INDUCTION OF CHONDROGENESIS FROM MSCs BY BMP7 AND TGFβ1 IN LOW OXYGEN TENSION: EXPRESSION OF SUPERFICIAL ZONE PROTEIN.

A Project

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Abstract

of

INDUCTION OF CHONDROGENESIS FROM MSCs BY BMP7 AND TGFβ1 IN LOW OXYGEN TENSION:

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Osteoarthritis (OA) is the most common form of arthritis, afflicting 12.1% of the United States population. OA is characterized by the degeneration of articular cartilage, thus its synonym degenerative arthritis. Articulating ends of bone found within a synovial joint are lined with articular cartilage. Articular cartilage imparts extremely important mechanical properties that give the joint's ability to function properly. Even under high loads, articular cartilage slide together creating near frictionless motion comparable to ice-on-ice. Due to the avascular nature of articular cartilage, the oxygen tension of the tissue is as low as 1%, and makes delivering pharmaceuticals via the blood stream nearly impossible. OA patients have limited treatment options, mainly focusing on treating inflammation and pain. My goal is to differentiate human bone marrow derived mesenchymal stem cells (hBM-MSCs) into articular cartilage with morphogens BMP-7 and TGFβ1. Differentiating stem cells into chondrocytes in hypoxic conditions has been shown to increase chondrogenesis. I am comparing the expression of superficial zone

protein (SZP) by MSC differentiated at atmospheric (27% O₂) and hypoxic (3% O₂) oxygen concentrations. The degree of differentiation of MSCs into articular cartilage will be assessed on the pellets expression of articular cartilage proteins (collagen, aggrecan, superficial zone protein etc.) and glycosaminoglycans. Differentiated MSCs are evaluated through histologically using Toluidine Blue staining for glycosaminoglycans, and immunohistochemically for the expression of superficial zone protein of the cells in pellet cell culture is quantified using qPCR.

Results show no increase of chondrogenesis or SZP expressed in MSC pellets differentiated in hypoxic environment. Toluidine blue stain shows no increase glycosaminoglycans, a key element in articular cartilage matrixes. Although western blot showed more SZP secreted in the media of pellets in low oxygen environment, both immunohistochemistry and qPCR show a decrease of SZP in pellets differentiated in hypoxic environment. Other chondrogenic markers such as Sox9 and aggrecan were also shown to be decreased in the hypoxia differentiated pellets.

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Date

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INTRODUCTION

Arthritis is a disease that affects more than 21% of the population (Helmick et al., 2008). Osteoarthritis is the most common form of arthritis afflicting 12.1% of the United States population between the ages of 25-74, amounting to 27 million Americans (Lawrence et al., 2008). Not only is OA the most common musculoskeletal disorder, but its incidence increases drastically for people over 60, making it the most common disability for that age group (Buckwalter et al., 2004). According to a cost analysis the total cost of care in 2001 due to OA was \$89 billion dollars (in 5 industrialized countries; Australia, Canada, France, UK, and US) (Bitton, 2009). In addition, chronic arthritis has been projected to increase to 25% of the adult population by 2030 (Hootman & Helmick, 2006).

The condition of osteoarthritis is defined by the Center for Disease Control as degeneration of cartilage, exposing the underlying bone within the joint. Adjacent bones in an articulating synovial joint are lined with a smooth layer of articular cartilage. Articular cartilage, a type of hyaline cartilage, provides the smooth lubricated gliding surface important in allowing friction-free movement of the joint. Studies show that OA correlates to increased friction in the load bearing areas of a joint, as well as increased expression of superficial zone protein (a key protein in the synovium, described later) (Neu et al., 2010). The progression of OA can lead to lesions as deep as subchondral bone. These degraded lesions are irreversible, displaying the need for a regenerative approach to OA treatment (Jorgensen et al., 2004). Degeneration is compounded when the cartilage physically detaches as the lesion becomes larger (wider and deeper). The

break-up and detachment of cartilage fragments can cause further damage, leading to total cartilage loss and bone exposure (Stockwell, 1991). In addition to degeneration of cartilage, as OA progresses bony out-growths (osteophytes) begin to form around the joint which wears on the surrounding ligaments ("Center for Disease Control and Prevention," 2010). These physiological manifestations lead to the symptoms of pain, inflammation and joint stiffness (Stockwell, 1991). The joints more often used and with the highest loads show higher incidences of OA, as a result feet, hips, knees, hands, and spine are most often affected (Hunter, 2007). The combination of pain and stiffness during locomotion can greatly limit the range of motion, often reducing the quality of life as compared to those with full joint mobility.

In a normally functioning joint, articular cartilage lines bone surfaces and give the joint the ability to move freely. This function is made possible by the three layers that make up articular cartilage tissue: superficial zone, middle zone, and deep zone. Each layer has a distinct cellular morphology as well as a different gene expression profile that allows articular cartilage to function correctly (Temenoff & Mikos, 2000). The middle and deep layers secrete Type II collagen and Aggrecan (a proteoglycan) respectively (Lee et al., 2008). Type II collagen and Aggrecan act as important structural elements of articular cartilage and serve as markers for identifying these two layers. The superficial zone is characterized by flattened cells in a parallel arrangement that secrete superficial zone protein (also named Lubricin) (Lee et al., 2008). This protein is essential for normal joint function as it reduces the friction coefficient and acts as a boundary lubricant

between the two surfaces. Studies have linked OA to a reduced expression of superficial zone protein and loss of function (Elsaid et al., 2007).

Superficial Zone protein (SZP) is a key glycoprotein found in many tissues of the synovial joint (synovial membrane, tendon, meniscus, and infrapatellar fat pad) (Lee et al., 2010; Rees et al., 2002; Schumacher et al., 1999; Schumacher et al., 2005). The 345 kDa protein is encoded by the PRG4 gene located on chromosome 1q25 and contains several functional domains. The domain responsible for lubrication is an extensive mucin like region, which is highly substituted by O-linked oligosaccharides that produce repulsive forces which reduce friction (Jay et al., 2001). SZP is the critical proteoglycan for providing boundary lubrication, giving the joint a near frictionless environment to move as well as inhibiting overgrowth of chondrocytes (Chan et al., 2010; Rhee et al., 2005). Comparing the zones of articular cartilage, SZP is highly expressed by the cells within the superficial zone, and can be found secreted into the synovial fluids as well as bound to macromolecules of the surface zone (Jones et al., 2007; Schmid et al., 2001). Research shows that early stage OA results in decreased SZP expression with increased friction, while late stage OA shows an increase in SZP expression (Elsaid, et al., 2007; Neu, et al., 2010; Young et al., 2006).

It is important to note that articular cartilage is not innervated by vascular tissue. Not only does this impart an extremely low oxygen tension, 1% in deep zones, but it also makes it hard for the body to regenerate cartilage, or clinically deliver treatment via the bloodstream (Nakagawa et al., 2009). For this reason, current treatment is aimed at elevating the quality of life for OA patients, allowing movement with less pain, but making no repairs to the underlying cause. Conservative treatments are aimed at life style changes including weight-loss and mobility aids such as canes, crutches, and braces to alleviate pressure to the affected joints, in addition to physical therapy (Hunter, 2007). Pharmaceutical treatments begin with nonprescription medication such as acetaminophen, to control pain and inflammation of the affected joints. When acetaminophen is insufficient, prescription non-steroidal anti-inflammatory drugs (NSAIDs) can be used to control swelling due to inflammation. Extreme treatments include injection of corticosteroids directly into the joint, replacing synovial fluid with synthetic synovial fluid, or surgery. Both corticosteroids and synthetic joint fluid work well for reducing inflammation and pain but these effects are short lived and must be administered several times a year. Surgery is used to excise damaged cartilage, fuse joint bones together (spinal OA), or replace the joint altogether with a prosthesis. All treatments fall short of permanent relief of symptoms associated with OA. For this reason continued research must be done on methods to regenerate and replace the damaged articular cartilage.

Komárek and colleagues were among the first to treat damaged, focal lesions of articular cartilage with autologous chondrocytes (Brittberg et al., 1994). Autologous cellular transplant (ACT) by definition treats the patient using cells from their own body. In the case of articular cartilage focal defects, clinicians have used chondrocytes taken from the healthy non-load bearing regions of cartilage, cultured ex-vivo, and then transplanted to the degraded cartilage lesion of the same patient. The technique of using one's own cells in treatment of any disease has the advantage of minimizing immune system reactivity since the transplanted tissue will be recognized as "self." The results of these studies show a decrease of pain and increase in range of motion (Nelson et al., 2010). Limitations of this treatment to small focal regions are due to the decrease in proliferation capacity of mature cells (chondrocytes) with increased donor age, the transplanted cells can only divide a limited amount of time. Because OA is primarily a disease of the elderly, the constraint of age related autologous cells division greatly limits ACT as a treatment for the geriatric community.

Mesenchymal stem cells give us a possible solution to the limited chondrocyte proliferation. Much attention has been focused on mesenchymal stem cells (MSCs) for treatment of many diseases and traumas. MSCs represent a potential strategy for therapeutic applications of stem cells due to their defining characteristics of self renewal and ability to differentiate into functional cell types. Bone marrow aspirates are a main source of MSCs, though they are also found in adipose, skeletal muscle, synovium, and periosteum (Sakaguchi et al., 2005). Because they are readily available throughout the body, these cells can be obtained directly from the patient for the patient's own therapeutic use (Baksh et al., 2004). Moreover, it has been shown that stem cells treated with morphogens or transcription factors will induce chondrogenic differentiation and subsequent chondrogenesis (Barry et al., 2001; Friedenstein et al., 1966; Jorgensen et al., 2004; Pereira et al., 1995; Raghunath et al., 2005; Temenoff & Mikos, 2000; Wang et al., 2005; Williams et al., 2003). MSCs therefore, offer a legitimate route to the treatment of OA.

With the goal of inducing chondrogenesis, researchers have used many growth factors and morphogens to successfully differentiate stem cells down the path of chondrocyte lineage. Previous research has used bone morphogenetic protein 7 (BMP7) and transforming growth factor beta1 (TGF β 1) to induce hESCs to express the articular cartilage phenotype (Nakagawa et al., 2009; Shen et al., 2010). BMPs are a conserved sub-family of TGF β , and are expressed in chondrocyte differentiation, and their receptors expressed in distinct cell types within cartilage (Pathi et al., 1999). BMPs act as growth factors, binding to cell receptors type I/type II serine/threonine kinase receptor complexes, and TGF β -activated kinase 1 (TAK1) activate signal transduction pathways ultimately leading to activation of transcription factors R-SMAD5, R-SMAD8, SMAD-4 (via type I/type II serine/threonine kinase receptor binding), p38 MAPK (via TAK1 binding), Sox9, and Brachyury which enter the nucleus and regulate transcription of targeted genes (Jorgensen et al., 2004; Shen et al., 2010). Sox9 and Brachyury induce downstream expression of specific genes, such as aggrecan and collagen II (Jorgensen et al., 2004). Transcription of these targeted genes results in enhanced mRNA expression or protein expression of chondrogenic markers (Aggrecan, Collagen) and extracellular matrix proteoglycan synthesis.

Recently, the conditions in which MSCs are cultured and differentiated have been a major focus to better control their chondrogenic differentiation. It is common practice in cell culture to use atmospheric concentrations of oxygen gas, 21%, along with 5% carbon dioxide at a temperature of 37° C. A carbon dioxide concentration of 5% serves as a buffer, and represents the *in vivo* concentration (Csete, 2005). However, the 21% O₂ is

far more oxygen than chondrocytes are exposed to in vivo (as low as 1% in deep zones) (Murphy & Polak, 2004). Therefore many studies have been oriented at the effects of hypoxic conditions during chondrogenesis. When differentiating stem cells, the best results in obtaining large, homogeneous populations of a specific cell type is obtained when the developmental environment is mimicked as precisely as possible (Murry & Keller, 2008). Studying the development of bone and cartilage, the first steps are the differentiation of MSCs into chondrocytes that form a hyaline cartilaginous matrix, which will serve as the growth plate for the forming bone. This happens during an avascular period and therefore a hypoxic environment. As the bone is vascularized and the tissue is no longer hypoxic, osteoblasts arise from the local MSCs and begin to form bone. Therefore chondrogenic differentiation using low oxygen could be critical to the differentiating stem cells. Through the stabilization of hypoxic inducible factor 1α (HIF- 1α), cells and tissues are able to monitor the changes in available O₂ and transcribe hypoxic inducible genes (Csete, 2005). This relatively novel area of research has generated a limited number of published articles concerning the effect of hypoxia on MSC chondrogenic differentiation (Das et al., 2010). One study examined the effect of hypoxia on MSCs and found MSCs have increased proliferation in hypoxic conditions (Wang et al., 2005). Still other studies have found that chondrogenic differentiation of MSCs in hypoxic conditions $(1-5\% O_2)$ increased the expression of aggrecan, collagen type II, as well as activation of transcription factor Sox9 (Kanichai et al., 2008; Martin-Rendon et al., 2007; Robins et al., 2005; Wang et al., 2005). In addition to increasing expression of articular cartilage proteins, the low oxygen environment has been shown to

decrease collagen type X, an unwanted form (in articular cartilage) that is associated with fibrillation and scaring (Betre et al., 2006).

Mesenchymal stem cells provide us with multi-potent, self-renewing cells that are abundant in the human body. Their ability to differentiate into connective tissues makes them a prime candidate for articular cartilage regenerative medicine. However, though MSCs are regularly differentiated into chondrocytes, and induced to undergo chondrogenesis, the expression and secretion of SZP, the crucial lubricant proteoglycan, has yet to be achieved. Knowing that the development of articular cartilage occurs in a hypoxic environment, more research needs to be done concerning stem cell chondrogenic differentiation in hypoxic conditions. I hypothesize that chondrocytes differentiated from bone marrow derived MSCs in hypoxic conditions (3% O₂) will produce more SZP than those differentiated at atmospheric oxygen tension (21% O₂).

METHODS

Isolation of MSCs from human whole bone marrow. Human bone marrow MSCs (hBM-MSCs) were obtained from Dr. Jan Nolta (Institute for Regenerative Cures, UC Davis Health Sciences, Department of Internal Medicine). The IRC received the sample as whole bone marrow, purchased commercially (AllCells Inc.). To isolate the MSCs, nucleated cells were recovered from a 25mL sample using a Ficoll-Paque density gradient and were resuspended in complete culture medium (CCM). After 24 hours non-adherent cells were discarded and adherent cells were washed twice with PBS, and resuspended in CCM. CCM contains α MEM modified media (Hyclone) supplemented with 20% premium select fetal bovine serum (Atlanta biologicals, lot selected for maximum growth), 1% penicillin/streptomycin (HyClone), and 1% L-glutamine (HyClone). For freezing, 5x10⁵ cells are frozen in 5% DMSO in CCM solution.

Maintenance of hBM-MSCs. hBM-MSCs were plated at $1.75 \times 10^5 \cdot 2.5 \times 10^5$ cells per T-175 flask in 25mL MSC media (MSCM). MSCM was composed of α MEM supplemented with 20% FBS and 1% Glutamax. Cells were allowed to attach and grow in a 37°C incubator, containing 21% O₂, 5% CO₂, media was changed twice a week. Once the cells in the T-175 flasks were 70-80% confluent, the media was siphoned off, and the cells rinsed with 25mL room temperature PBS. After PBS removal, cell were lifted by adding 5mL .083% Trypsin/EDTA solution and allowed to incubate for 5 minutes at 37°C, 21% O₂ and 5% CO₂. Microscopy confirmed the cells were lifted and floating based upon their spherical morphology and movement. Trypsination was halted by addition of 10mL MSCM (20% FBS). Cells were then plated again at 1.75×10^{5} -2.5x10⁵ cells per T-175. In this way the hBM-MSCs were kept in an undifferentiated state.

Functional differentiation of hBMD-MSCs into chondrocytes. For chondrogenic differentiation of MSCs, 3-dimensional aggregate cultures were carried out. To form a pellet, 4×10^5 cells were collected in 15-ml polypropylene conical tubes (Becton Dickinson, Franklin Lakes, NJ) and centrifuged at 200g for 5 minutes. Serum free chondrogenic medium (CM) consisting of high-glucose DMEM containing 0.1 µM dexamethasone (Sigma), 50 µM L-ascorbic acid 2-phosphate (Sigma), 40 µg/ml proline (Sigma), 1 mM sodium pyruvate (Gibco), 1% ITS+ Premix (6.25 µg/ml, 6.25 µg/ml transferrin, 6.25 ng/ml selenious acid, 1.25 mg/ml bovine serum albumin, and 5.35 ug/ml linoleic acid) (Becton Dickinson) in the presence or absence of 10 ng/ml TGFB1 (R&D Systems) and/or 300 ng/ml BMP7. Morphogen concentrations were based on pilot experiments and previous work (Lee et al., 2008). The pellets were then divided into 2 groups: Low Oxygen (hypoxic) and Atmospheric Oxygen (normoxic). Within the two main groups, the pellets were further divided into 4 groups; Culture Media (CM) alone, CM with TGF^β1, CM with BMP7, and CM with TGF^β1 and BMP7. All groups were incubated for 21 days. There were a total of 4 pellets per group, and medium replaced every 3-4 days.

Histologic and immunohistochemistry analysis for chondrogenic proteins and SZP. After 3 weeks of differentiation, pellets were measured with a microscope using a 500 µm scale. Volume was calculated using ellipsoid volume: $4/3\pi r_1 r_2 r_3$, where the depth (r_3) was equal to the smallest r value. After measuring radius of the pellets, 2 pellets from each group were fixed in Bouin's fixative (Sigma) overnight, washed and dehydrated in ethanol, and embedded in paraffin. Histology sections (5µm sections), mounting, and Toluidine blue staining was performed by UC Davis Veterinary Histology. For immunohistochemistry (IHC), a Vectastain Elite ABC Kit and ImmPACT DAB Peroxidase Substrate (Vector, Burlingame, CA) were used on the 5µm sections. The sections from the pellets were then treated with 0.3% hydrogen peroxide in methanol for 20 minutes, rinsed in H₂O, and treated with 5% normal horse serum for 20 minutes. Sections were then incubated overnight at 4°C with anti-human superficial zone protein (SZP) mAb (S6.79) at a 1:1000 dilution. After washing in H_2O , the sections were then incubated with biotinylated secondary antibody for 30 minutes at room temperature, then rinsed and incubated with ABC reagent for 30 minutes at room temperature. After further washings in $H_{2}O_{1}$ sections were permanently mounted. As a negative control no primary antibody was added to duplicate samples.

Western Blot for secreted SZP in chondrogenic media. After 21 days of incubation, changing the media twice a week, the media (1mL) was collected from each pellet to analyze any SZP secretion. Samples were prepared using LDS sample buffer, Betamercaptoethanol, and dH₂O. Bovine synovial fluid (SF) was used as positive control, H₂O as a negative control, samples and controls were run along side BioRad Precision Plus Protein Dual Color Standards (161-0374).

Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) for chondrogenic genes. The pellets were homogenized with disposable pestles and total RNA was extracted using an RNeasy Mini Kit with DNase I (both from Qiagen, Valencia, CA) [Nakagawa, 2009]. In an attempt to increase the RNA yield, 2 pellets were used per sample. Total RNA was reverse transcribed with a random primer using a Superscript first-strand kit (Invitrogen). Real-time RT-PCR was performed using the TaqMan Gene Expression Assay and ABI Prism 7700 Sequence Detection System according to the instructions of the manufacturer (Applied Biosystems, Foster City, CA). Primers used can be found in Table 1. The level of each target gene was normalized to GAPDH levels and expressed relative to the control culture levels ($\Delta\Delta C_t$ method; Applied Biosystems).

Statistical analysis. For all experiments the values were presented as the mean \pm standard deviations. All quantitative data were analyzed by one way ANOVA analysis of variance. *P* values below 0.05 were considered statistically significant.

Table 1. Primers used for quantitative reverse transcriptase PCR

Gene	Forward	Reverse
gapdh	ATGGGGAAGGTGAAGGTCG	TAAAAGCAGCCCTGGTGACC
col1	GCCTGGTGTCATGGGTTT	GTCCCTTCTCACCAGCTTTG
sox9	ACGCCGAGCTCAGCAAGA	CACGAACGGCCGCTTCT
szp	TTGCGCAATGGGACATTAGTT	AGCTGGAGATGGTGGACTGAA
aggrecan	TCGAGGACAGCGAGGC	TCGAGGGTGTAGCGTGTAGAG

RESULTS

Pellet size, morphology. To qualitatively determine the degree of chondrogenesis the volume and morphology of the differentiated pellets was observed. Pellets that have achieved chondrogenesis are a light-brown, beige color with a high sheen to the surface. Normoxic pellets (Figure 1) show a more constant spherical shape than hypoxic (Figure 2). In addition, the hypoxic pellets show variability in their color, however it should be noted this was due to a change in lighting during the picture taking process while pellets where under the microscope (orangish pellets are due to nearby blinds being open instead of closed). The normal color is seen throughout the normoxic pellets and the majority of hypoxic pellets, a beige tan color which is standard in chondrogenesis of pellet culture. In addition the glossiness is an important observation of the pellets.

The reflecting light from pellet surfaces suggests a hyaline cartilage being generated similar to en-vivo hyaline cartilage which is a result in part of an extremely smooth surface. The normoxic pellets show a consistent glossiness on each pellet, while the hypoxic pellets have variable glossiness. The change of lighting also affected the observable glossiness of hypoxic pellets. For example Low oxygen control 1 and low oxygen BMP-7 1 (LC1 and LB1 respectively) both showed a glossiness expected in chondrogenic pellet culture, however the resulting photo shows the orange matte. However even in the low oxygen treated pellets that show normal coloration, a rough bumpiness can be seen in several of the pellets (e.g. LBT1, LBT2, LB2, and LB1)

The third pellet treated with low oxygen, TGF β -1 alone (LT3), is absent from the presentation of hypoxic pellets (Figure 2). This is due to the pellet disintegrating during

it's handling after 3 weeks differentiation, while attempting to take it's photo and measure its diameters. This is most likely due to disruption of the pellet aggregate immediately after its creation, perhaps during media exchange.

An increase of chondrogenesis should also be manifested by increased pellet size. By calculating the average volumes of each pellet group, the sizes can be compared. Figures 1 and 2 shows the pellets laying on a scale of 500 μ m, which was used to find the long and short diameters of each pellet (assuming the third diameter, the z-axis, was equal to the short diameter). The average sizes of pellets differentiated in the low oxygen environment seem to be larger (Figure 3). This trend is seen in all groups except for pellets treated with TGF β -1 alone, which shows a larger volume average for the pellets differentiated in a normoxic environment. However with a sample size of n=4, no statistical difference was found between pellets differentiated in normal oxygen and low oxygen environments.



Figure 1. Pellets differentiated in normoxic environment for 21 days. Normoxia (N) contains 21% O_2 . Four groups include control (C, no growth factors), BMP7 (B), TGF β 1 (T), or combined BMP7+TGF β 1 (BT). Background lines are scaled 500 μ m.



LC4LB4LT4LBT4Figure 2. Pellets differentiated in hypoxic environment for 21 days. Hypoxic (L) conditions contain 3%O2. Four groups include control (C, no growth factors), BMP7 (B), TGFβ1 (T), or combinedBMP7+TGFβ1 (BT). Background lines are scaled 500µm.

Average Pellet Volume



Figure 3. Histogram of average pellet volumes. Volume calculated at day 21 of differentiation. Volumes are in cubic millimeters, error bars are standard deviations. No significant differences were found ($p \le 0.05$ to be significant)

Histology toluidine blue staining. The degree of chondrogenesis can be seen in the toluidine blue staining. Toluidine blue is a metachromatic stain that turns a pinkish color upon binding to negatively charged macromolecules such as aggrecan and it's glycosaminoglycans (GAGs), a major constituent of hyaline cartilage. Figures 4 and 5 show the toluidine blue staining of 5μ m sections taken from 2 different pellets differentiated in Normoxic and Hypoxic conditions respectively with type of growth factor treatment.

Normoxic pellets show dark blue staining throughout pellet sections, including pellet surface and centers (Figure 4). The second pellets treated with BMP7 alone and combined BMP7 & TGF β 1 show the most metachromasia of the normoxic treated groups (NB2 and NBT2 respectively). These two pellets show a slight pinkish hue throughout the pellet, included in both surface and center. The cellular morphology of these two pellets also shows the most consistency which can be seen on the higher power pictures. The mature chondrocytes is a plump, spherical cell which is more common in these two pellets center than in other pellets of normoxic treated pellets. In addition the cells change morphology and density at a more fluid and constant rate as they move out toward the surface. Surface cells should be flattened and more densely packed than center cells. Indeed, this change is more consistent in these two pellets. The pellets that show a dark blue stain have a more irregular cellular morphology, larger at the center (though not thoroughly spherical), and flattened at the surface, however the change from large to flattened cells is not at a constant gradient around the surface of the pellets.

The hypoxic pellets show slightly more metachromasia than normoxic pellets. In the low oxygen treated pellets a light pinkish hue, can be seen in both pellets of BMP7 alone, the second pellet of TGF β 1 alone, and the second pellet of BMP7 & TGF β 1 combined (LB1, LB2, LT2, and LBT2). As with the normoxic treated pellets that showed signs of metachromasia, the hypoxic pellets that showed metachromasia have a more chondrogenic like morphology. These pellets, LB1, LB2, and LT2 show a more spherical morphology in the center of pellets, while the surface has a more flattened morphology packed with more cells. This might be true for LBT2, however it is broken up and therefore consistency of morphology in this pellet is hard to judge. Again, the pellets that show a darker staining pattern show a more inconsistent cellular morphology, though the number of pellets in the hypoxic group is slightly less than the normoxic group.



Figure 4. Toluidine blue staining of pellets differentiated in normoxic conditions. Normal oxygen level used is $21\% O_2$. NC = normal O_2 , control (no growth factors). NB = normal O_2 and BMP7. NT=normal O_2 and TGFB1. NBT= normal O_2 with BMP7+TGFB1. 2 pellets of each group represented at 2 different magnifications (10x and 20x objectives), 5µm sections mounted and stained. Scale on whole pellet is 100μ m, 50µm on higher magnification.



Figure 5. Toluidine blue staining of pellets differentiated in hypoxic conditions. Hypoxic oxygen level used is $3\% O_2$. LC = low O_2 , control (no growth factors). LB = low O_2 and BMP7. LT=low O_2 and TGFB1. LBT= low O_2 with BMP7+TGFB1. 2 pellets of each group represented at 2 different magnifications (10x and 20x objectives), 5µm sections mounted and stained. Scale on whole pellet is 100µm, 50µm on higher magnification.

Western blot. To determine if differentiated MSCs secreted SZP, the differentiation media from days 18-21 was collected and analyzed for SZP via western blot. Figure 6 shows the SZP content of the media found through western blot. The positive controls (Bovine synovial fluid, SF) display strong signal, and the negatives are empty.

The normoxic (atmospheric oxygen) treated samples shows that BMP-7 treated pellets have weak signal for SZP, seen in normoxic BMP-7 alone samples 1 and 3 (NB1 and NB3). In addition, the normoxic TGF β -1 treated pellets also show mild signal for SZP (NT1, NT2, NT3), though not as clear as the normoxic BMP-7 treated pellets. The normoxic pellets treated with combined BMP-7 and TGF β -1 do not appear to have secreted any measurable amount of SZP (NBT1, NBT2, NBT3).

Pellets differentiated in low oxygen show more SZP secreted into the media. In the hypoxia-treated samples, the greatest SZP synthesis occurred in pellets treated with TGF β -1 alone (LT1, LT2, LT4). The SZP secreted by 2 out of the 3 hypoxic pellets with combined BMP-7 and TGF β -1 treatment seems to be at the same level as the TGF β -1 alone (LBT2 and LBT3). LT3 media was decidedly left out of this western blot due to the malformation/differentiation of this pellet. Indeed in an earlier blot of media from LT3, no SZP was found to be secreted from this pellet (not shown).

(A) Normoxic





Immunohistochemistry. To determine SZP in the pellets bound extracellularly, immunohistochemistry for SZP was performed on 5µm sections of pellets. A secondary antibody conjugated to HRP gives a clear brown signal when treated with the appropriate substrate (DAB). It is important to note that when preparing the controls for immunohistochemistry the bovine synovial tissue did not adhere to the slide properly and therefore detached during the staining and rinsing process. Therefore we are limited to comparing within the experimental samples.

SZP expressed by pellets differentiated in normoxic conditions is shown in Figure 7. Several of these pellets show clear brown signal towards the surface layer of the pellet. Within this group of pellets differentiated in normoxia, TGFβ1 alone is the more consistently on the surface, rather than spotted throughout. Combined BMP7 & TGFβ1 also shows strong signal around the surface of the pellet. The normoxic pellet treated with BMP7 alone is broken up, so it is difficult to determine its staining pattern, though it seems like it is more spotted and random throughout the pellet.

In hypoxic cultured pellets SZP signal seen is in the pellets treated with TGF β 1 alone and BMP7 alone (Figure 8). Hypoxic BMP7 alone SZP signal can be seen on the very surface of the pellet, and lightly spotted throughout the pellets center. The TGF β 1 pellet alone is torn through the center, but the staining can still be seen on the surface of the pellet. Comparing the two groups, normoxic and hypoxic differentiated pellets, more brown signal is seen in the normoxic pellets. Each treatment group in normoxia compared to the same group in hypoxia has much more signal indicating more SZP present. The most drastic difference is seen in the pellets treated with combined growth factors BMP7 and TGF β 1, where normoxic has strong signal throughout the pellet surface, and hypoxic has next to zero signal can be found anywhere in the pellet.



BMP7

 $BMP7 + TGF\beta1$

Figure 7. Immunohistochemistry of pellets differentiated in normoxic conditions. Normoxic conditions used were 21% O_2 . Sections are 5µm thick. Primary antibody is mouse anti-SZP, secondary is hourse antimouse conjugated to HRP. Brown color is positive signal (DAB substrate). Scale bar is 100µm.



Figure 8. Immunohistochemistry of pellets differentiated in hypoxic conditions. Hypoxic condition used contains $3\% O_2$. Primary antibody is mouse anti-SZP, secondary is horse anti-mouse conjugated to HRP. Brown color is positive signal (DAB substrate). Scale bar is $100\mu m$.

qPCR of expressed mRNA. Four chondrogenic gene transcripts were isolated and quantified using qPCR: Aggrecan, Collagen1, PRG4 (SZP) and Sox9. GAPDH expressed in normoxic conditions was used to normalize all other genes expressed in normoxic and hypoxic conditions. Figure 9 displays the fold change in mRNA expression of these 5 genes. A trend of down regulation of genes is seen in hypoxic conditions.

In normal oxygen treated pellets, PRG4 transcript coding for SZP decreased drastically in TGFβ1 alone and combined BMP7 & TGFβ1 growth factor treatments. This was the only gene to show such a noticeable decrease in expression at this oxygen level, the other three genes either increased slightly or stayed at the same expression level with the various growth factor treatments in normoxia. Out of these three genes, Aggrecan and Sox9 change noticeably within normal oxygen conditions. Aggrecan and Sox9 increased almost 1.5 fold in combined growth factors, and slightly in BMP7 alone. Collagen1 is the only gene to show a decrease in normal oxygen BMP7 alone, while increasing slightly over TGFβ1 alone and combined BMP7 & TGFβ1.

Hypoxic conditions show an overall decrease in expression of all four probed genes. Out of all four genes SZP was the most drastically decreased, which was barely in the range of detection. Aggrecan and Collagen1 increased expression in low oxygen treatment from control and BMP7 alone, to TGFβ1 alone and combined growth factors, though Collagen1 increased much more noticeably than Aggrecan. Sox9 stayed at a constant level at this oxygen concentration, though showed the greatest change (increased expression) in the TGFβ1 alone. SZP mRNA transcript was at its highest in BMP-7 differentiated cells in normoxic conditions. Not only did this expression decrease drastically in the normoxic TGF β -1 and combined BMP-7 & TGF β -1 treatments, but SZP transcripts in hypoxic conditions was nearly non-existent, though increased slightly in combined BMP-7 & TGF β -1 treatment.



Figure 9. qPCR data normalized to normal oxygen pellet's GAPDH gene. Fold change of aggrecan, collagen1, PRG4, and Sox9 genes.

DISCUSSION

Minimal chondrogenesis was achieved in the differentiated pellets of both normoxic and hypoxic treatments. Histology of the differentiated pellets shows only very weak metachromasia of the toluidine stain which color shifts from blue to pink in the presence of GAGs which is a key glycoprotein of cartilage matrixes. The lack of a strong pinkish hue in the toluidine blue histological pellet sections shows minimal GAG secretion into the extracellular matrix, and therefore only slight chondrogenesis achieved. Also, mature chondrocytes have been seen using these growth factors and culture method in the past. No significant GAG increase along with no mature chondrocytes morphology does not agree with results already published (Nakagawa et al., 2009), which shows an increase of GAGs secreted and mature chondrocytes using the same growth factors and culture methods at "normal" oxygen levels.

The size of pellets differentiated by the various growth factors is also in disagreement with the previously published results using these growth factors. Previous research with H9 ESCs show combined growth factors BMP7 & TGF β 1 to be the largest pellets compared to no growth factors, BMP7 alone, and TGF β 1 alone. Our results here show the opposite, the only group that was smaller than our combined growth factor treatments was TGF β 1 alone. Instead of control (no growth factors) showing the smallest pellets, our control group was the largest pellet size on average. The present study used MSCs, therefore differences could be due to variations and differences in the cell lines used. Western blot results of the media taken from differentiated pellets shows more SZP in the media of hypoxic treated pellets than in the normoxic treated pellets. The western blot shows that more SZP was found consistently in media of hypoxic pellets with TGF β -1 and combined BMP-7 & TGF β -1 treatments than control and BMP-7 alone. This single result led us to believe we were on the right track in expressing SZP and creating articular cartilage, however more analysis of other methods showed otherwise.

Immunohistochemistry for SZP shows articular cartilage was not generated. Although positive controls for this analysis did not develop properly, comparing the normoxic and hypoxic conditions of the various treatments shows a clear decreased expression of SZP in the hypoxic treated pellets. In the pellets differentiated in normoxic conditions, BMP-7 and combined BMP-7 & TGF β -1 showed the most SZP expression. The lack of SZP shown through immunohistochemistry does match the results of the experiments previously discussed using ESCs at normal oxygen concentration, which shows these growth factors and culture method insufficient at inducing expression of SZP (Nakagawa et al., 2009).

qPCR confirms the previous results of lack of SZP increase in the immunohistochemistry of pellets. The results show a decrease in all genetic markers used in the MSC pellets differentiated in low oxygen. The only increase seen in the hypoxia treated cells is Collagen1 transcripts of differentiated MSCs treated with BMP-7 alone. These qPCR results agree with our immunohistochemistry, in that they show no increase of SZP in the pellets differentiated in low oxygen. However, the other genes we have data for (Sox9, Aggrecan, and Collagen1) should have all increased in low oxygen differentiations according to previously published results (Kanichai et al., 2008; Martin-Rendon et al., 2007; Robins et al., 2005; Wang et al., 2005).

Although articular cartilage was not generated using hypoxia, much more work needs to be done in the field of induced MSC chondrogenesis in low oxygen environments. In this specific experiment, an increased population of pellets would be the primary focus, to increase the statistical relevance. In several of our quantitative and molecular methods, statically relevant data could not be gathered due to small pellet population. For example qPCR results did not agree with published works, which could be associated with the problems we had isolating high quality and quantity of nucleic acids. Collecting nucleotides from pellet remains a difficult method; therefore a streamlined approach to acquire nucleotides would need to be developed and applied in order to harvest an increased quality and quantity of mRNA transcripts. In addition more primers for chondrogenesis can be used for quantifying chondrogenesis. Obtaining a larger, cleaner population of nucleotides would better confirm or argue the previously published results. More oxygen tension points would also be beneficial; we chose $3\% O_2$ as hypoxic conditions because of its point between 1% and 5% which are the most common hypoxic conditions used in the literature. Next quantification of chondrogenic proteins is also a necessary step. ELISA would be a powerful tool to quantify data of expressed proteins secreted in media, and it might be beneficial to analyze the media for secreted proteins throughout the differentiation instead of only the last 3 or 4 days. Finally, GAG concentrations would be beneficial using standardized using sGAG dye binding assay.

Although this research has not changed the current status of obtaining stem cell derived articular cartilage, this data as well as copious amounts of published work shows low oxygen does affect stem cells during differentiation. In applying stem cells in treating articular cartilage defects, obtaining a large number of chondrocytes and genesis of hyaline cartilage is just a starting point. In order for stem cell derived articular cartilage to be applicable in treating lesions of damaged tissue en-vivo, the resulting engineered tissue needs to have the same mechanical properties as articular cartilage envivo, including the production and expression of SZP. When this is accomplished a viable long term treatment may be available for large injury defects of articular cartilage and osteoarthritis.

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