ferris et al. 2009) and human patients(Centeno et al. 2008; Centeno et al. 2011; Davatchi et al. 2011) provide additional evidence for the value of direct intra-articular injection of MSCs for the treatment of OA. This has led to phase II clinical trials utilizing allogenic cultured MSCs for the treatment of knee OA (NCT01448434). However, an equine model investigating the effect of adipose derived stromal vascular fraction versus bone marrow derived MSCs found no treatment effect other than improvement in synovial PGE2 levels in bone marrow derived MSC treated joints.(Frisbie et al. 2009) Enhanced stem cell function through gene transduction of cells in

culture, may allow the benefits of cell and gene overexpression to normalize the joint environment and support cell repopulation of fibrillated cartilage.

Anti-cytokine therapy has proven effective in the treatment of rheumatoid arthritis(Elliott et al. 1993) and may also be useful in OA(Malemud 2010). In OA, IL-1 and TNF-α upregulate the inflammatory, apoptotic and destructive events as well as downregulate matrix synthesis(Bondeson et al. 2006; Fischer et al. 2000; Goldring 1999; Singh et al. 2003). However, controlling IL-1 activity through administration of recombinant IL-1Rα has failed to significantly improve OA symptoms in some double blind placebo controlled trials.(Chevalier et al. 2009) It may be that overproduction of IL-1 or modification of IL-1 receptors within the OA joints has led to IL-1Rα failure. If this is the case, non-competitive gene silencing through post-transcriptional gene knockdown of IL-1 may be effective where IL-1Rα has failed. (Elliott et al. 1993)

Dual axis gene therapy using a combination of anti-cytokine therapy and an anabolic growth factor may provide synergistic effects not apparent in single target manipulation. Growth factors from the transforming growth factor (TGF) superfamily may enhance chondrocyte function with increased production the (Gunther et al. 1994)of large proteoglycans(Malemud et al. 1991), reduced production of MMPs(Hui et al. 2001) and augmentation of TIMP synthesis(Hui et al. 2003)(Gunther et al. 1994; Hui et al. 2003). The TGF superfamily may also induce chondrogenesis of endogenous progenitors and trangene MSCs.

The purpose of this study was to investigate the in vivo potential of intra-articular injection of MSCs overexpressing the anabolic TGF-β3 gene concurrent with a long hairpin construct coding an RNA interference motif to suppress the catabolic IL-1β and TNF-α

cytokines, in an equine model for treatment of osteoarthritis (OA). The horse was selected as a model because spontaneous and traumatic OA in the equine athlete are similar to human OA.(McIlwraith et al. 2010) Additionally, there is published evidence for the efficacy of intra-articular MSC therapy in clinical equine joint disease,(Frisbie et al. 2007) similar to that reported for human OA patients.(Centeno et al. 2008; Centeno et al. 2011; Davatchi et al. 2011) We hypothesized that transgene overexpression in MSCs would reduce progression of OA compared to placebo after acute osteochondral fragmentation in the middle carpal joint of the horse.

MATERIALS & METHODS

Study Outline

Thirteen 3-7 year-old Thoroughbred horses that had previously been race trained and conditioned were utilized. All horses had radiographic and clinical evaluation confirming lack of pre-existing carpal disease prior to inclusion in this study. Bone marrow was harvested for MSC isolation and expansion, and horses were acclimated to daily treadmill exercise. One forelimb from each horse was randomly assigned to receive arthroscopic radial carpal osteochondral fragmentation for OA induction (OA joint; n=13). The contralateral forelimb received sham arthroscopic surgery without fragmentation (no OA joint; n=13). Each horse was randomly assigned to a treatment group [MSC (group 1) or placebo (group 2); Fig 6.1]. Two weeks after arthroscopic surgery for OA induction, OA joints were injected with transduced MSCs (n=6; gp1) or placebo (n=7; gp 2). Sham joints from group 1 and 2 horses were injected with placebo only. Forty-eight hours after treatment injection horses resumed treadmill exercise 5x/weekly. Seventy days after osteochondral fragmentation, horses were euthanized and tissues were collected. All procedures were approved by the university's Institutional Animal Care and Use Committee.

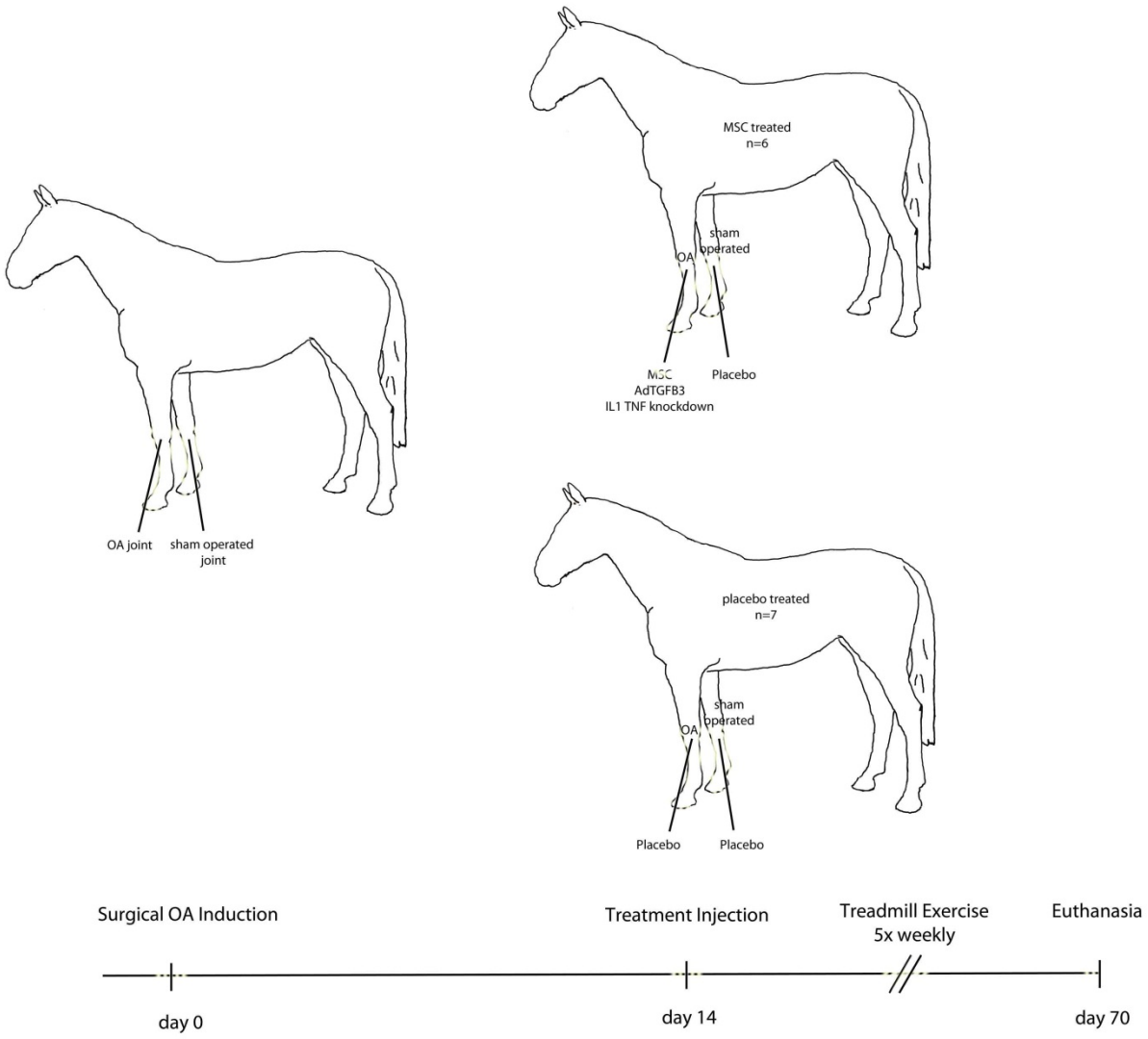

Figure 6.1. Outline of study timeline. One randomly selected middle carpal joint was operated for OA induction and the contralateral joint was sham operated in all horses. Two weeks later, treatment injections to the OA joints with either transgene MSCs or placebo were performed. Contralateral noOA joints were all injected with placebo. Thereafter, weekly clinical examinations and synovial fluid cytology was performed between day 14 and 70 and horses were euthanized for analysis and tissue collection on day 70.

Stem Cell Isolation

Bone marrow aspirates were obtained from the sternum of each horse.(Fortier et al. 1998) Local anesthesia and light sedation was used for bone marrow collection. Bone marrow biopsy needles (Jamshidi, VWR Scientific, Bridgeport, NJ) were used to aspirate bone marrow into four 60-mL syringes containing heparin (10,000 units/ml; APP Pharmaceuticals, LLC; Schaumburg, IL 60173), for a final concentration of 1,000 units/ml. Each 60 ml was collected from a separate site with advancement of the Jamshidi needle after each 15 ml of marrow had been drawn. Bone marrow aspirate was diluted 1:1 in growth medium (Dulbecco's modified Eagles' media; 1,000 mg/L glucose; 2 mM L-glutamine; 100 units/ml penicillin-streptomycin; 1 ng/ml bFGF; 10% stem cell tested fetal calf serum) and 60 ml was plated to T-175 tissue culture flasks each. Non-adherent cells were removed through daily feeding. Once colony formation was evident, adherent cells were passaged using trypsin and replated at 20,000 cells/cm^2, and fed every other day. Monolayer cultures were passaged a second time when plates were 80-90% confluent. When passage 2 cultures were 80-90% confluent, adherent MSCs were cryopreserved for later injection.

Long Hairpin RNA Generation

Preliminary studies designed and assessed five RNAi ribo-oligonucleotide constructs for ability to post-transcriptionally diminish IL-1β and TNF-α mRNA levels. Two DNA fragments of the coding sequence for antisense (19-nt) and sense (19-nt) RNA strands, joined by a 9-nt hair-pin loop, and capped by a 5T termination sequence were selected to knockdown IL-1β and TNF-α. Constructs were synthesized (IDT) and ligated with U6 promoter of RNA polymerase III in the pSIREN shuttle vector (Clontech). Clonally isolated plasmids were confirmed by

sequencing. Plasmids were then tested by transfection into equine MSCs via electroporation (Amaxa nucleofector; or BTX Electroporator) followed by LPS stimulation.

Adenovirus Generation

A recombinant, replication deficient (E1 and partial E3 deleted from the Ad serotype 5 genome) adenovirus was constructed via Cre-lox recombination with cDNA encoding equine TGF-β3 and GFP in the human embryonic kidney cell line (HEK 293 cells)(Hardy et al. 1997). The construct contained the cytomegalovirus early promoter enhancer, the transgene of interest and the SV 40 polyA tail. The construct for AdTGF-β3 was made in combination with a GFP reporter. Virus was purified by centrifugal separation in a cesium chloride gradient and salts were removed by overnight dialysis in a sucrose buffer. The viral titer was estimated spectrophotometrically, and infectious titer carrying the transgene estimated to be 1% of this total.

OA Induction

After routine preparation for arthroscopic surgery under general anesthesia the OA designated joint was explored, digitally photographed, and an 8 mm dorsomedial osteochondral fragment off the radial carpal bone was created as previously described (Fig 6.2).(Howard et al. 1994) The fragment was not free within the joint and remained attached to joint capsule dorsally. A motorized arthroscopic burr was used to debride parent subchondral bone to create a 15 mm defect with lack of congruity between the fragment and parent bone. Debris was retained within the joint and the joint was not flushed prior to routine closure. The sham operated joint (no OA) was arthroscopically explored, lavaged, and digitally photographed through similar arthroscopic portals. Postoperatively, limbs were maintained in bandages until 5 days after treatment injection.

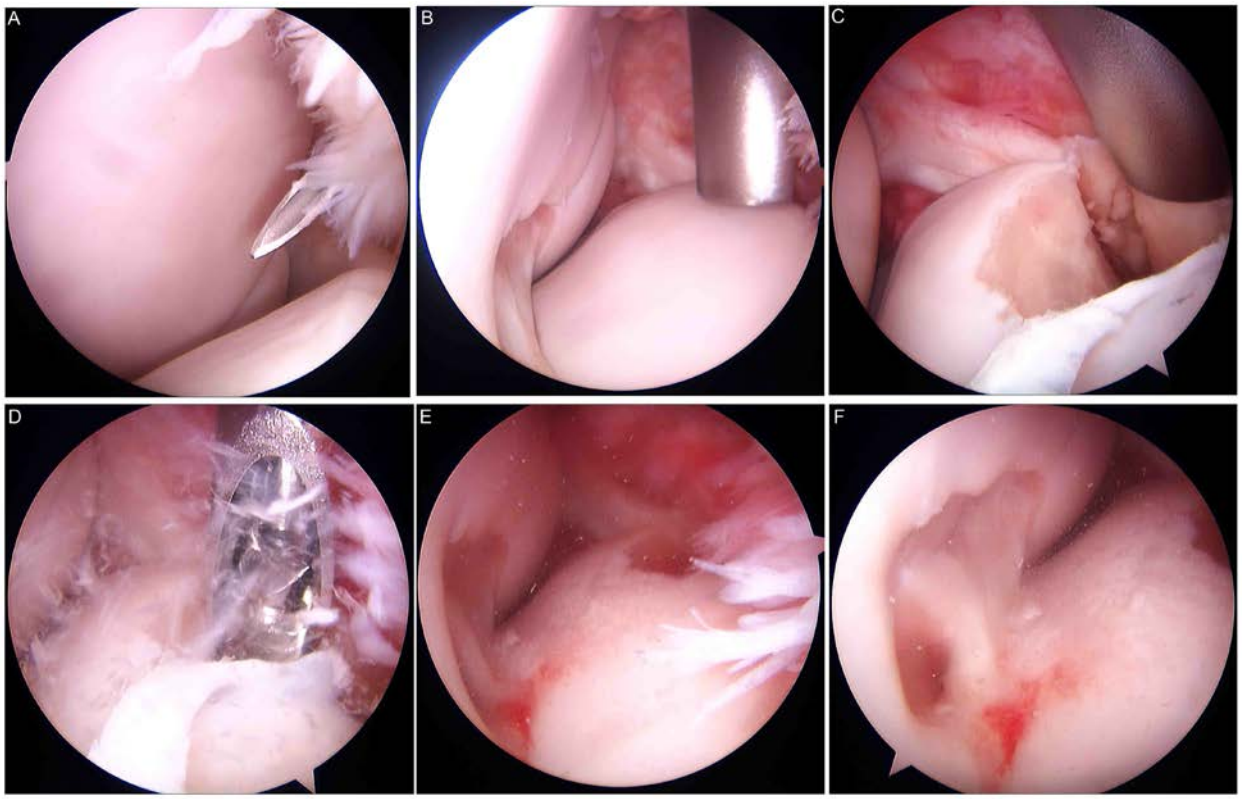

Figure 6.2 Arthroscopic osteochondral fragmentation of the dorsomedial surface of the radial carpal bone: A) Joints were explored and digitally photographed; B & C) an 8 mm gouge was used to create the osteochondral fragment; D) an arthrobur was used to debride the parent subchondral bone to a defect width of 15 mm; E & F) debris was retained within the joint.

Exercise

Prior to OA induction, horses were acclimated to treadmill exercise (1 mile of walk, 2 minutes of trot, 2 minutes of gallop, 2 minutes of trot and a 1 mile cool down at walk). For 2 weeks after OA induction, horses were not exercised, to allow arthroscopic portals to heal. Horses resumed treadmill exercise 48 hours after treatment injection and were continued on daily exercise 5x/weekly until termination at 70 days.

Cell Preparation

Cryopreserved MSCs were thawed, washed once in serum free balanced salt solution and plated in growth medium at 20,000 cells/cm^2. Forty eight to 72 hours later, growth medium was exchanged for antibiotic free growth medium for a minimum of 2 hours. MSCs were collected via trypsinization and resuspended to $2.5x10^6$ cells/mL in OptiMEM. Ten µg of sterile filtered Long Hairpin DNA in Tris EDTA was dispensed to 2 mm gap electro-cuvettes (VWR® North American, West Chester, PA). MSCs in OptiMEM were mixed 1:3 in cytosalts (10 mM K_2HPO_4/KH_2PO_4, 120 mM KCl, 0.15 mM $CaCl_2$, 5 mM $MgCl_2$) and immediately dispensed to cuvettes, followed by electroporation (550 V, 150 µsec pulse length, 2 pulses, 100 msec pulse interval) or sham electroporation (no pulse). Post-electroporation and sham electroporation cuvettes were placed on ice for a minimum of 5 minutes. Cells were collected from cuvettes, a portion was used for viability assay by fluorescein staining and propidium iodide exclusion, and the remainder were plated at 40,000 live cells/cm^2 in growth medium on 175 cm^2 tissue culture flasks.

The following day, medium was aspirated and adherent MSCs were washed twice with serum free balanced salt solution. Three ml of high glucose DMEM with 10,000 infectious adenovirus particles per cell (100 MOI), or control with no virus, was applied in T175 tissue culture flasks. Cells were incubated at 37°C for 90 minutes without agitation. After 90 minutes, 30 ml of serum free transduction medium (high glucose DMEM; 12.5 ml/500 ml HEPES buffer; 100 nM dexamethasone; 50 µg/ml ascorbate 2 phosphate; 100 µg/ml sodium pyruvate; 40 µg/ml proline; 1x ITS+; 100 IU/ml penicillin-streptomycin) was added to monolayers and flasks were returned to the incubator for an additional 24 hours.

Joint Injection

Labeled cells were collected by trypsinization, washed once in phosphate buffered saline and washed twice in Modified Eagles' Medium (MEM). A portion of MSCs and control MSCs (sham electroporated and mock transduced) were collected for RNA and DNA isolation. Cells were prepared in MEM ($5x10^6$ cells/ml) containing 40 µg/ml gentamicin for 2 mL injection per joint to horses in group 1. Sham operated joints (from groups 1 and 2) and placebo injected OA joints (group 2) were injected with a similar volume of cell free MEM and gentamicin.

Horses were sedated for joint injection. Injection to the middle carpal joint was made medial to the tendon of the extensor carpi radialis muscle. Horses were administered a non-steroidal anti-inflammatory drug at the time of joint injection (phenylbutazone; 4.4 mg/kg, IV) and once daily for 3 days after joint injection (phenylbutazone; 2.2 mg/kg, PO). Joints were maintained under a clean bandage for 5 days after joint injection. Horses were housed individually in box stalls without forced exercise until 48 hours after joint injection at which time daily treadmill exercise was resumed (5x/week).

Reaction to Injection

Effusion, range of motion (ROM), and lameness evident at a walk were assessed daily for the first week after treatment injection. Effusion was rated on a scale of 0-4 (0, no effusion; 1, slight effusion; 2, mild effusion; 3, moderate effusion; 4 marked effusion). Range of motion was rated on a scale of 0-4 (0, no reduction; 1, <25% reduction; 2, 25-50% reduction; 3, 50-75% reduction; 4, >75% reduction in ROM). Lameness was scaled using an 11 point scale(Watts et al. 2011b).

Clinical Exam

Weekly clinical examination was performed throughout the study. Effusion and ROM was graded 0-4 as described above for reaction to injection. Lameness was graded on a 0-5 scale,(American Association of Equine Practitioners 1991) and lameness reaction to joint flexion was graded on a 0-4 scale (0, no change in lameness; 1 - 4, slight - severe worsening of lameness).

Synovial Fluid

Synovial fluid was collected at the time of surgery and weekly thereafter. Cytologic examination was performed by a board certified veterinary clinical pathologist and total protein, total nucleated cell count and cellular differential was recorded. Synovial fluid was stored at -80°C for assay of glycosaminoglycan content.

Joint Tissue Processing & Analysis

Horses were euthanized by pentobarbital overdose 70 days after OA induction. Joints were opened, articular cartilage was given a gross score (0-4 where 0=normal, 1=mild

fibrillation, 2=deep fibrillation, 3= ulceration, 4=eburnation; third carpal bone intermediate facet; third carpal bone radial facet; radial carpal bone; intermediate carpal bone) and synovial membrane was given a gross score (synovial membrane color where 0=normal, 1=red, 2=red-brown, 3=brown, 4=brown-black; synovial membrane thickness where 0=normal, 1=slightly thick, 2=moderately thick, 3=severely thick, 4=fibrotic) by two observers and articular surfaces were digitally photographed. Articular cartilage , synovial membrane and osteochondral sections (Fig 6.3) were collected and fixed in 4% paraformaldehyde prior to paraffin embedding. Osteochondral sections were decalcified in citrate buffered formic acid. Cartilage sections were stained with toluidine blue, osteochondral sections were stained with safranin-O, and all sections were stained with hematoxylin and eosin, examined and digitally photographed (50 x magnification). Synovial membrane, cartilage and osteochondral sections were subjectively evaluated.

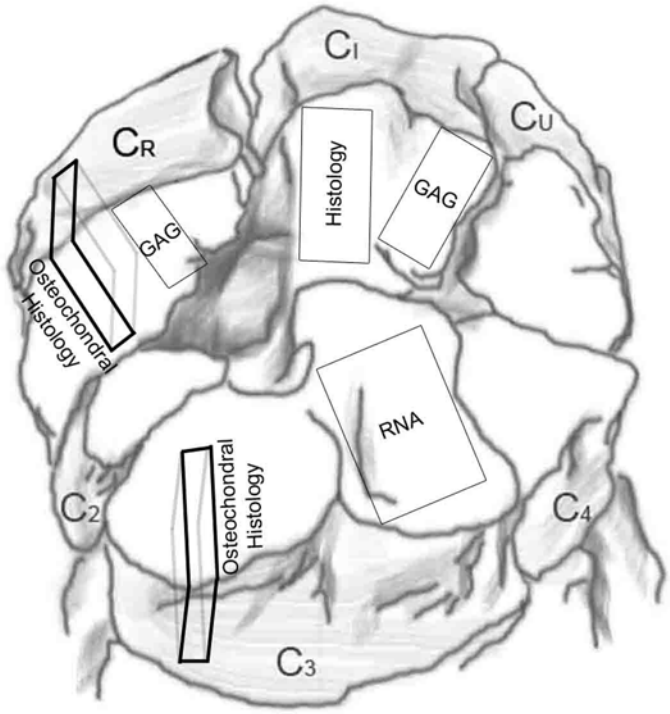

Figure 6.3 Tissue collection sites from the radial carpal bone (C_R), intermediate carpal bone (C_I), intermediate facet of the third carpal bone (C_3), and radial facet of the third carpal bone, for total glycosaminoglycan (GAG) content, gene expression (RNA), and routine histology of articular cartilage (histology) and osteochondral sections (osteochondral histology).

Biochemical Analysis

Cartilage samples were pulverized in a freezer mill and lyophilized. For total glycosaminoglycan and total DNA assay, cartilage samples were digested in papain (1 mL papain [0.5mg/ml]/10 mg lyophilized cartilage) at 65°C for 4 and 24 hours, respectively. For total glycosaminoglycan of synovial fluid, samples were digested in papain (1 mL papain [0.5mg/ml]/ ml synovial fluid) at 65°C for 4 hours. The samples were mixed with dimethylmethylene blue dye for glycosaminoglycan quantification by colorimetric assay,(Farndale et al. 1986) and bisbenzimide compound for DNA quantification by fluorometric assay(Kim et al. 1988) in duplicate aliquots.

Gene Expression

Total cellular RNA was isolated from freezer mill pulverized tissue and MSCs collected prior to treatment injection using a commercially available RNA extraction kit (PerfectPure RNA Fibrous Tissue Kit, 5 Prime, Gaithersburg, MD). Genomic DNA was isolated from MSCs collected prior to treatment injection using a commercially available genomic DNA extraction kit (PureLink Genomic DNA kit, Invitrogen, Carlsbad, CA). Genomic DNA was removed from RNA samples prior to PCR, by DNase I digestion. RNA and genomic DNA quality was assessed by spectrophotometry at 260:280 nm and by 1% agarose gel electrophoresis (data not shown). Total RNA was reverse transcribed and amplified using the One-Step RT-PCR technique and the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Life Technologies, Carlsbad, CA). The qPCR program included reverse transcription at 48°C for 30 minutes and denaturing at 95°C for 10 minutes, followed by 40 cycles of 90°C for 15 seconds and 60°C for 1 minute. For gene expression and DNA quantitation, each well of the qPCR plate was loaded with 10 ng of RNA or DNA in 20 µl. Other than *18S*, a standard curve was

generated from equine specific plasmid DNA for each gene at known concentrations to allow copy number estimation. The primers and dual-labeled fluorescent probe [6-FAM as the 5' label (reporter dye) and TAMRA as the 3' label (quenching dye)] were designed using Primer Express Software version 2.0b8a (Applied Biosystems, Life Technologies, Carlsbad, CA) using equine specific sequences published in Genbank. Samples were run in duplicate on the qPCR plate and total copy number per loaded RNA of each gene was obtained from a standard curve and normalized to *18S* gene expression. Sequences for qPCR included:

18S-Fwd CGGCTTTGGTGACTCTAGATAACC *18S*-Rev CCATGGTAGGCACAGCGACTA;

COL2b-Fwd CGCTGTCCTTCGGTGTCA, *COL2b*-Rev CTTGATGTCTCCAGGTTCTCCTT;

ACN-Fwd GATGCCACTGCCACAAAACA, *ACN*-Rev GGGTTTCACTGTGAGGATCACA;

SOX9-Fwd CAGGTGCTCAAGGGCTACGA; *SOX9*-Rev GACGTGAGGCTTGTTCTTGCT;

ADAMTS4-Fwd CCCTGGTCTCCGAAACCTCTA; *ADAMTS4*-Rev TATTCACCATTGAGGGCATAGGA;

ADAMTS5-Fwd CAGACGTTGGGACCATATGCT; *ADAMTS5*-Rev TGCGTGGAGGCCATCAT;

IL1b-Fwd CGTCTCCCAGAGCCAATCC; *IL1b* -Rev CACCAGGCTGACTTTGAGTGAGT;

TNFa-Fwd CAGCCGCTTAGCTGTCTCCTA; *TNFa* -Rev GTGTGGCAAGGGCTCTTGAT;

MMP13-Fwd TGAAGACCCGAACCCTAAACAT; *MMP13* -Rev GAAGACTGGTGATGGCATCAAG;

IL10-Fwd CTTGTCFFAFATGATCCAGTTTT; *IL10* –Rev AGTCCCCAGGCTGAGAACCACG;

TGFB3-Fwd GCCTGGCGGAGCACAAT, *TGFB3*-Rev CGGAATTCTGCTCGGAACA.

Statistical Analyses

Continuous synovial fluid variables (cytology, GAG concentration) were reported as a mean and SEM. Comparisons were made with 1-way ANOVA within each timepoint. Continuous gene expression variables were not normally distributed and were reported as a median and inter-quartile range. Comparisons were made with Wilcoxon Rank Sum test for difference between MSC and placebo treated OA joints and with Wilcoxon Signed Rank for

differences between OA and no OA joints. Scored data (clinical exam, gross exam) were reported as a mean and SEM and tested for differences between MSC and placebo treated OA joints by Wilcoxon's Rank Sum test. Observer scores were averaged for statistical testing. Statistical analysis was performed using commercially available software (Statistix 9). For all tests a $p \leq 0.05$ was considered significant.

RESULTS

Surgical OA Induction

Osteochondral fragmentation was accomplished in all OA joints. However, fragment configuration was variable amongst the horses. A lateral slab fracture occurred during fragmentation in 1 OA MSC injected horse and 1 OA placebo injected horse and the fragment was very loosely attached and fractured itself in 1 MSC OA horse.

Clinical Reaction to Injection

No horses developed lameness apparent at a walk in the week following injection. OA joints had significantly worse effusion and ROM scores on all days compared to sham operated joints. Range of motion and joint effusion were not different between MSC and Control injected OA joints on the day of injection (day 14). During the following week, MSC injected OA joints had an initial trend toward improvement and then significantly improved ROM scores on day 17 and 20. Similarly, effusion scores were significantly lower (improved) on day 15, 16, and 18-21 (Figure 6.4).

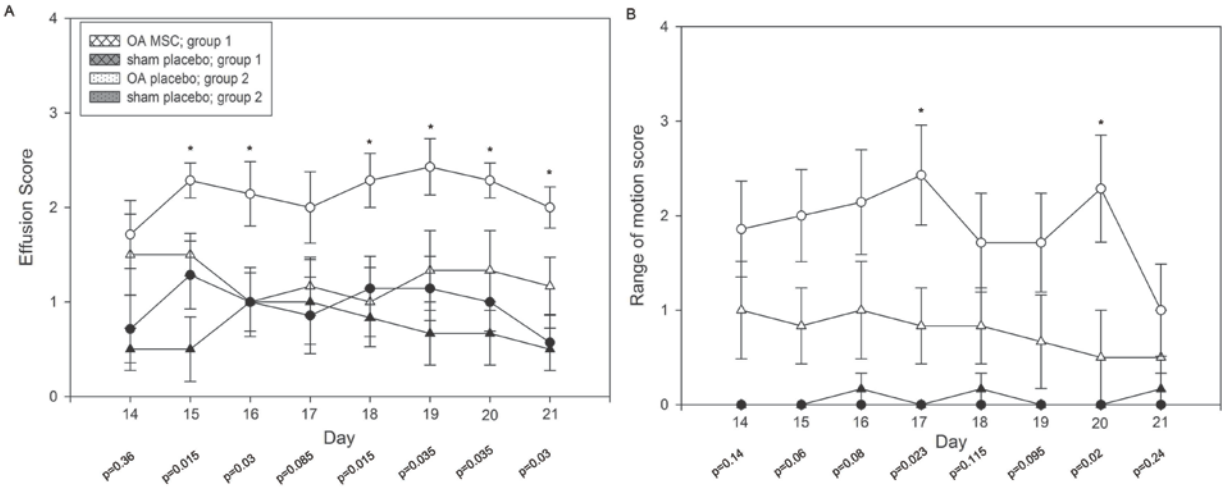

Figure 6.4 Clinical reaction to treatment injection: A) effusion score and B) range of motion score. Joint effusion and range of motion were not different between OA MSC and OA Control joints on the day of injection (day 14). A) During the following week, OA MSC joints had significantly (*) lower (improved) effusion scores on day 15, 16, and 18-21. B) Range of motion scores from OA MSC joints had an initial trend of improvement and then significantly (*) lower (improved) range of motion scores on day 17 and 20.

Clinical Exam

When comparing OA joints to the sham operated opposite carpal joint, OA joints had significantly increased lameness scores at all time points except day 49 ($p=0.06$), significantly increased effusion scores at most time points including day 7 ($p=0.005$), 14 ($p=0.002$), 21 ($p=0.003$), 35 ($p=0.05$), 42 ($p=0.01$) and 70 ($p=0.05$), significantly increased lameness response to flexion scores at all time points except day 42 ($p=0.065$) and day 63($p=0.065$) and significantly increased (worse) ROM scores at all time points. When comparing OA joints treated with MSC to OA placebo injected joints, lameness, effusion, and range of joint motion were consistently improved by MSC injection, (Fig 6.5) however, scores only achieved statistical significance for effusion on day 49 only (Fig 6.5).

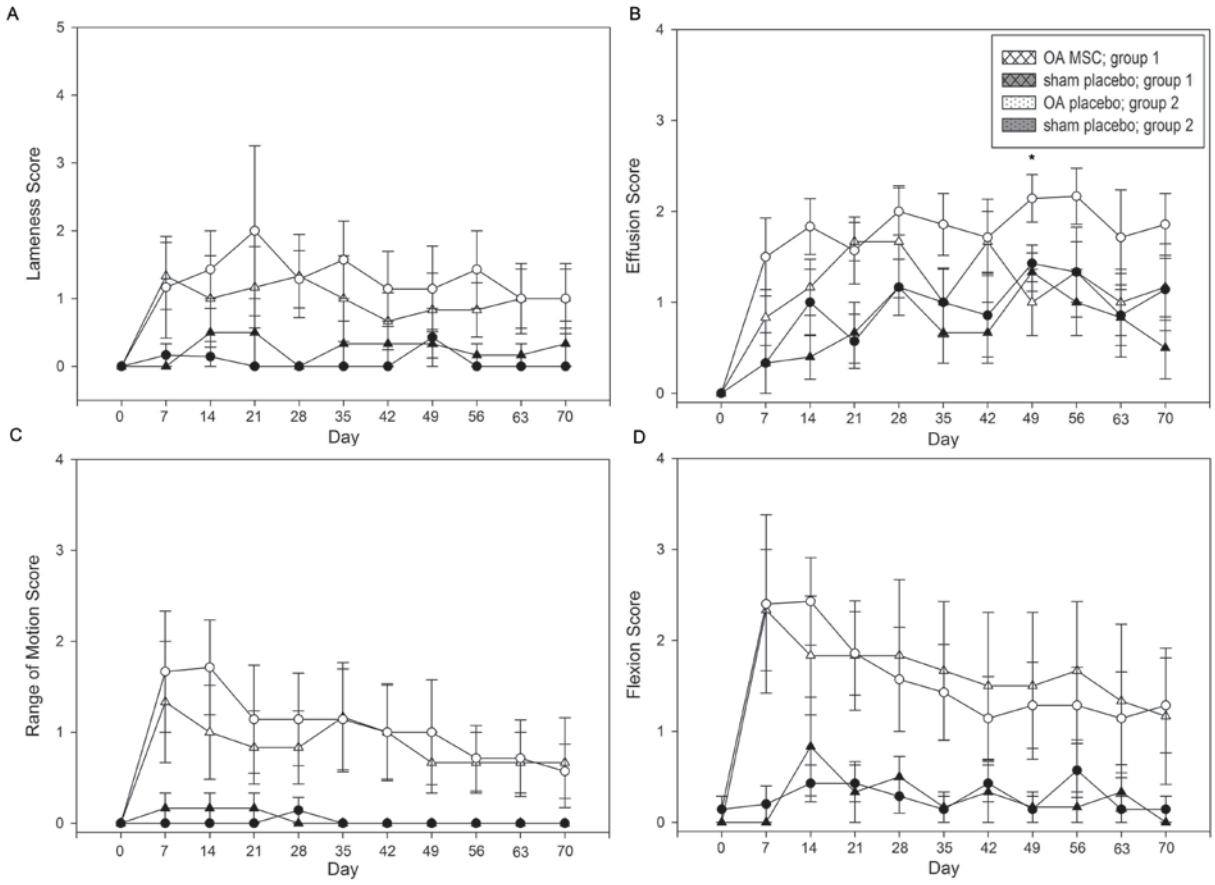

Figure 6.5 Clinical examination scores: A) lameness, B) effusion, C) range of motion and D) lameness response to flexion. When comparing OA joints receiving MSC to OA placebo injected joints, there were no significant differences other than significantly lower (better) effusion score from MSC OA joints (*) on day 49.

Synovial Cytology

Joints with induced OA had significantly increased TNCC on day 14 (p=0.0009), 21 (p=0.0001), 42 (p=0.0002), 56 (p=0.035) and 63 (p=0.027) compared to normal sham operated joints. Total protein from OA joints was also significantly increased on day 7 (p=0.017), 14 (p=0.015) and 21 (p=0.025) (Table 6.1).When comparing OA joints treated with MSC to OA placebo injected joints, OA MSC joints had significantly increased small mononuclear cell percentage on day 21 (p=0.01) and 28 (p=0.045) and turbidity score (p=0.017). Otherwise, there were no significant differences.

Synovial GAG

Synovial fluid GAG content was significantly increased in OA joints on day 7 (p=0.005), 28 (p=0.009), 42 (p=0.03), and 56 (p=0.007) compared to normal joints (Fig 6.6). In the joints with induced OA, MSC injection resulted in significantly increased total GAG content on day 7 (p=0.035) and day 14 (p=0.04; Fig 6.6) compared to placebo treated OA joints.

			day 0		day 7		day 14		day 21		day 28		day 35		day 42		day 49		day 56		day 63		day 70	
Variable	Group		Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
TNCC x1000/µl	1	OA	**167**	**67**	**700**	**126**	**867**	**176**	**950**	**214**	**533**	**152**	**500**	**159**	**567**	**92**	**517**	**70**	**700**	**203**	**367**	**49**	**683**	**190**
		sham	200	63	833	260	500	124	367	88	383	65	467	67	300	45	800	424	917	580	467	230	867	226
	2	OA	**214**	**40**	**650**	**205**	**543**	**43**	**757**	**169**	**443**	**57**	**543**	**97**	**771**	**278**	**429**	**61**	**443**	**84**	**457**	**75**	**771**	**233**
		sham	214	26	1017	416	257	48	314	55	471	130	500	170	271	52	300	58	243	48	286	26	386	77
% neutro-phils	1	OA	**0.6**	**0.6**	**25.7**	**8.7**	**21.8**	**9.1**	**14.8**	**5.3**	**7.2**	**2.8**	**2.0**	**1.3**	**5.2**	**2.3**	**7.0**	**3.7**	**4.2**	**2.5**	**7.8**	**4.4**	**3.3**	**2.2**
		sham	0.8	0.8	12.3	5.7	6.7	2.4	4.2	2.0	6.0	2.8	2.8	1.1	2.8	1.3	7.3	5.2	6.0	3.5	6.2	4.3	4.3	3.0
	2	OA	**1.0**	**0.5**	**23.7**	**10.8**	**16.9**	**4.7**	**20.9**	**8.3**	**11.9**	**5.6**	**7.1**	**3.6**	**12.9**	**8.8**	**3.3**	**1.1**	**3.6**	**1.3**	**5.9**	**3.4**	**2.0**	**0.7**
		sham	5.3	3.9	17.7	7.3	6.0	2.1	2.0	0.6	7.9	3.8	2.0	1.1	0.4	0.4	0.6	0.6	1.4	1.1	3.3	2.0	0.4	0.3
% small mono-nuclear	1	OA	**22.5**	**5.1**	**13.0**	**5.0**	**16.8**	**4.4**	**18.5**	**4.2**	**24.0**	**4.6**	**21.5**	**4.8**	**25.3**	**4.6**	**23.3**	**5.0**	**15.8**	**3.4**	**21.7**	**4.8**	**22.3**	**3.5**
		sham	20.7	2.9	11.8	3.0	23.8	3.1	21.7	6.8	23.0	6.5	25.5	4.2	27.0	6.7	26.7	4.5	19.0	2.7	18.0	4.7	27.7	5.5
	2	OA	**15.6**	**3.9**	**16.5**	**3.6**	**14.9**	**5.5**	**8.6**	**1.5**	**16.3**	**2.7**	**18.0**	**4.9**	**18.1**	**5.0**	**17.1**	**2.8**	**18.7**	**4.9**	**14.4**	**3.4**	**15.9**	**3.1**
		sham	20.9	4.1	14.2	5.7	23.1	4.3	24.1	2.8	22.0	3.5	18.6	4.2	22.0	4.5	15.1	2.2	24.0	4.6	16.7	3.8	14.7	3.3
& large mono-nuclear	1	OA	**75.3**	**5.0**	**60.2**	**11.9**	**61.3**	**11.5**	**70.0**	**6.8**	**68.8**	**5.3**	**76.5**	**5.2**	**69.5**	**3.7**	**69.7**	**4.5**	**79.8**	**4.0**	**70.2**	**7.7**	**74.3**	**5.5**
		sham	77.3	3.7	75.8	5.2	69.5	2.9	74.8	7.2	71.0	6.3	71.7	3.6	70.2	7.8	65.5	6.6	75.0	5.3	75.8	3.2	68.5	7.3
	2	OA	**83.4**	**4.3**	**59.7**	**8.1**	**68.3**	**5.9**	**70.6**	**7.5**	**71.9**	**4.6**	**74.9**	**5.1**	**69.0**	**8.1**	**79.6**	**2.0**	**77.7**	**4.2**	**79.7**	**4.4**	**82.1**	**3.6**
		sham	74.4	5.5	68.2	10.2	70.9	6.3	74.3	3.1	70.1	4.8	79.4	4.4	77.6	4.8	84.3	1.9	74.0	3.8	81.9	3.9	84.9	3.4
TP g/dL	1	OA	**2.52**	**0.02**	**3.75**	**0.44**	**3.27**	**0.40**	**3.10**	**0.19**	**2.68**	**0.12**	**2.60**	**0.07**	**2.80**	**0.20**	**2.72**	**0.12**	**2.62**	**0.08**	**2.52**	**0.02**	**2.62**	**0.12**
		sham	2.52	0.02	2.77	0.27	2.87	0.20	2.95	0.21	2.58	0.05	2.65	0.11	2.65	0.10	2.57	0.07	2.70	0.15	2.65	0.10	2.68	0.12
	2	OA	**2.54**	**0.03**	**3.28**	**0.28**	**3.10**	**0.17**	**3.00**	**0.15**	**2.61**	**0.10**	**2.76**	**0.15**	**2.66**	**0.11**	**2.60**	**0.10**	**2.57**	**0.07**	**2.66**	**0.16**	**2.51**	**0.01**
		sham	2.71	0.18	3.03	0.41	2.56	0.06	2.50	0.00	2.50	0.00	2.66	0.16	2.56	0.06	2.50	0.00	2.50	0.00	2.50	0.00	2.50	0.00

Table 6.1 Synovial fluid cytology for total nucleated cell count (TNCC), percent neutrophils of TNCC, percent small monocytes of TNCC, percent large monocytes of TNCC and total protein (g/dl). When comparing OA MSC to OA placebo joints, OA MSC joints had significantly increased small mononuclear cell percentage on day 21 (p=0.01) and 28 (p=0.045). Otherwise, there were no significant differences.

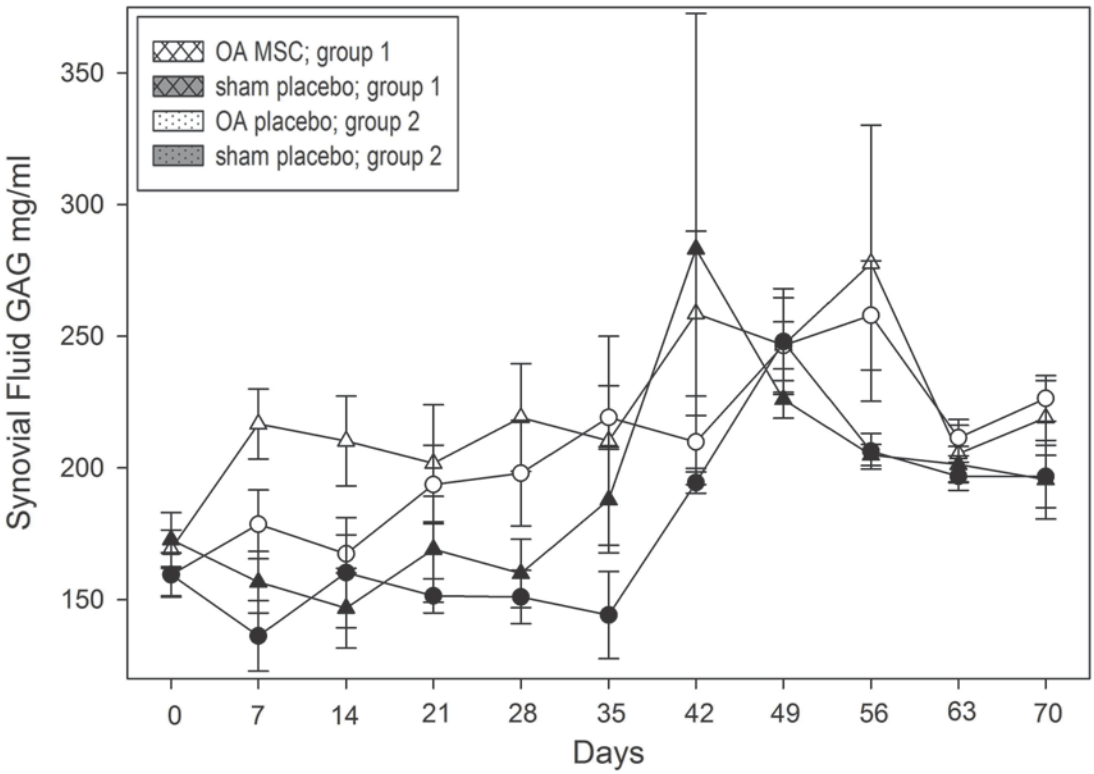

Figure 6.6 Synovial fluid total glycosaminoglycan content. Joints with induced OA had significantly elevated total GAG content on day 7 (2-tailed p=0.005), 28 (p=0.009), 42 (p=0.03), and 56 (p=0.007). Treatment with MSC expressing TGF-β3 and lhpRNA*i* resulted in significantly elevated GAG on day 7 (2-tailed p=0.035) and day 14 (p=0.04), compared to placebo injected OA joints.

Gross Scoring

The osteochondral fragment was apparent at gross examination in all horses (Fig 6.7). Gross scores for cartilage fibrillation/eburnation and synovial membrane changes from OA joints injected with MSCs were similar to OA joints getting placebo injection. (Fig 6.8). Gross scores were significantly worse for all parameters in OA joints compared to normal sham operated joints.

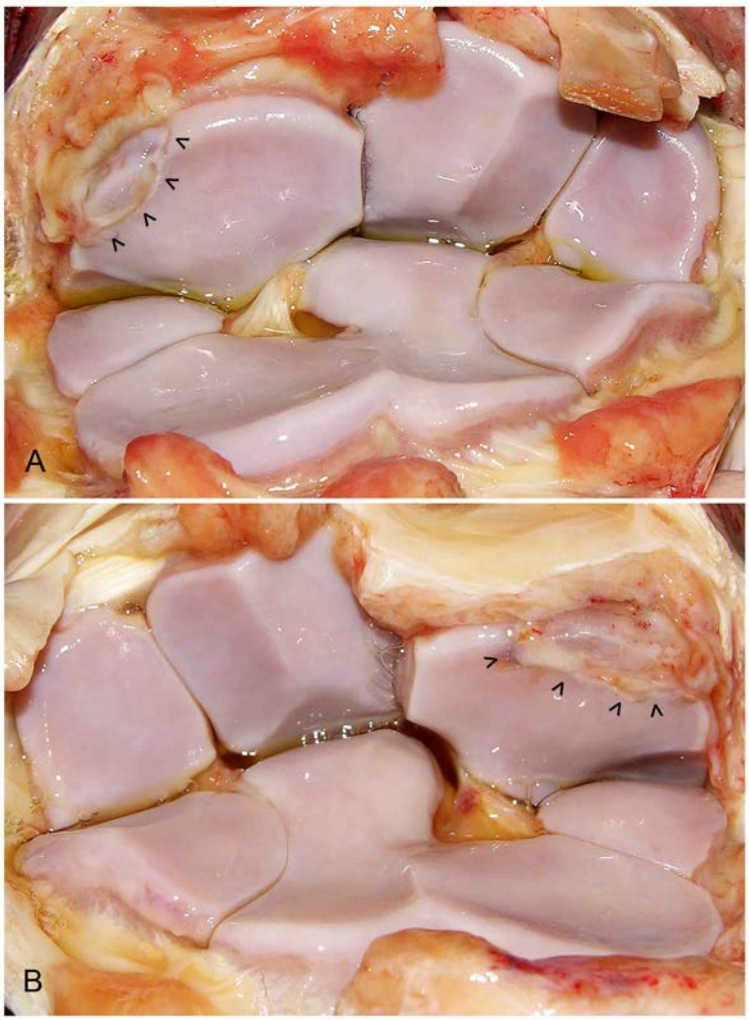

Figure 6.7 Photographs of the articular surface of the middle carpal joint at necropsy 70 days after osteochondral chip fracture of the radial carpal bone. A) Right OA joint after receiving MSCs overexpressing TGF-β3 and *lhp*RNA*i*, and B) left OA placebo injected joint, 70 days after chip fragmentation. ^ outline the osteochondral fragment of the dorsomedial radial carpal bone.

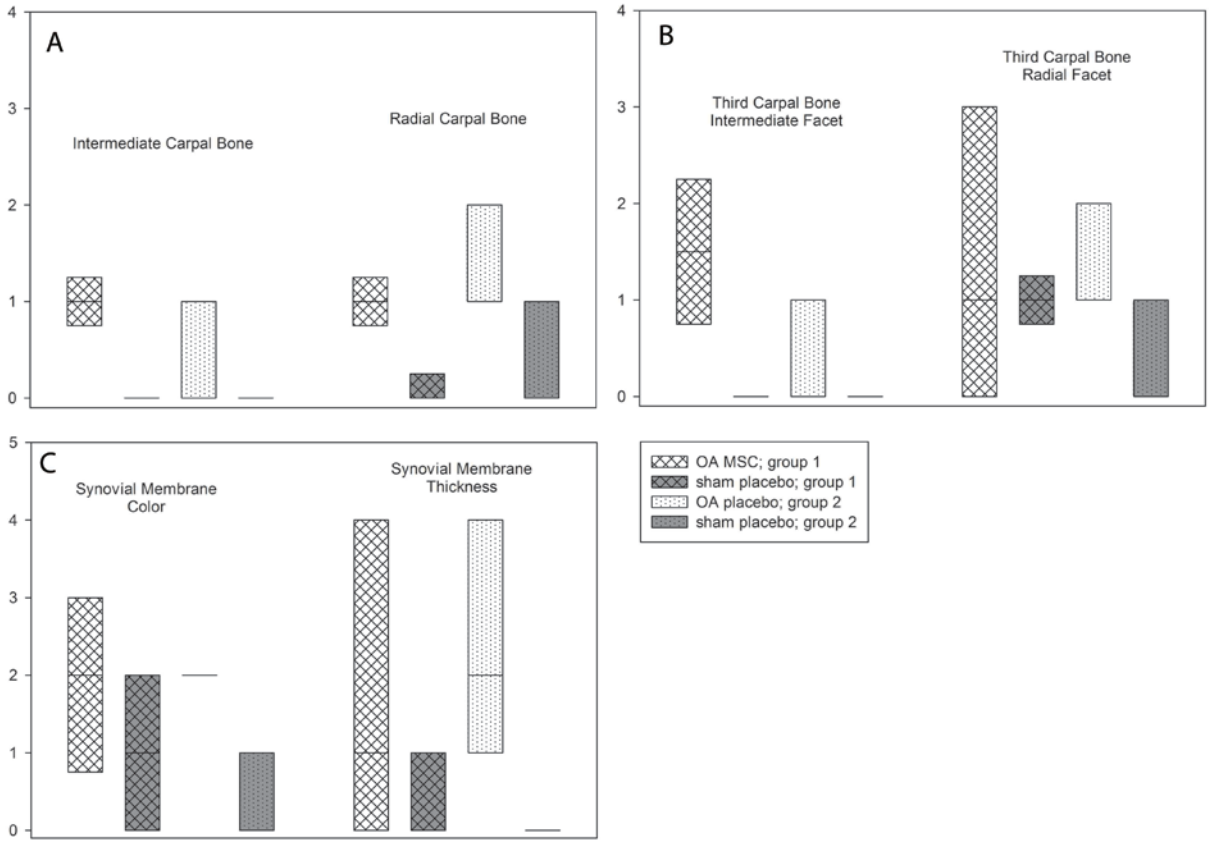

Figure 6.8 Gross articular cartilage and synovial membrane scores at necropsy. There were no significant differences in scores between OA joints injected with MSCs and those receiving placebo.

Cartilage Biochemical Analysis

Total GAG content was not significantly different when comparing OA to normal joints or when comparing OA joint injected with MSCs or placebo. There was a trend toward increased total GAG in cartilage from the intermediate carpal bone of OA joints injected with transduced MSCs, compared to OA joints injected with placebo ($p=0.053$; Fig 6.9).

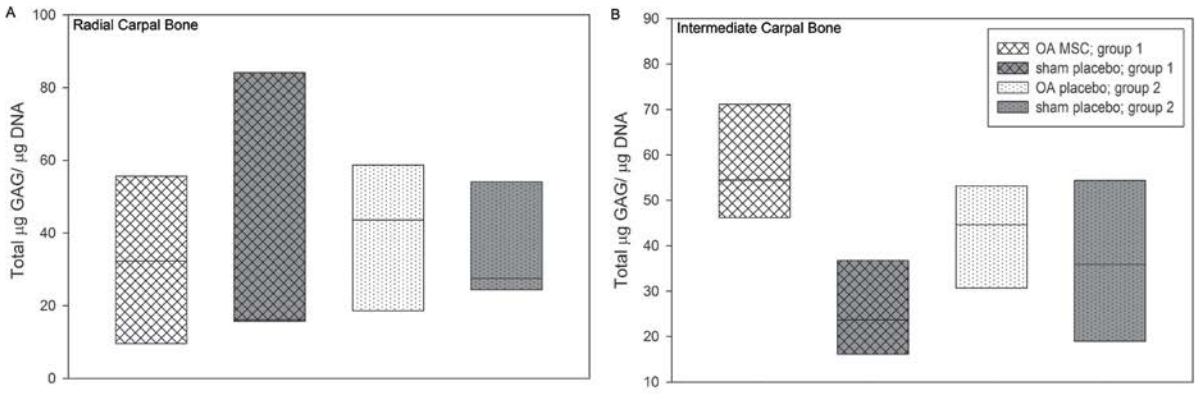

Figure 6.9 Total glycosaminoglycan (GAG) normalized to total DNA content of the articular cartilage from the A) radial carpal bone and B) intermediate carpal bone. Total GAG/DNA content was not significantly different when comparing OA to sham operated joints, or when comparing OA joints injected with transduced MSC to placebo injected. There was a trend toward increased total GAG/DNA of the intermediate carpal bone B) from OA joints injected with MSC compared to placebo injected OA joints ($p=0.053$).

Gene Expression

Gene expression of MSCs prior to joint injection confirmed significantly increased expression of TGF-β3 in transduced cells (Fig 6.10). Additionally, PCR of genomic DNA confirmed intra-ceulluar presence of long hairpin RNA plasmid constructs (Fig. 6.10).

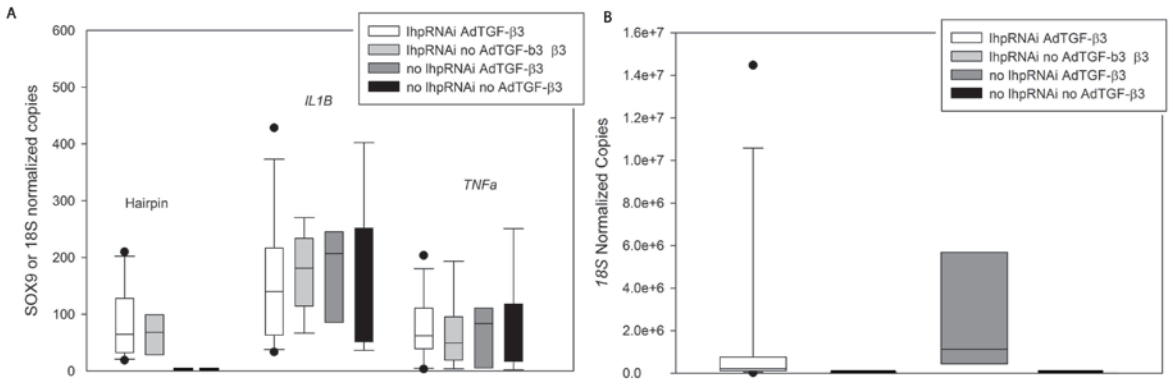

Figure 6.10. Normalized rt-PCR copies per ng of loaded RNA or DNA from MSCs prepared for treatment injection for A) presence of the lhpRNAi plasmid DNA and gene expression of IL-1β and TNF-α and B) gene expression of TGF-β3. MSCs for treatment injection to group 1 are the white bars and had been treated with lhpRNAi and AdTGF-β3. Corresponding controls with and without plasmid transfection and/or adenoviral transduction are included.

In post mortem assays, when comparing OA to normal sham operated joints, gene expression of *ACN* ($p=0.046$) and *COL2b* ($p=0.017$) was significantly lower in cartilage from OA joints and synovial membrane expression of ADAMTS4 was significantly higher ($p=0.003$). Gene expression of all other matrix and catabolic genes were not significantly different between OA and normal joints. Injection of MSCs expressing TGF-β3 and the cytokine silencing long hair-pin construct significantly reduced cartilage MMP-13 expression ($p=0.04$) and IL-1β expression ($p=0.05$) in the synovial membrane (Fig 6.11).

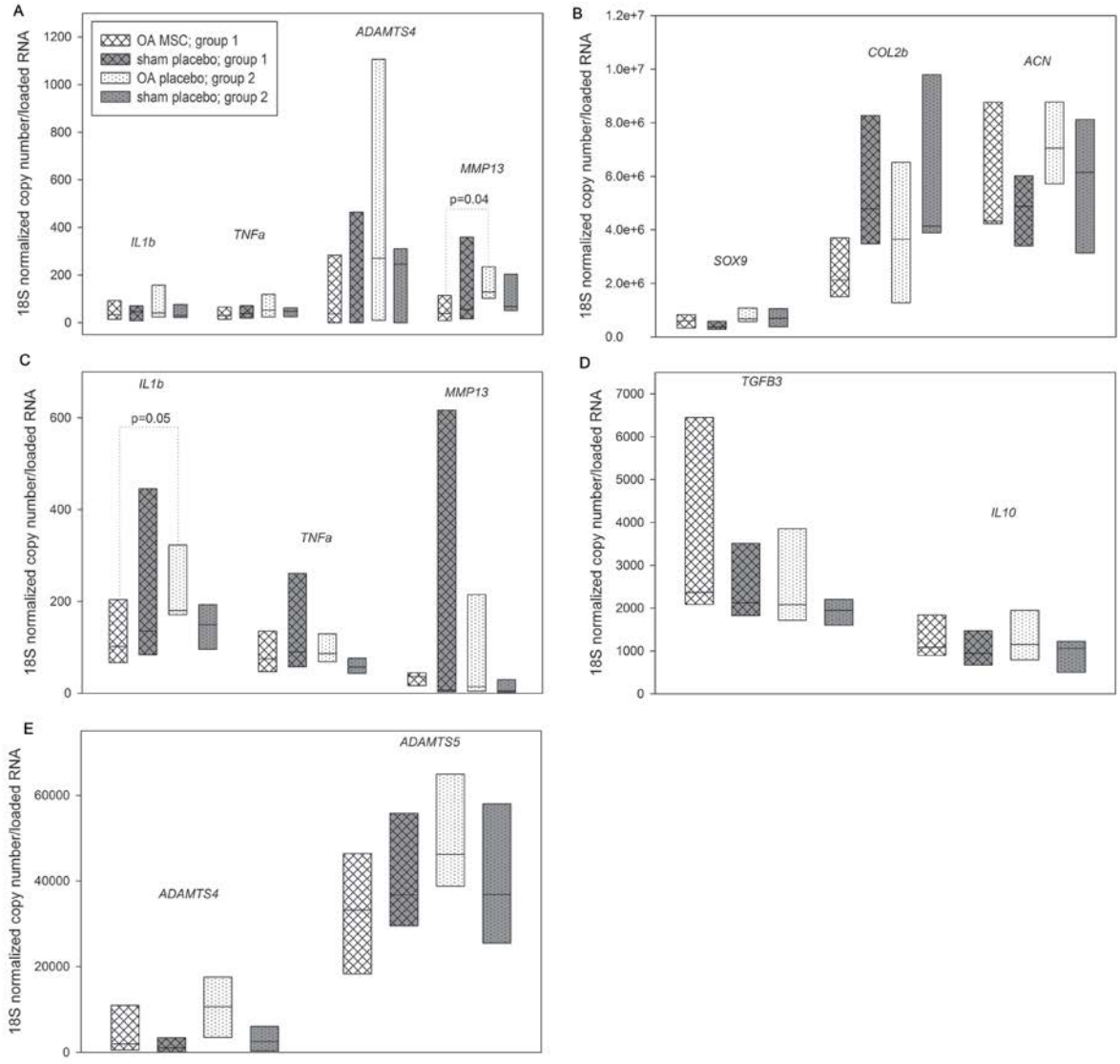

Figure 6.11 Gene expression from A, B) articular cartilage and C, D, E) synovial membrane. A) Proinflammatory cytokines in cartilage including IL-1β and TNF-α, were unchanged by MSCs expressing IL-1β and TNF-α silencing elements. However, ADAMTS4 was reduced ($p<0.1$) and MMP-13 was significantly suppressed by MSC gene transduction. B) Matrix genes (Col2b and ACN) were not influenced by MSC treatment. C) Synovial membrane expressions of catabolic genes was suppressed by MSCs expressing the IL-1 and TNF-α silencing construct, while D) TGF-β3 was elevated but not to significant levels compared to placebo injected joints. E) Synovial ADAMTS-4 and -5 were reduced in MSC injected joints compared to placebo and sham joints, but not to significant levels.

Histology

Synovial sections from placebo injected OA joints had increased thickening and fibrosis compared to OA joints injected with MSC. Cartilage sections from OA joints injected with MSCs or placebo appeared similar (Fig 6.12).

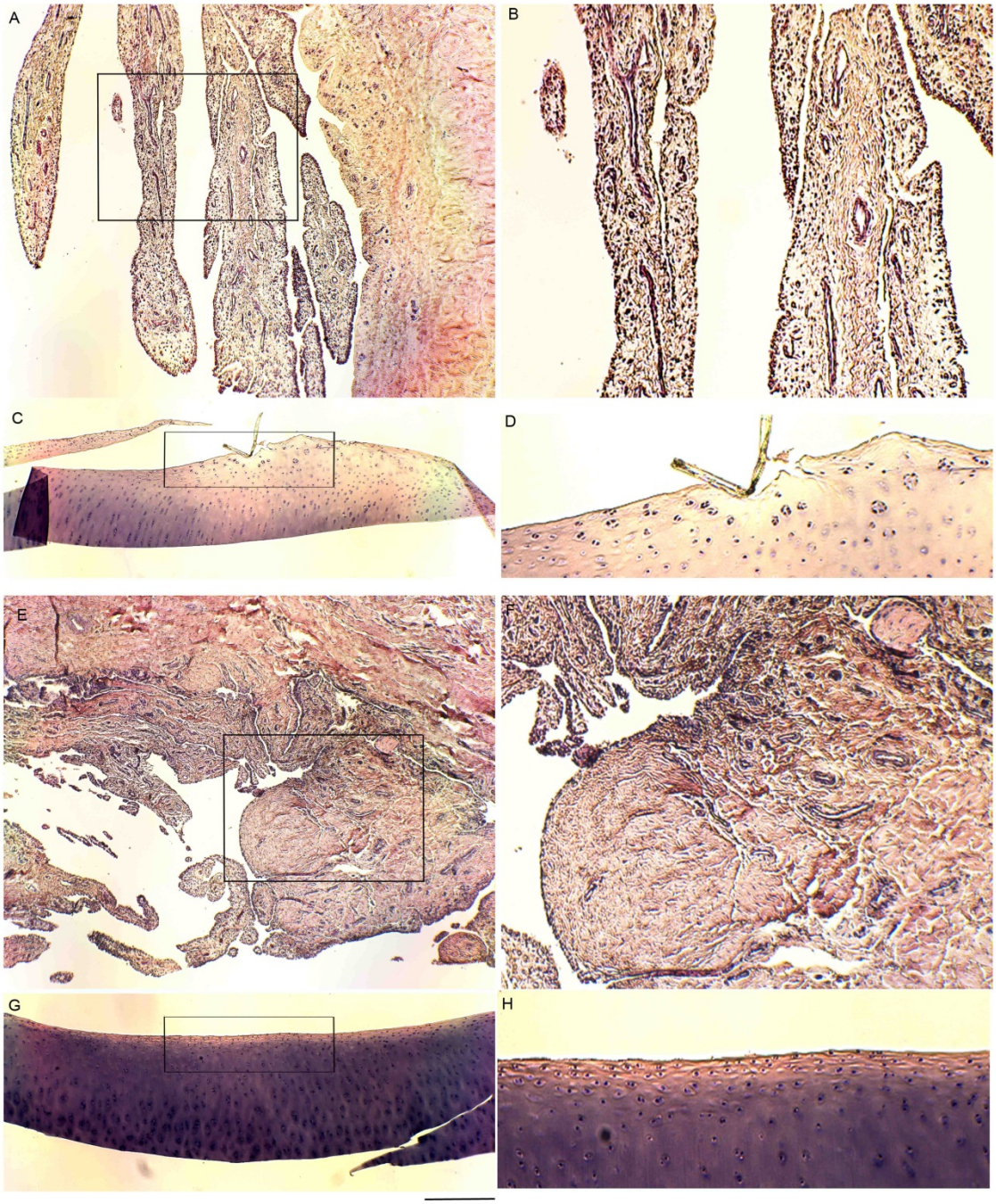

Figure 6.12 Synovial membrane sections (A,B,E,F) and cartilage sections (C,D,G,H) from OA joints treated by MSC injection (top panels; A,B,C,D) or placebo injection (lower panels: E,F,G,H) The black box outlines area magnified in right hand images. Left hand images made at 50x magnification. Scale bar = 200µm. Hematoxylin and eosin stain.

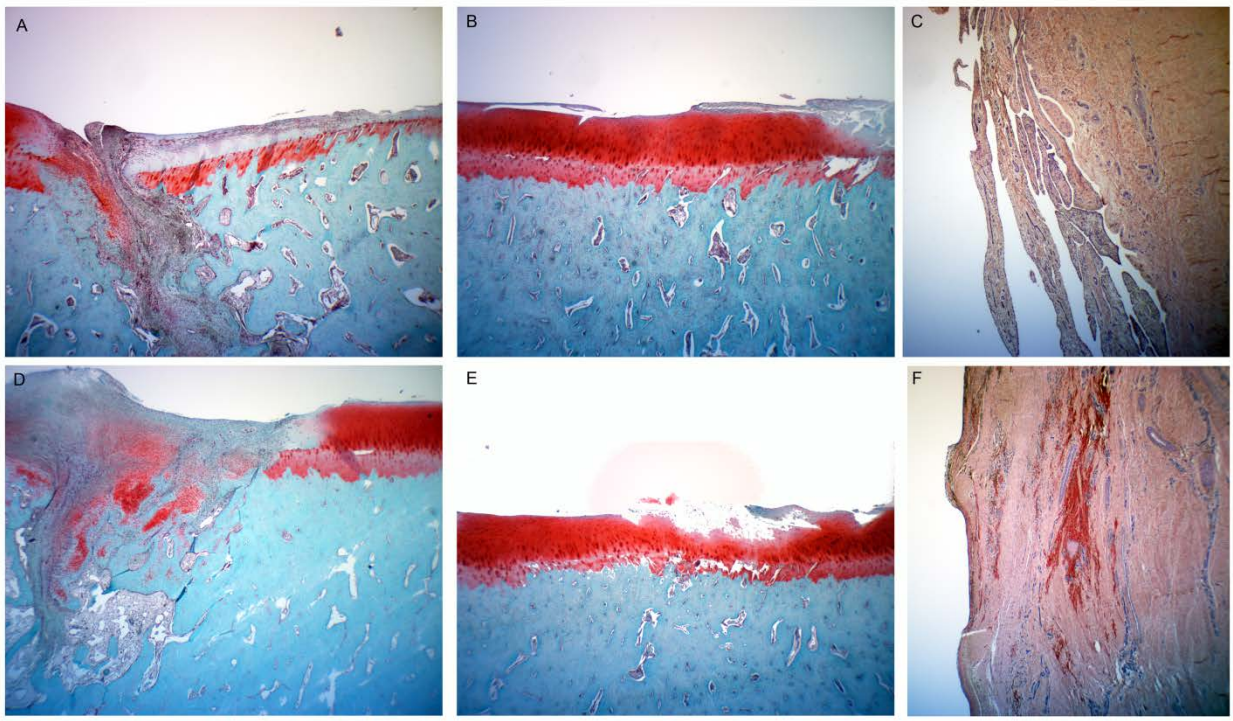

Figure 6.12. Photomicrograph (25x magnification) of the radial carpal bone (A and D), the opposing third carpal bone (B and E) and the adjacent synovial membrane. Osteochondral sections are stained with safranin-O and fast green and synovial sections are stained with hematoxylin and eosin.

DISCUSSION

This study indicates the intra-articular injection of MSCs overexpressing TGF-β3 and simultaneously suppressing IL-1β and TNF-α through RNA interference may be an effective treatment for OA. No adverse treatment effects were observed and there were significant reductions in gene expression of MMP13 in cartilage and IL-1β in synovial membrane, along with a strong trend toward increased total GAG content of articular cartilage from the intermediate carpal bone and reduced synovial fibrosis seen histologically. Importantly, range of motion and effusion were significantly improved in the week following treatment of joints by MSC injection.

Given the low rate of MSC adherence to articular cartilage after intra-articular injection(Watts and Nixon 2011), it seems unlikely that injected stem cells will contribute directly to significant cartilage repair. However, intra-articular injection of MSCs has resulted in significant experimental(Lee et al. 2007; McIlwraith et al. 2011; Murphy et al. 2003; Toghraie et al. 2011) and clinical(Black et al. 2007; Black et al. 2008; Centeno et al. 2008; Centeno et al. 2011; Davatchi et al. 2011; Frisbie et al. 2007) reduction of clinical symptoms and progression of joint disease. This may be the result of a paracrine interaction between injected MSCs that have populated the synovial membrane, and endogenous repair mechanisms from the synovium, subchondral bone, and progenitors within the joint.(Jing et al. 2008; Murphy et al. 2003) This paracrine interaction may occur through one of several mechanisms known to occur with MSCs: modulation of the immune response; stimulation of growth of host cells; recruitment of endogenous progenitors; or prevention of an inappropriate fibrotic response.(Caplan 2009)

Previous studies using a similar experimental OA model in the horse, indicated intra-articular injection of MSCs had little treatment effect.(Frisbie et al. 2009) Therefore, we wanted to augment the MSC paracrine action through dual axis gene therapy induced transgene expression of anabolic TGF-β3 and knockdown of catabolic IL-1β and TNF-α. In this manner, the MSC could affect endogenous repair through their own mitotic, anabolic and anti-inflammaotry properties(Caplan 2009), in addition to action as a gene therapy vehicle for anti-catabolic and pro-anabolic transgenes.

In OA, IL-1 and TNF-α upregulate the inflammatory, apoptotic and destructive events as well as downregulate matrix synthesis(Bondeson et al. 2006; Fischer et al. 2000; Goldring 1999; Singh et al. 2003; Elliott et al. 1993). In a rabbit model of OA, the combination of IL-1 and TNF-α blockade, compared to blockade of either alone, enhanced the therapeutic effect to both cartilage and synovial membrane regions. The authors concluded that blockade of multiple inflammatory cytokines is more efficacious than either alone.(Wang et al. 2006) The reduced MMP13 expression from articular cartilage and reduced IL-1β abundance in synovial membrane in our study indicates that MSC engraftment to the synovial membrane with transgene expression of TGF-β3 and dual knockdown of IL1b and TNF-α was able to shift the intra-articular molecular balance away from catabolism.

We selected the anabolic growth factor TGF-β3 for these experiments, as the TGF-β family of growth factors plays an important role in chondrogenesis in vivo(Ballock et al. 1993) and in vitro(Johnstone et al. 1998), in cartilage repair with increased proteoglycan synthesis in OA(Malemud et al. 1991), and in regulation of the inflammatory milieu within the joint(Hui et al. 2001; Redini et al. 1993; van Beuningen et al. 1993; van Beuningen et al. 1994). We chose TGF-β3 over TGF-β1 or –β2 because it better prevents terminal differentiation(Watts et al.

2011a) and most importantly, does not induce synovial fibrosis and scarring and may even reduce connective tissue scar formation(Shah et al. 1995) compared to TGF-β1 and -β2.

Clinically, hyaluronic acid has been used as a carrier for intra-articular injection of MSCs(Frisbie et al. 2009), similar to the vehicle used in a caprine OA study.(Murphy et al. 2003) However, in this study, we used MEM as the MSC carrier. This obviated the possible reaction to HA, which has been described in the horse,(Kuemmerle et al. 2006) and in humans,(Goldberg and Coutts 2004; Magilavy et al. 2004; Roos et al. 2004) where intra-articular injection of HA alone can occasionally induce severe inflammatory joint reaction. We wanted to assess the synovial fluid for reaction to MSCs applied intra-articularly, as post-injection reaction to MSCs has been reported in the horse.(Carrade et al. 2011; Watts and Nixon 2011) Notably, in contrast to our experience with intra-articular injection of autologous MSCs(Watts and Nixon 2011) there was a reduction, rather than an increase, in effusion scores after transduced MSC injection to OA joints. There was also nearly immediate pain relief, with increased range of motion in the week following treatment injection.

The model induced a moderate synovitis with early cartilage changes and there were significant differences due to OA induction in several clinical parameters, synovial GAG concentrations, synovial cytology parameters, and molecular assays. However, there were limitations to the OA model in this study. Because we used previously trained racehorses, the subchondral bone density varied amongst the horses(Tidswell et al. 2008) which led to greater variability in the osteochondral fragment size and shape. The resultant spectrum of OA severity could have masked additional treatment effects. Notably, the OA MSC injected group was assigned a priori but happened to be more affected by larger osteochondral fragments, possibly unbalancing the experiment and masking additional benefit to gene transduced MSC injection.

An additional limitation was that the distribution and durability of injected MSCs was not characterized. Despite the obvious treatment effects demonstrated at day 70, these data do not confirm persistence of the MSCs or of their transgene expression. Methodology to confirm injected MSC distribution and long term engraftment would have improved this study. Our previous studies of short-term MSC distribution, using fluorescent quantum dot labeled cells injected to joints, do indicate a predominant synovial membrane homing after intra-articular injection(Watts and Nixon 2011). A final limitation to this study is the lack of positive control group where un-manipulated MSCs were utilized in treatment injections. This group was not included because of the significant cost of housing, buying and caring for these animals, and the strong emotional and ethical considerations in their use and sacrifice(Koch and Betts 2007).

Intra-articular MSC injection offers a simple regenerative therapy for modifying the structural progression of OA and reducing clinical symptoms by treating all tissues within the joint.(Chen and Tuan 2008) We utilized autologous MSCs for dual axis gene therapy, with overexpression of anabolic TGF-β3 and RNAi knockdown of catabolic IL-1β and TNF-α, for the treatment of osteoarthritis in an equine model. No adverse effects of transduced MSC injection were noted. Significant improvements were seen in OA joints treated with the gene transduced MSCs, including reduced MMP13 expression in cartilage and reduced IL-1β expression in synovial membrane, reduced effusion and improved range of motion in the week following injection, a strong trend toward increased GAG content in articular cartilage, o and reduced synovial fibrosis. Cell dosing and longer term studies are indicated to evaluate additional benefit of increased cell dose and more advanced OA following osteochondral chip fracture.

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CONCLUSION

The aim of the thesis project was to identify a growth factor that would enhance the in vitro chondrogenesis of the equine mesenchymal stem cell (MSC) and subsequently to assess the capacity of the growth factor expressing MSC to improve cartilage repair and osteoarthritis (OA) prevention in vivo.

Previous efforts in the lab on MSC enhanced cartilage repair have focused on implantation of MSCs to articular cartilage defects. This approach is only applicable to acute and focal articular cartilage injury and does not apply to the OA joint. Global joint disease, as in OA, is far more common than focal cartilage injury and no disease modifying therapies of regenerative therapies are currently available. In chapter 1, the short term tracking of fluorescent labeled autologous MSCs after intra-articular injection revealed that the majority of injected MSCs engrafted to the synovial membrane. In contrast to our hypothesis, there was not increased articular cartilage engraftment in joints with pre-existing joint disease and OA compared to normal joints. Despite the lack of efficient engraftment to the articular cartilage (either diseased or healthy) we still wanted to study growth factors that would enhance chondrogenesis. Once engrafted to the synovial membrane, MSCs expressing chondrogenic growth factors could have a profound effect on chondrogenesis through paracrine actions on endogenous healing.

Three-dimensional culture is required for the study of MSC chondrogenesis. Because long term 3-d culture of equine MSCs had been problematic in our laboratory, differing three-dimensional culture systems were tested and the results were described in chapter 2. Each three-dimensional culture system was tested for suitability for the study of in vitro chondrogenesis of MSCs from individual horses. In chondrogenic media, fibrin alginate culture and pellet culture

(500,000 cell pellets) were superior for chondrogenic induction when compared to agarose, alginate alone and 250,000 cells pellets. The 500,000 cell containing pellet was used for subsequent chondrogenic studies.

For in vitro MSC chondrogenesis, supplementation with an isoform of transforming growth factor beta (TGF-β) is required. TGF-β1, -β2 and -β3 have been used and although TGF-β1 is the most frequently reported both in vitro and in vivo, it is not clear which is superior for chondrogenic induction and prevention of hypertrophy. In chapter 3, the chondrogenic effect of the 3 isoforms was tested in MSC pellet culture where cultures were supplemented daily with recombinant human protein. All 3 isoforms resulted in MSC chondrogenesis, however, TGF-β3 had clear enhancement of prevention of hypertrophy.

Continuous growth factor supplementation in vivo is difficult to maintain because of the short half-life of injected or implanted growth factors. Gene therapy techniques to induce growth factor expression by injected or implanted MSCs would allow for continuous growth factor supplementation to the joint. In chapter 4, adenoviral vectors for expression of TGF-β1, -β2 and -β3 were tested in MSC pellet culture to confirm transgene expression and efficacy for chondrogenic induction in long term 3-dimensional culture. Adenoviral transgene expression of TGF-β3 resulted in chondrogenic induction and reduced progression toward hypertrophy compared to Ad TGF-β1 and -β2.

In chapter 1, short term tracking studies of intra-articular injection of autologous MSCs revealed that MSCs efficiently engraft the synovial membrane but not articular cartilage, whether normal or diseased. To enhance the paracrine effects of synovial engrafted MSCs within the OA joint, we wanted to test the effect of enhanced TGF-β3 in combination with gene knockdown of the inflammatory cytokines IL-1β and TNFα. In chapter 5, injection of growth factor enhanced

and anti-catabolic MSCs to the middle carpal joint of horses in the osteochondral fragmentation model of OA resulted in improved control of joint disease. This was evidenced by improved effusion scores and range of motion in the week following treatment injection, reduced cartilage *MMP13* and synovial *IL1b* expression, reduced synovial fibrosis and a strong trend of increased cartilage glycosaminoglycan content.