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Bilaminar CoCulture of Stem Cells and Instructive Cells for Tissue Regeneration

By

Aliza Apple Allon

DISSERTATION

Submitted in partial satisfaction for requirements of the degree of

DOCTOR OF PHILOSOPHY

in

Bioengineering

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO

and

UNIVERSITY OF CALIFORNIA, BERKELEY

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Abstract

Bilaminar Coculture of Stem Cells and Instructive Cells for Tissue Regeneration

By

Aliza Apple Allon

Doctor of Philosophy in Bioengineering

University of California, San Francisco and Berkeley

Professor Jeffrey C. Lotz, Chair

Tissue engineering is a growing and dynamic field with the potential to provide patients with minimally-invasive treatments that repair or replace dysfunctional musculoskeletal tissues. For the intervertebral disc, the goal is to re-establish pain-free motion by restoring the disc's physical and biochemical properties. Unfortunately, many of the biological processes involved remain unclear. Mesenchymal stem cells (MSCs) are an attractive component of disc tissue engineering given their ability to differentiate down multiple lineages. However, MSCs require a coordinated set of environmental cues to appropriately differentiate to a chondrogenic or disc-like cell.

Our goal is to identify mechanisms involved in disc regeneration and MSC differentiation to optimize disc tissue engineering strategies. We explored the benefits of coculturing nucleus pulposus cells (NPC) and adult mesenchymal stem cells (MSC) using a 3D system that exploits embryonic processes such as tissue induction and condensation.

The following dissertation reports on a novel “bilaminar cell pellet” (BCP) approach that structures cell-cell signaling between MSCs and instructive cells, in this case non-degenerative NPCs, in a 3D culture system. A spherical bi-layer pellet is used where one cell type forms an inner sphere enclosed within a shell of the other cell type. This technology has been patented by UCSF as of May 2008 (see Appendix).

The BCP system has been tested extensively under multiple conditions. We first tested the BCP under normal in vitro culture conditions and showed an increase in matrix production over controls. The BCP was subsequently tested in a bioreactor supplemented with inflammatory cytokines to simulate the degenerative disc environment. Again, the BCP proved to be resilient and produced more new matrix than controls. Finally, the BCP was evaluated in an in vivo rat tail model. Our data show that the beneficial behaviors previously reported in vitro translate to a more effective cell-based treatment in vivo. Future studies will explore the function of BCPs in larger discs that more closely mimic the human situation.

Our data show that spatial organization plays an important role in matrix production, and that structured communication between MSC and NPC enhances the efficacy of stem-cell-based strategies for nucleus regeneration beyond using MSCs alone.

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CH 1. Stem cells for disc repair

1.1 INTRODUCTION

1.1.1 The intervertebral disc

The intervertebral disc forms an avascular, fibrocartilaginous joint between adjacent vertebral bodies and provides flexibility while routinely supporting several multiples of body weight. The disc is composed of three major sub-tissues, the gelatinous nucleus pulposus (NP), the fibrous annulus fibrous, and cartilaginous endplates (Figure 1.1). The NP is centrally located and composed primarily of sulfated-glycosaminoglycan (GAG), type II collagen, and water. The NP serves as the osmotic mechanism that generates volume and hydrostatic pressure because the high GAG content makes the tissue very hydrophilic. The annulus fibrosus is firmly attached to the vertebral edges to serve both as a ligament to guide intervertebral movement, and as a barrier to contain nuclear swelling and thereby allow disc pressurization. The endplate is a thin (0.1 to 1.6mm) hyaline cartilage layer that separates the NP from the adjacent vertebra. The endplate functions as a semi-permeable membrane to allow diffusive communication between disc nuclear cells and vertebral vasculature, as well as to prevent large molecular weight GAG from leaving the nuclear space.

1.1.2 The clinical implications of disc degeneration

Pain of spinal origin afflicts most adults at some point in their lives: the annual US incidence of acute and/or chronic back pain is approximately 100 million [1].

Intervertebral disc degeneration underlies several painful low back disorders including intervertebral disc herniation (IVDH), degenerative spondylolisthesis (DS), spinal stenosis (SS), and degenerative disc disease (DDD). For the first three (IVDH, DS, SS), recent randomized prospective clinical studies have demonstrated advantages of surgical care compared with non-operative care [1-2]. However, DDD management remains the most difficult challenge because the underlying source of pain is unclear, causing uncertainty when developing guidelines for operative and non-operative care and therapies with improved efficacy [3]. As a result, estimates suggest there are between 1.5 and 4 million adults in the US with DDD-related chronic low back pain (CLBP) that have failed conservative management and await therapeutic intervention, of which there are few options beyond spinal fusion. [2-3]

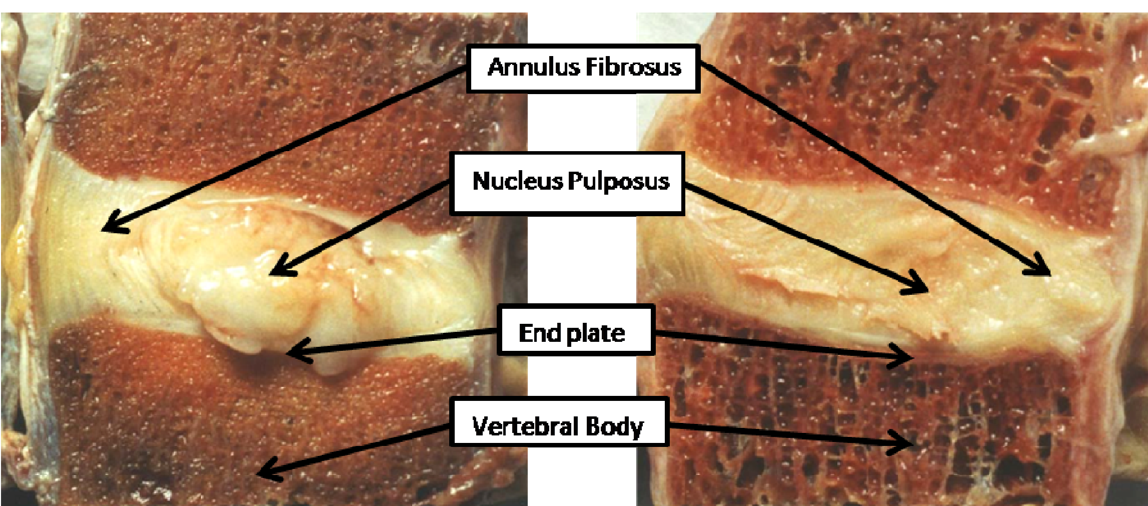

Figure 1.1: Mid-sagittal sections through a health (left) and moderately-degenerate (right) human disc. In the healthy state, the nucleus possesses a significant capacity to swell and support spinal forces. The early stages of degeneration are characterized nuclear fibrosis and annular fissuring.

CLBP is a common indication for spine fusion surgery, and more recently total disc replacement. Although success rates from spinal fusion are in the range of 70%, has several disadvantages including a decrease in spinal range of motion and acceleration of degeneration at adjacent levels [4,5]. Disc replacement (where a prosthetic disc is implanted between vertebrae) is an attractive alternative to fusion since it maintains near-physiologic movement, but there are currently barriers to widespread use, such as limited insurance reimbursement, and potential surgical and implant-related complications [6,7]. Spine fusion will likely remain the standard of care for advanced disease, where arthritic changes prevent return of normal function even with newer motion-sparing technologies. The concept of minimally-invasive biologic disc repair for less advanced cases of degeneration has grown in recent years. These include gene therapy, injection of various growth factors, and cell implantation (with or without scaffold).

1.2 THE DEMANDING INTERVERTEBRAL DISC ENVIRONMENT

The process of age-related disc degeneration can be considered chronic dysfunctional matrix remodeling in response to physical inputs such as impaired transport and/or abnormal mechanical loading, with the response to both likely modulated by yet undefined familial risk factors [58]. At an early age (before 10 years) there is a marked decrease in endplate vascularity and beginnings of structural disorganization. After age 20, the disc becomes sealed-off from the vertebral blood supply by the cartilage endplates and subchondral bone [59-60]. Thereafter, disc cell survival is dependent on diffusion from capillaries in the adjacent vertebra (for nuclear cells) and surrounding vascularized tissues (for annular cells) [24]. The capillaries within the vertebra terminate just above the hyaline cartilage endplate, providing a continuous capillary bed across the bone disc

interface [6]. Once nutrients reach the endplate, movement of small solutes (e.g. glucose and oxygen) pass through disc matrix primarily by diffusion [24-28] (larger solutes may also be influenced by convective fluid flow created my mechanical disc compression and recovery). Cells compete for nutrition, making it difficult to sustain high cell densities at the long distances from the nutrition source typical of human lumbar discs (approximately 8 mm)[25-28].

This disc transport limitation has several negative consequences. Tissue oxygen concentrations are low, in the range of 0.5% to 5% [61]. These hypoxic conditions inhibit matrix synthesis: sulfate incorporation at 1% oxygen is one-fifth that at 5% [24,61]. Because of limited oxygen, the nucleus pulposus cells produce energy through anaerobic glycolysis, which utilizes glucose and generates lactic acid as a byproduct [24,27]. Accumulation of lactic acid decreases disc pH (to near pH 6.3) and is detrimental to matrix as it decreases glycosaminoglycan production, tissue inhibitor of metalloproteinases (TIMP) production, and cell viability [62]. The dependence on anaerobic glycolysis for cell production of ATP makes glucose a critical nutrient. Disc glucose concentrations are typically considered to be in the range of 0.5 to 5 mM, where disc cells die within 24 hours at concentrations below 0.2 mM [27]. Other factors in serum are also important as serum deprivation results in decreased cell proliferation and increased cell senescence [63]. Moreover, the influence of these factors is not necessarily independent, since some research indicates hypoxia supports nucleus cell survival during serum withdrawal [63].

Another main feature of the disc nucleus is osmolality. The disc functions biomechanically by using a high osmotic pressure (generated by proteoglycan fixed

charge) to attract water and produce physical pressure to support spinal compression. This osmotic stress (in the range of 250 to 450 mOsm) causes changes in cell volume and stimulates cell behavior via cytoskeleton rearrangement [27]. Under hypo-osmotic conditions (250 mOsm) disc cells increase gene expression for aggrecan and type II collagen, while at hyper-osmotic conditions (450 mOsm) there is a down-regulation of biglycan, decorin and lumican in nucleus pulposus cells [28].

1.3 EVALUATING A CELL-BASED THERAPY

1.3.1 In vitro outcome measures and culture models

Since disc degeneration is considered to initiate in the nucleus, regenerative strategies target nucleus regeneration. As a results, stem cell based therapies focus their efforts on replicating the characteristics of the native NPCs. Stem cell performance is typically evaluated by characterizing gene expression and the matrix synthesis. In order to assess differentiation stage and the cell fate, the gene expression levels are measured for the positive chondrogenic marker Sox9, as well as several negative markers including the fibroblastic marker Collagen 1, the hypertrophic marker Collagen X, and the osteogenic marker Runx2. Major matrix proteins aggrecan and Collagen 2 are often evaluated to assess the cells' level of protein synthesis. In addition, matrix metalloproteinase (MMP) genes that are responsible for degrading tissue are generally measured, in particular MMP-2, MMP-9 and MMP-13 that are commonly measured in degenerate discs [49]. At the protein level, proteoglycans are typically measured using a dimethylmethylene blue assay [52,53]. Both proteins can be also be assessed

qualitatively using histologic staining with Safranin-O or immunohistochemistry techniques [52].

Biological efficacy should be demonstrated in models of increasing complexity. Two and three-dimensional cell culture systems can be useful for initially demonstrating cellular effects, dosing, and toxicity. Three-dimensional systems are preferable to maintain cell phenotype, and augmenting stimuli to include other disc mimetic conditions such as pressure, hypoxia, and inflammation are important as these factors can significantly influence cell function [47]. The degenerative disc conditions will vary with the degree of degeneration, however, the cells implanted in a human degenerative disc will generally experience hypoxia (about 4% O2), high pressure (350 KPa at rest), inflammatory cytokines (particularly TNF-alpha and Il-2b), and a drop in pH (as low as pH 6.7). To simulate those conditions, a number of different methods can be used. The oxygen content can be controlled with a hypoxic incubator and a pressurized environment can be stimulated with the aid of a bioreactor. In addition, the inflammatory cytokines and pH levels can be replicated in the culture media. Therefore, the in vivo degenerative disc environment can be mimicked in vitro and should be considered during testing and optimization.

1.3.2 Models for efficacy and safety: in vivo Pre-clinical models

Ultimately small animal studies are a critical next step because of the important in situ interactions between disc cells and spatially-varying host features unique to the healing disc environment: pressure, hypoxia, degraded matrix, cytokines, other stromal and inflammatory cells, plus systemic factors. Along with an increasingly complex model comes challenges in response interpretation. Outcome measures should be coupled to the

designed treatment mechanisms, but should also include overall indices of disc quality such as histology and biomechanics. Time dependence of the outcomes is critical to establish whether the therapeutic response is persistent above the background degenerative response typically triggered by the therapy delivery. Design of experiments (DOE) techniques for study design and statistical analyses can help establish sample size and efficiently optimize treatment parameters [48].

Safety also needs to be established in pre-clinical models. The avascular nature of the disc environment can lead to persistence of active therapeutic agents, secreted cytokines, as well as carrier degradation products. Consequently, even though a growth factor or carrier has an established use track record in other tissues, they need to be evaluated in the unique NP environment. Adverse reactions can manifest through interdiscal toxicity, inflammatory cell recruitment, and matrix erosion. For example, cytokines and scaffold degradation products can diffuse from the disc and incite a sclerotic reaction in the adjacent vertebral endplates, along the delivery wound site, or outside the annulus fibrosus [49]. Equally important is to establish the reaction to extradiscal placement of the therapeutic materials and delivery vehicles. It is likely that these can escape from the disc during surgery or early in the post surgical period. Inflammation and the mass effect induced by these materials can adversely affect adjacent nerve roots and other paraspinal tissues [50]. The safety of injected materials or cells should also be confirmed under the worst case scenario to avoid a catastrophic event, such as the one seen for chemonucleolysis [51].

After efficacy and safety are established in vitro and small animals, large animal studies are required to motivate clinical use, principally because of size effects on disc

transport [26] and biomechanics. Typical animals used for this purpose include goats, sheep and mini-pigs. As with other pre-clinical models, efficacy may be difficult to establish due to a lack of relevant starting points and clinical metrics that match the intended patient population. Yet, biologic plausibility should be supported as well as safety through histological, biochemical, and biomechanical assays. Comparisons to negative controls (surgical procedure without treatment delivery) and untreated levels can help judge effect size and potential clinical relevance.

1.4 NON-STEM CELL BASED REGENERATION STRATEGIES

1.4.1 Gene therapy and growth factors

Several groups have published promising results using either in vivo or in vitro gene therapy [31]. Growth factors such as TGF-β, BMP2; transcription factor Sox9; inhibitors IL1Ra (interleukin-1 receptor anatagonist) and TIMPs have been successfully delivered to nucleus pulposus cells (NPCs). Another approach that modulates cells at the gene level is RNA interference which is designed to silence genes that are the potential cause of disc degeneration without needing a viral delivery vehicle [32].

The most straight-forward acellular biologic strategy is to inject growth factors into the disc. Several studies have reported in vivo effects of OP-1 (osteogenic protein-1), TGF-β (transforming growth factor-b), GDF-5 (growth differentiation factor 5) that have led to an increase in disc height and GAG content [33]. Although promising results have been reported using these techniques, the relative acellularity of human degenerated discs raises the concern that the patient's own disc cells may be insufficient to mount a

therapeutic response. Other concerns regarding these strategies include the activation of the host immune response due to the presence of viral vectors during gene therapy and the short half-life of bioactive molecules used in growth factor therapy.

1.4.2 Autologous NPC

The introduction of cells capable of surviving within the intervertebral disc and producing appropriate amounts of matrix is an important component of disc tissue engineering. One type of cell considered is an autologous (derived from the patient) NPC cell-line [34]. Even with some promising data, there is legitimate clinical concern over donor-site morbidity, since harvesting the patient's own cells requires damage to an adjacent disc, which will likely induce degeneration in that level. Also, disc acellularity will require a slow in vitro cell-culture expansion step to obtain sufficient cell numbers. Furthermore, autologous cells will be similarly aged to the diseased level and potentially limited in their ability to mount therapeutic repair response.

1.5 STEM CELLS FOR DISC REPAIR

1.5.1 Cell Carriers

Carriers for cell-based disc regeneration strategies fulfill multiple roles. They serve as delivery vehicles to attain acute cell retention in the high-pressure disc nucleus. They preserve nucleus volume and defend against scar tissue encroachment by adjacent annular tissue during early healing. By augmenting nuclear volume, carriers also serve to enhance acute biomechanical stability. In addition to these biomechanical functions, carriers need to provide an environment that supports the desired biological activity of

the delivered cells. Unfortunately, biomechanical and biological roles may create conflicting design constraints, with stiffer materials being more suitable for biomechanical retention and stability, and porous, pliant materials being more appropriate for nutrient transport and a 3D milieu conducive to a disc cell phenotype. Importantly, these materials, which may or may not degrade over time, have to be synergistic will cell function over the long term. If non-degrading, they need to be biocompatible and non-migratory under complex loading/pressures. If degrading, the degradation kinetics should ideally be timed with cell matrix synthesis. Also, because of disc size and avascularity, degradation products may have a longer persistence and achieve higher concentrations than observed in other applications.

Many types of carriers have been used that include gels with or without porous solid scaffolds. Examples include synthetic polymers such as poly(lactide-co-glycolide) (PLGA) [35], polyglycolide (PGA) [36], polylactide (PLA) [37]. Many forms of natural scaffolds are available, such as hyaluronan [38], collagen/atelocollagen [39], and chitosan [40], alginate [41], agarose [42], calcium polyphosphate [43] demineralized bone particles [44], fibrin sealant [35,45], and small intestine submucosa (SIS) [46] – a natural extracellular matrix. So-called smart scaffolds can contain bioactive agents such as growth factors, cytokine inhibitors, or antibiotics.

1.5.2 Autologous vs allogenic

Adult mesenchymal stem cells (MSCs) are attractive for disc tissue engineering since they can differentiate into a variety of cell types, including NPC-like cells.

Depending on the therapy, the MSCs can be autologous or allogenic (derived from a donor). In the case of autologous transplantation, the patient would have a preliminary outpatient procedure where MSCs would be harvested from bone marrow or adipose tissue. Since MSCs represent only a small percentage of the cells in either of these donor tissues, the MSCs would need to be separated and expanded in vitro to have sufficient numbers desired for the therapy. The second implantation procedure would be performed several weeks later. In the case of an allogenic transplant, the patient would be treated with MSCs from an organ donor in a one-procedure cost-effective approach. While host rejection of allogenic cells is a concern, several studies have indicated that MSCs are immunoprivileged and do not elicit a rejection response.

1.5.3 Differentiation of stem cells before implantation

A primary concern regarding the use of MSCs for disc repair is whether they survive the harsh in vivo conditions and appropriately differentiate in situ. Although the NPC lineage is not fully characterized, it is generally agreed to closely match that of chondrocytes [8,9]. While MSCs are known to readily differentiate into this cell type under controlled conditions in vitro, it must still be established whether will spontaneously differentiate and thrive in situ, or alternatively require augmentation with supplemental, differentiation factors.

Environmental cues can provide MSC with important differentiation signals. Several studies have traced labeled MSC implanted within discs and observed that they persist, integrate with host tissue, and differentiate over time [10,11]. Similarly, in vitro

studies have shown that MSC cultured in 3D scaffolds also exhibit some levels of differentiation [12,13]. Yet, several studies have shown greater persistence and matrix deposition with MSC that are first pre-differentiated or implanted along with stimulatory factors [14]. Consequently, the introduction of key bioactive molecules is commonly used to enhance desired MSC differentiation.

The use of adenoviral vectors encoding chondrogenic growth factors in MSCs is one possible strategy to ensure the differentiation and sustained performance of implanted MSC. Gene transfer therapy enables the sustained synthesis of the encoded bioactive transgene products that may be effective since these factors would not likely be present in a degenerative in vivo environment. The transfer of key genes including TGF-β1 and BMP-2, to MSCs in vitro have lead to the sustained upregulation of key matrix production and differentiation genes [15,16]. Transfected cells can be implanted alone or in combination with untransfected cells [17]. However, the use of viral vectors and genetically modified cells represent important safety hurdles when considered for clinical application [18]. These safety concerns will likely slow clinical adoption.

Exposing MSCs to key growth factors either before or during implantation is a more popular strategy. The most common approach is to culture MSC in 3D alginate bead culture with TGF-b1 supplemented media, where MSCs have robust differentiation and matrix synthesis [19]. This method is also convenient because the cells can easily be released from alginate using sodium citrate washes and re-implanted without compromising cell viability. Controlled release of growth factors such as TGF-β1 and other such molecules can also be incorporated into scaffolds that are seeded with cells and implanted [20,21]. The main concern regarding TGF-β induced differentiation of

MSC is the progression of MSC towards hypertrophy whereby the MSC begin secreting collagen X and MMP-13 [22]. This is a concern since the mechanical properties of matrix secreted by hypertrophic cells do not match those desired for disc regeneration [23].

1.5.4 Co-culture techniques

A newly emerging technique is to co-culture MSCs with mature instructive cells. This approach was originally explored to indentify interactions between implanted stem cells and host cells. However, when synergistic effects were observed, co-culture was investigated for potential therapeutic benefits. In this context, co-culture involves creating 3D cell pellets that allow for contact between the two cell types. This synergy has been shown to increase overall matrix production and promote differentiation of MSC without leading to hypertrophy [52]. The NPC are thought to provide sustainable signaling cues to the MSC and the MSC are also thought to be providing the NPC with stimulatory signals. The combination of these two effects is very attractive therapeutically in creating a self-sustaining implant that does not require external cues.

Yamamoto et al report that cell-cell contact between MSC and NPC had synergistic effects in monolayer, however, it remained unclear whether the MSC were differentiating or acting as feeder cells to reactivate the NPC [77]. Ultimately, Richardson et al employed a similar two-dimensional co-culture system and demonstrated that NPCs cause MSCs to differentiate into an NP-like phenotype as assessed by gene expression after FACS sorting [78]. They observed that a 75% NPC / 25% MSC ratio was optimum for MSC differentiation, as indicated by SOX9, collagen 2, and aggrecan gene expression. Another study has since made similar observations in 3D

culture using a randomized mixture of MSCs and degenerative NPCs [57]. These studies highlight the beneficial effects of recreating a condensation shape and the unique signaling arising from co-culturing. However, none have reproduced the key embryonic induction process where two layers of different cell types communicate both with heterogeneous signaling across the interface and homogeneous signaling within the layer of the same cell type.

Our current strategy seeks to regenerate the disc using a novel bilaminar cell pellet (BCP) (Figure 1.2). The BCP is a co-culture pellet composed of an inner sphere of MSC enclosed in an outer shell of NPC. The cell composition ratio is 75% MSC and 25% NPC with a total of 500 000 cells and roughly a 1mm diameter. The bilaminar structure allows for homotypic interactions between cells of the same type within the layer and for heterotypic interactions between different cell types across a defined interface. This organization mimics the developmental processes of condensation, where cell aggregates form, and induction, where a mature layer of tissue directs the differentiation of a naïve one. The two cell types provide one another with stimulatory signaling which eliminates the need for growth factors or genetic manipulation since the BCP provides self-sustaining cues.

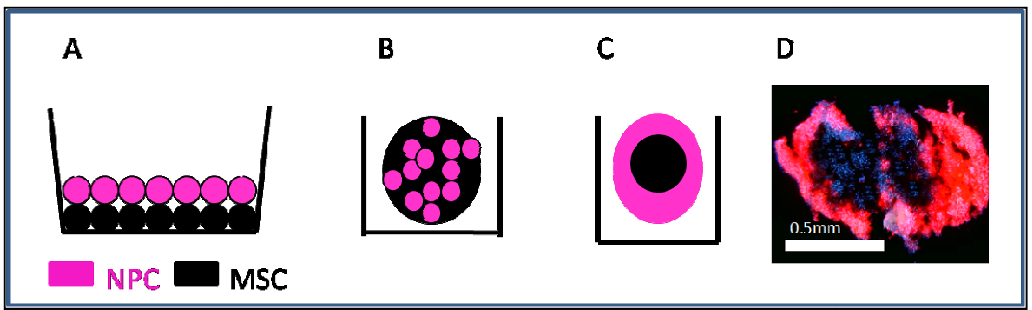

Figure 1.2: Coculture experiments. A) Coculture in monolayer by Yamamoto [55] and Richardson [56]. B) Random 3D coculture pellet [46,57]. C) Bilaminar coculture pellet (BCP) [52,54]. D) Frozen section histology of BCP with MSC dyed with DiI (red) [52,54].

Synergistic interactions are apparent within the BCP suggesting NPCs direct MSC differentiation. After three weeks in culture, MSCs in the BCP exhibit significantly higher gene expression of aggrecan (2 fold), collagen II (675 fold), and SOX9 (175 fold) and a significant downregulation of MMP13 (3 fold) and ColX (8 fold) over MSC controls [52]. In conjunction, NPCs in the BCP exhibit significantly lower levels of expression of aggrecan and collagen II (both 2 fold) but a similar level of SOX9. Spatial and temporal gene expression patterns also provide clues to the nature of cellular interactions within the BCP. At early culture times (one week), the expression of both the aggrecan and collagen II is primarily on the BCP periphery, where the NPCs are located. As time progresses, there is increased aggrecan gene expression by MSCs at the BCP center [52]. Taken together, these results indicate that MSC are differentiating due to their interaction with the NPC within the BCP.

BCP culture results in a 30% increase in proteoglycan production after three weeks as compared to single cell type controls [52]. Consistent with gene expression

patterns, early aggrecan production is primarily restricted to the outside layer presumably by the NPCs. By three weeks of culture, the aggrecan staining is widespread, indicating that the MSCs have begun synthesizing the protein (Figure 1.3).

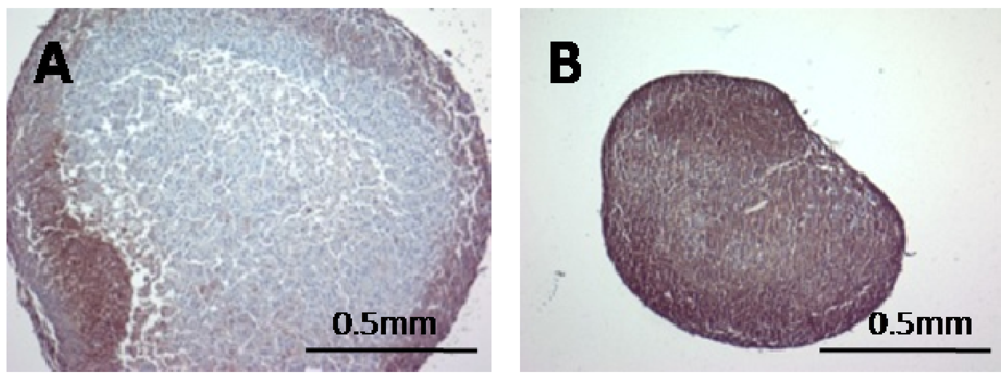

Figure 1.3: Immunohistochemistry staining for aggrecan (brown) on paraffin sections of BCP. A) BCP at 1 week time point. The aggrecan staining is localized to the outer layer where the NPC are present. B) BCP at 3 week time point. The aggrecan staining is throughout the pellet including the center of the pellet where the MSC are located. This indicates a progression in MSC differentiation towards an NPC phenotype between 1 and 3 weeks.

Importantly, BCPs demonstrate superior performance when cultured under conditions that mimic those anticipated for the degenerate disc environment: hypoxia (4% O2), pressure (350KPa), and inflammation (10 ng/ml of TNF-alpha and interleukin 1-beta). As expected, single-cell type pellets consisting of NPCs produce more matrix than MSCs alone or BCPs under physiologic disc conditions of hypoxia and pressure. However, when cultured in the presence of cytokines, BCPs and MSCs produce significantly more proteoglycan than NPCs alone. In this setting, the NPC performance was dramatically reduced indicating their high sensitivity to the inflammatory environment. When hypoxia, pressure, and inflammation are combined to simulate the

pathologic disc environment, BCPs produce significantly more proteoglycan than MSCs and NPCs [53]. These results demonstrate the sensitivity of cell performance to culture conditions, highlighting the importance of mimicking the anticipated in situ environment during in vitro optimization of cell-based tissue engineering strategies.

The resilience of BCPs to the simulated pathologic disc environment in vitro suggests advantages for in vivo application. This is borne out by preliminary studies in rat caudal discs. Two weeks after implantation using a fibrin carrier into denucleated discs, cell retention and survival was increased by xxx with BCPs versus MSCs or NPCs alone. [54]. At 5weeks, the BCP-treated discs demonstrated significantly better disc-morphology (assessed histologically by a blinded-scoring-scheme) than either untreated or fibrin-only groups, and trended toward better scores than MSC and NPC-only conditions. The BCP-treated discs uniquely exhibit histologic evidence of proteoglycan synthesis, and tended to better maintain disc height than the other groups (Figure 1.4).

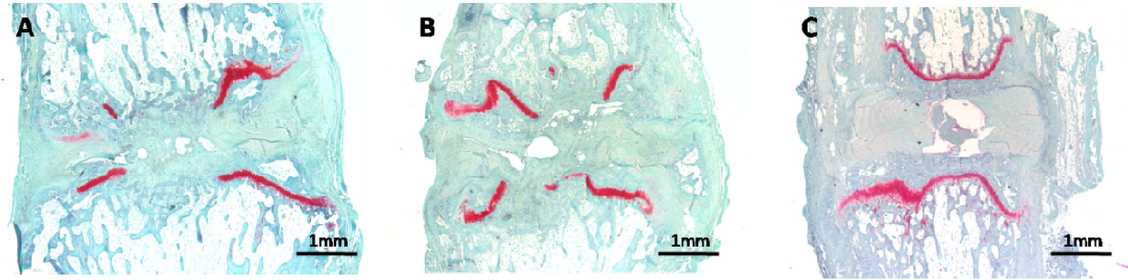

Figure 1.4: Rat disc paraffin section histology with Safranin-O staining 5 weeks after surgery. A) This disc was a control disc with no treatment. B) This disc was injected with the fibrin carrier alone. In images A & B, the disc has collapsed and there is no proteoglycan in the disc space. The end plate and growth plate are severely disrupted. C) This disc was treated with a BCP and fibrin carrier. The disc height is maintained with some proteoglycan staining in the disc space. The end plate and growth plate are both continuous.

These BCP studies indicate the value of adapting inductive strategies utilized during normal joint development to guide appropriate MSC differentiation in the challenging wound healing environment. As opposed to using a single growth factor supplement or genetic manipulation, this method leverages the totality of NPC signaling to program appropriate response in MSCs. This programming inhibits hypertrophy and promotes resistance to inflammation. BCPs synthesize substantially more disc-like matrix than either NPCs or MSCs alone. Thus, BCP are a promising stem cell-based approach for disc repair.

1.6 CONCLUSION

The successful design of cell-based treatments for low back pain is confounded by ambiguities of disease and pain mechanisms in patients, and lack of consensus regarding ideal pre-clinical models. In particular, the primary clinical endpoint – pain relief – is currently not directly testable in animals. Yet, these therapies can be advanced by establishing biologic plausibility of efficacy and safety using models of increasing complexity, starting with cell culture, small animals (rats and rabbits), then large animals (goat and mini-pig) that more closely mimic nutritional, biomechanical, and surgical realities of human application. Ultimately, success will hinge on carefully designed clinical trials with well-defined patient selection criteria and objective outcome metrics that demonstrate significant benefits relative to gold-standard control treatments, such as spinal fusion.

CH2. Structured co-culture of stem cells and disc cells in a bilaminar cell pellet enhances matrix synthesis and stem cell differentiation

2.1 Introduction

Harnessing the power of stem cells is an important strategy for regenerative medicine. In particular, understanding the differentiation of stem cells into the desired cell type has been an area of active research in the hopes of eliciting a therapeutic result. Our structured co-culture pellet approach seeks to direct the behavior of stem cells by mimicking embryonic processes underlying cartilage and intervertebral disc development.

Cell pellet implantation strategies may have benefits in the context of cell-based therapies as they can selectively recapitulate aspects of the embryonic microenvironments for regenerative purposes [70]. During embryonic development, cartilage and disc formation begins with the aggregation of progenitor cells. These cell condensations then progress towards differentiation via the process of induction where a layer of more mature cells instructs a naïve layer to differentiate and to secrete the appropriate matrix [72-75]. We hypothesize that tissue regeneration will be enhanced by mimicking the embryonic environment through engineering a tissue induction interface between the stem cells and differentiated cells.

Regenerative medicine is a growing and dynamic field with the potential to provide patients with minimally-invasive treatments that repair or replace dysfunctional musculoskeletal tissues. We are investigating a spinal intervertebral disc application where the goal is to re-establish pain-free motion by restoring the physical and biochemical properties of the tissue. Each disc is composed of a peripheral, ligament-like annulus fibrosus and a central nucleus pulposus (NP). The NP contains chondrocyte-like nucleus pulposus cells (NPC) embedded in a matrix of proteoglycan and type II collagen that is highly hydrophilic, which causes the tissue to swell and resist compression hydrostatically [64]. This high-pressure environment is incompatible with blood vessel maintenance, and consequently the disc is the largest avascular tissue in the body as well as a challenging environment for cell function and survival. For these reasons, disc degeneration is a common and an underlying cause of various spinal disorders [65].

Current regenerative strategies for the disc include stimulating host cells to resume matrix synthesis (particularly aggrecan) and/or introducing new, more synthetically-active cells. Mesenchymal stem cells (MSCs) are attractive for this purpose because they can differentiate into a variety of cell types, including chondrocytes, and are a ready source of undifferentiated autologous cells [66]. The feasibility of this approach has been demonstrated in several *in vivo* animal studies [67-69]. However, long-term functional regeneration of adult discs has not been achieved which has lead several groups to investigate co-culture as a method for reliably differentiating MSC into NPC.

Previous co-culture studies have demonstrated the potential benefits of MSC/NPC co-culture. Paracrine signaling has shown advantages in inducing the differentiation and proliferation of MSC and NPC [76]. However, those effects have been shown to be

greater with cell-cell contact [77,79]. Yamamoto et al report that cell-cell contact between MSC and NPC had synergistic effects in monolayer, however, it remained unclear whether the MSC were differentiating or acting as feeder cells to reactivate the NPC [77]. Ultimately, Richardson et al employed a similar two-dimensional co-culture system and demonstrated that NPCs cause MSCs to differentiate into an NP-like phenotype as assessed by gene expression after FACS sorting [78]. They observed that a 75% NPC / 25% MSC ratio was optimum for MSC differentiation, as indicated by SOX9, collagen 2, and aggrecan gene expression. Several studies have since made similar observations in 3D culture using a randomized mixture of MSCs and degenerative NPCs [79-83]. These studies highlight the beneficial effects of recreating a condensation shape and the unique signaling arising from co-culturing. However, none have reproduced the key embryonic induction process where two layers of different cell types communicate both with heterogeneous signaling across the interface and homogeneous signaling within the layer of the same cell type.

We report here on a novel bi-layer pellet approach that structures cell-cell signaling between MSCs and non-degenerative NPCs in a 3D culture system. We expect that signaling interactions between MSCs and NPCs regulate cell differentiation and stimulate matrix production. To this end, we have designed a spherical bi-laminar cell pellet (BCP) where one cell type forms an inner sphere enclosed within a shell formed by the other cell type. We find that spatially organizing these two cell types into a spherical bi-layer enhances matrix production in comparison to single cell-type controls or to pellets with a random organization of co-cultured cells.

Adapting inductive mechanisms from development in order to trigger differentiation and ultimately restore diseased tissue has many advantages. As opposed to strategies that require growth factor supplements or genetic manipulations, our method is self-sustaining and targeted. In addition, BCP can be administered therapeutically with a minimally invasive injection. Thus, BCP have the potential to become a very attractive cell-based system for skeletal tissue engineering.

2.2 Materials and methods

2.2.1 Cell Culture

Bovine NPCs were isolated from caudal discs of healthy adult cows within 48 hours (hrs) of sacrifice. The NP tissue was carefully separated by gross dissection and digested in 0.5% collagenase/dispase and 2% antibiotic/antimycotic in low glucose Dulbecco's Modified Eagle Medium (DMEM) at 37°C for 4-6hrs with constant stirring. The cells were then plated in tissue culture flasks and expanded to the fourth passage in NPC Media (DMEM with 1% antibiotic/antimycotic, 1.5% 400 mOsm, and 5% Fetal Bovine Serum (FBS)) at 37°C with 5% CO_2. Culture media was changed twice a week.

Commercially available human MSCs were purchased (Lonza, Switzerland) and expanded to the seventh passage in growth media (DMEM low glucose, 1% antibiotic/antimycotic, and 10% FBS) at 37°C with 5% CO_2. Culture media was changed twice a week.

Human nucleus pulposus samples were obtained from a consenting 55-year-old female patient undergoing surgery for scoliosis. The tissue was digested and the cells

expanded. In addition, bovine MSC were isolated from femur tissue and the cells were expanded.

2.2.2 Bi-Layer Pellet formation

After trypsonization, cells were counted and pelleted. Three different types of pellets were formed, each consisting of 500,000 cells total: pellets of 100% one cell type, pellets of MSCs and NPCs with randomized organization, and pellets of MSCs and NPCs organized into a bi-layer. The pellets containing both MSCs and NPCs were formed with three different cell number ratios of 25/75, 50/50, and 75/25 respectively (Figure 2.1). To produce the 100% one cell type pellets, 500,000 cells were pipetted into a 15mL polypropylene tube and centrifuged at low speeds (300g) for 5min.

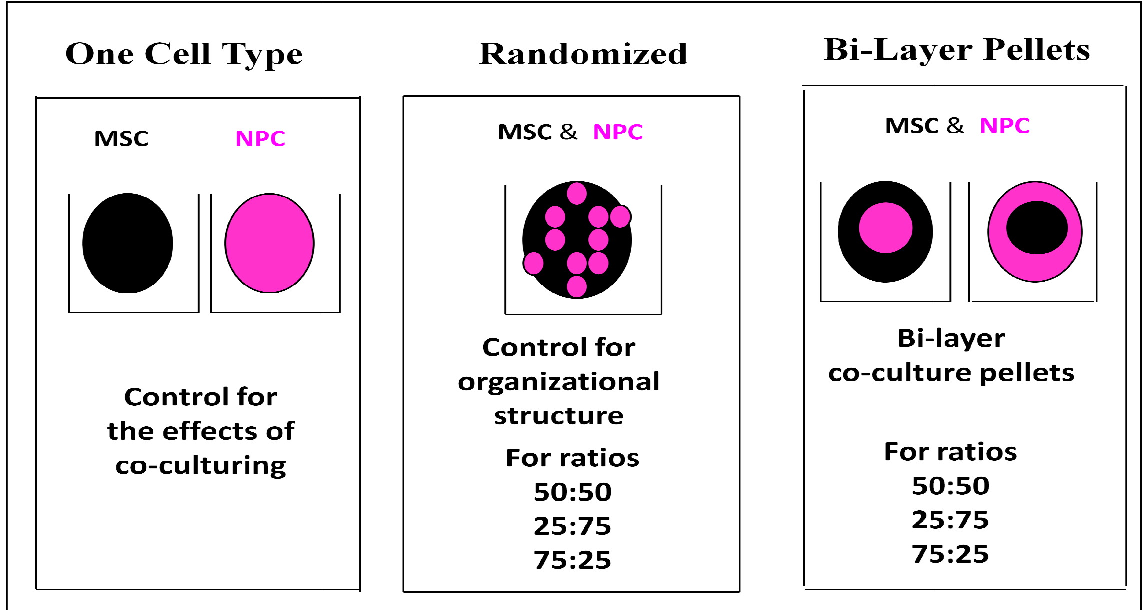

Figure 2.1: Schematic of the eleven different experimental groups indicating their compositions, structures, and ratios.

To create the randomized pellets, both cell types were added to the same tube, pipetted to ensure thorough mixing, and centrifuged at low speed for 5 min. In order to form the bi-layer organized pellets, the cell type that would form the inner sphere of the pellet was added to a 15mL polypropylene tube and centrifuged at low speed for five minutes. Subsequently, the second cell type that would form the outer shell was gently added to the same tube. The cells were then centrifuged again at low speed for 5 min.

Organized pellets were formed for all three ratios with MSCs on the inside (MSCin) and NPCs on the outside (NPCout) and vice versa with MSC on the outside (MSCout) and NPC on the inside (NPCin). All pellets were cultured in 2 mL of growth media for three days with caps loosened to allow for gas exchange. After three days, the pellets became spherical and were transferred to ultra-low attachment 24 well plates (Corning) for the remainder of their culture time. Media was changed three times a week.

To control for the effects of FBS, pellets were also cultured in serum-free media (High glucose DMEM, 1% Antibiotic / Antimitotic, insulin–transferrin–selenious acid mix (ITS) (BD Biosciences, Bedford, MA), 100 μg/ml sodium pyruvate, 40 μg/ml L-proline, 50 μg/ml L-ascorbic acid 2-phosphate, in the presence of 100 nM Dexamethasone.

2.2.3 Sulfated glycosaminoglycan (GAG) and DNA content

Pellets were removed from culture media and digested in papain (20U/mL in PBS) and incubated at 60°C overnight. Digested pellets were assayed with a Quant-iT

PicoGreen kit (Invitrogen, CA) to measure DNA content. Measurements were made using a spectrophotometer, with the excitation at 488nm and absorption at 525 nm.

Digested pellets were also assayed with a dimethylmethylene blue (DMMB) assay to quantify GAG content. A standard curve was made with chondroitin sulfate isolated from bovine trachea (Sigma, MO). Absorption was measured at 525nm using a spectrophotometer.

Overall, 11 pellet groups were generated (Figure 2.1) and each was analyzed after 3 culture times (1, 2, and 3 weeks). For all groups, sample size n = 10 or higher.

In addition, 100% MSC pellets were cultured for three weeks supplemented with 5ng/mL of TGF-beta1 (Peprotech, NJ). These pellets were compared to 75% MSC inside and 25% NPC outside pellets grown both in growth media and TGF-beta supplemented media. The pellets were cultured for three weeks and evaluated by DMMB.

2.2.4 Histology

At the end of the culture time, the pellets were fixed in 10% Buffered Formalin, embedded with paraffin, and sectioned at 7nm thickness. The sections were stained with Safranin-O and Hall-Brunt Quadruple stain in order to qualitatively assess matrix deposition. The sections were also stained using immunohistochemistry techniques. The human specific antibodies Lamp1 and Lamp2 were used to detect the MSC (Abcam, MA) and an aggrecan specific antibody was used to detect the protein (Santa Cruz Biotechnoloy, CA). In all cases, the sections were then counter-stained with hematoxylin.

2.2.5 In Situ hybridization

In situ hybridization was performed [148]. Sections adjacent to those used for histological and immunohistochemical analyses were hybridized with ^{35}S-labeled human riboprobes to aggrecan and collagen II-a1 (fibrillar collagen). Sections were counterstained with Hoechst dye (Sigma, MO). Hybridization signals were detected using darkfield and the nuclear stain with epifluorescence.

2.2.6 Quantitative RT-PCR

After three weeks of culture, 100% MSC, 100% NPC, and 75% MSCin & 25% NPCout were harvested for RNA extraction. In order to have sufficient amounts of RNA, 5 pellets of the same type were pooled together for an n=1. There was a total n=5 for each group. RNA was extracted from pellets using a QIAshredder kit and an RNeasy Mini Kit (Qiagen, Germany). The RNA was reverse transcribed using iScript (Bio-Rad Laboratories, CA). The samples were analyzed using Taqman species specific primers for the following human and bovine genes: beta-2-microglobulin (B2M), sox9, aggrecan, collagen I, collagen II, collagen X, and matrix metalloproteinase 13 (MMP13) (Applied Biosystems, CA).

2.2.7 Data Analysis

All statistical analyses were performed using JMP statistical software (Version 5.0). Standard analysis of variance procedures (ANOVA) were performed to compare group means and to estimate the effect of the specimen group variables (pellet type, pellet structure, and cell number ratio were entered as categorical predictors, and culture time as a continuous predictor) on the measured parameters of interest (GAG and DNA content). The Tukey-Kramer test was used to determine pair-wise statistical differences.

2.3 Results

2.3.1 DNA Content

After the first week of culture, structure and ratio alone were not significant predictors of DNA content (Figure 2.3). Although, two conditions did show significantly higher DNA content. 100%MSC pellets had statistically significant higher cell numbers than pellets with random 25%MSC / 75%NPC, pellets with 25%MSCin / 75%NPCout, and pellets with 50%MSCout / 50%NPCin ($p<0.001$). Also, pellets with 75%MSCin / 25%NPCout had significantly higher cell numbers than pellets with 25%MSCin / 75%NPCout and pellets with 50%MSCout / 50%NPCin ($p<0.001$).

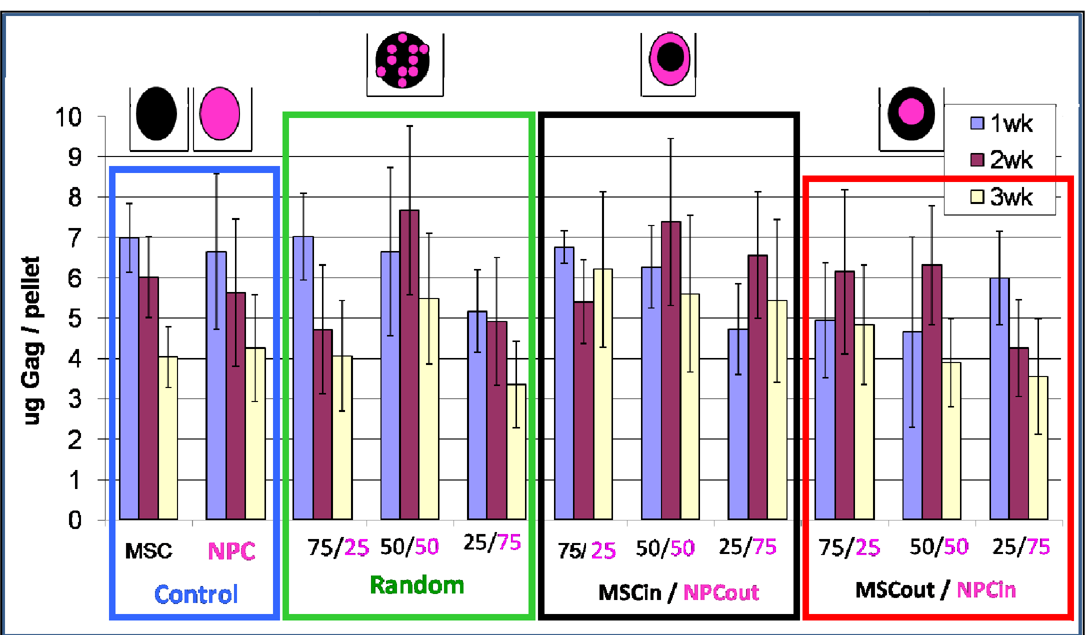

Figure 2.3: Graph ug GAG per pellet for each structure, conformation, and ratio. The effects of co-culturing are strong, as are the effects of structural organization.

By the second week of culture, the pellets of 100%MSCs or 100%NPCs did not experience any significant increase in cell numbers. The co-culture pellets had a significantly higher DNA content than the single cell type pellets ($p<0.0001$). In addition, the ratio of 50/50 significantly increased cell numbers over the ratio of 25%MSC / 75%NPC ($p<0.0001$). However, structure did not play a statistically significant role in DNA content

By the third week of culture, structural organization and ratio were both significant predictors of cell numbers. All three structures were significantly different from one another with MSCin / NPCout having the most DNA content followed by MSCout / NPCin, and finally random structure having the least ($p<0.0001$). The ratio of 25%MSC / 75%NPC and the ratio of 50/50 were statistically significantly higher than the single cell type pellets ($p<0.0001$). The single cell type pellets and the randomized pellets experienced a four-fold increase in cell number as compared to 1 week. By contrast, the structured pellets experienced a ten-fold increase in cell number, with the 50%MSC-inside/ 50%NPC-outside reaching a fourteen-fold increase. Overall, the pellets with 50%MSCin / 50%NPCout had significantly more DNA content than all other pellets except for pellets with the same structure at a ratio of 25%MSC / 75%NPC.

2.3.2 GAG Content

After one week of culture there was no clear trend in GAG production (Figure 2.3). Two groups (25%MSCin / 75%NPCout and 50%MSCout / 50%NPCin) had an average of 4.69ug which was statistically significant lower GAG production than the

random 75%MSC 25%NPC pellets at 7.01ug ($p<0.0002$). There were no other statistically significant differences between the groups.

After two weeks of culture, pellet structure and ratio together were significant predictors of GAG production ($p<0.0001$). While the single cell type pellets experienced a slight decrease in GAG content, the co-culture pellets demonstrated both significant increases and decreases. The ratio of 50/50 did show statistically significant higher GAG production than all other ratios ($p<0.0001$). The two highest GAG producing pellets both had a cell number ratio of 50/50, one with a random organization (7.67 ug) and the other with the MSCs inside and the NPCs outside (7.38 ug). For the structure with MSCs outside and NPCs inside, a ratio of 50/50 increased GAG production by 33% over pellets with the ratio of 25%MSC / 75%NPC ($p<0.0001$).

Though structure alone was not a statistically significant predictor, the data reveal a trend whereby pellets with the MSCs inside and NPCs outside produced more GAG at all ratios. This structure produced an average of 6.44ug of GAG compared with 5.76ug for the randomized and 5.57ug for the MSCout and NPCin. In particular, for a ratio of 25%MSC and 75%NPC, a structure with the MSCin and the NPCout significantly increased GAG production by 35% (from 4.256ug to 6.558ug) over the inverse structure of MSCout and NPCin ($p<0.0001$).

After three weeks of culture, the overall highest GAG producing pellets were those with 75%MSCs inside and 25%NPCs outside at 6.02 ug. This was significantly different from all other pellets except for those with similar structure but different ratios and 50% higher than the average of the controls ($p<0.0001$) (Figure 2.4).

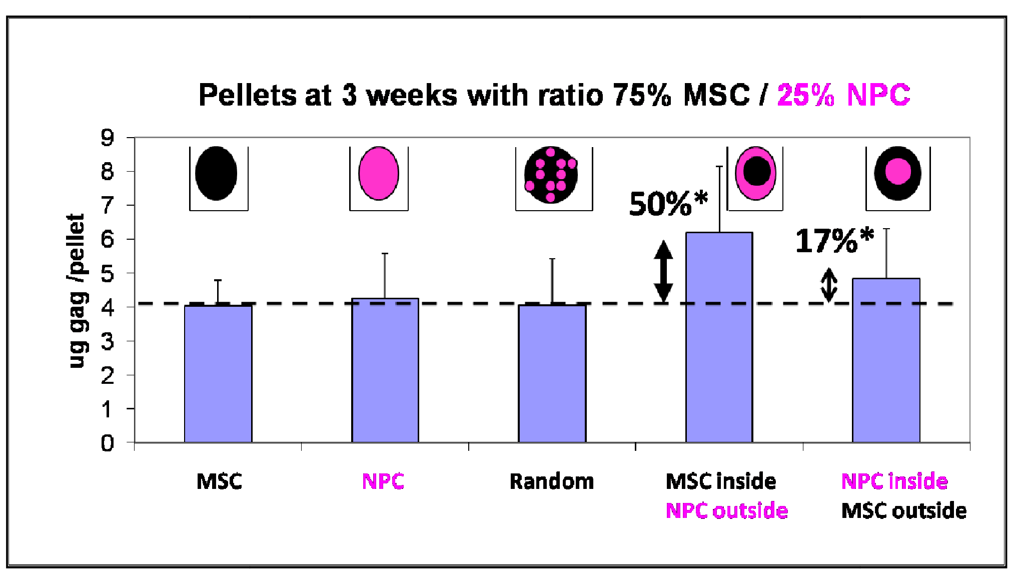

Figure 2.4: Graph of ug of GAG/pellet with a ratio of 75%MSC & 25%NPC culture for 3 weeks. The MSCin and NPCout group produced 50% more GAG than the average of the controls (dotted line). The NPCin and MSCout group produced 17% more than controls (p<0.0001).

The ratio of the cells was not a statistically significant predictor of GAG production at three weeks. Though there were cases where the ratio affected GAG production, there was no clear trend. For example, with the random structure, the ratio of 75%MSC / 25%NPC was statistically significantly lower than the 50/50 ratio (p<0.0001). However, this was not a trend that was observed for the other structures.

The structure of the pellets was a statistically significant predictor of GAG production at three weeks (p<0.0001). The structure of MSCs inside and NPCs outside produced statistically significantly more GAG at 5.74 ug compared with 4.30ug for random structures and 4.09 ug for MSC on the outside and NPC on the inside (p<0.0001). In fact, for a ratio of 25% MSC/ 75%NPC, having MSCin and NPCout

increased gag production by 34% over the opposite structure, by 38% over the randomized pellets, and by 31% over controls (Figure 2.5). The structure of having MSCs outside and NPCin was not statistically different than the random structure.

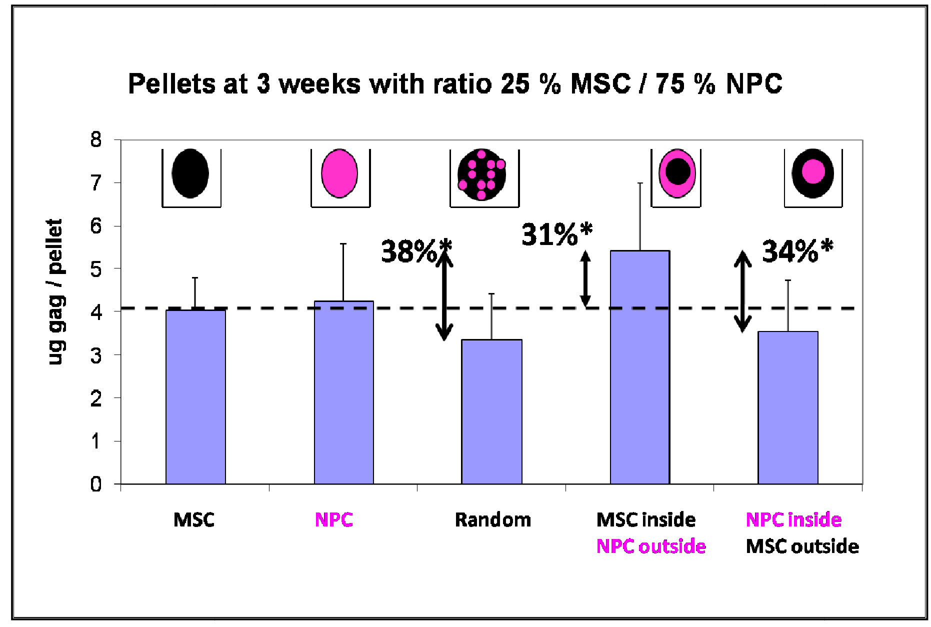

Figure 2.5: Graph of ug of GAG/pellet for pellets with a ratio of 25%MSC & 75%NPC culture for 3 weeks. The pellets with MSCin and NPC outside produced 31% more GAG than the average of the controls (dotted line), 34% than pellets with the opposite structure, and 38% more than randomized pellets ($p<0.0001$).

At three weeks, the single cell type pellets experienced a further drop in GAG production, down 42% from the first week for MSCs and down 36% from the first week for NPCs ($p<0.0001$). Perhaps most interestingly, the MSCin and NPCout group sustained GAG production levels at similar rates to the first week with only a 3% decrease (there was no statistically significant difference between 1 week and 3 week values). Between 1 week and 3 week measurements, GAG production rates fell 21% for MSCout and NPCin, though this was not a statistically significant decrease. They also

fell significantly by 31% for the randomized group ($p<0.0001$). In fact, 25%MSCin/ 75%NPCout was the only pellet that increased GAG production at three weeks over the one week rate by 14% though this was not a statistically significant increase.

At three weeks, the pellet with 75% MSC inside and 25% NPC outside were compared to 100% MSC cultured with TGF-beta. These structured co-culture pellets without TGF-beta had significantly higher GAG than the MSC with TGF-beta pellets. In addition, when the structured co-culture pellets were cultured with TGF-beta, they made 7 times more GAG than MSC with TGF-beta (data not shown).

2.3.3 Controls for cross species interaction and the effects of FBS

In order to control for the species interaction between bovine and human cells, we made co-culture pellets using MSC and NPC of the same species to be run in parallel with cross species pellets that use human MSC and bovine NPC. Bilaminar pellets were made with human MSC combined with human NPC and pellets were also made with bovine MSC combined with bovine NPC. After three weeks of culture, the pellets were evaluated for GAG and DNA content. We saw no statistically significant differences in the GAG or DNA content between co-culture pellets that were all human, all bovine, or cross species (data not shown).

In addition, two sets of pellets were grown in parallel using different media in order to assess whether any factors in the FBS were influencing GAG and DNA content. One set of pellets was cultured in growth media (with 10% FBS) and one set was culture in serum-free media. The pellets were cultured for three weeks and evaluated GAG and DNA content. We saw no statistically significant differences between the two sets of

pellets (data not shown). This is an indication that the potential growth factors in FBS are not significantly influencing the behavior of the cells in the pellet.

2.3.4 Histology and In situ hybridization

The formalin fixed paraffin embedded sections were stained with both Safranin-O and HBQ which qualitatively detect the presence of proteoglycan with red staining for the former and blue for the latter. In figure 2.6, Images C and F of the MSC in NPC out pellet are clearly staining more vibrantly red and blue respectively which is consistent with the GAG content data.

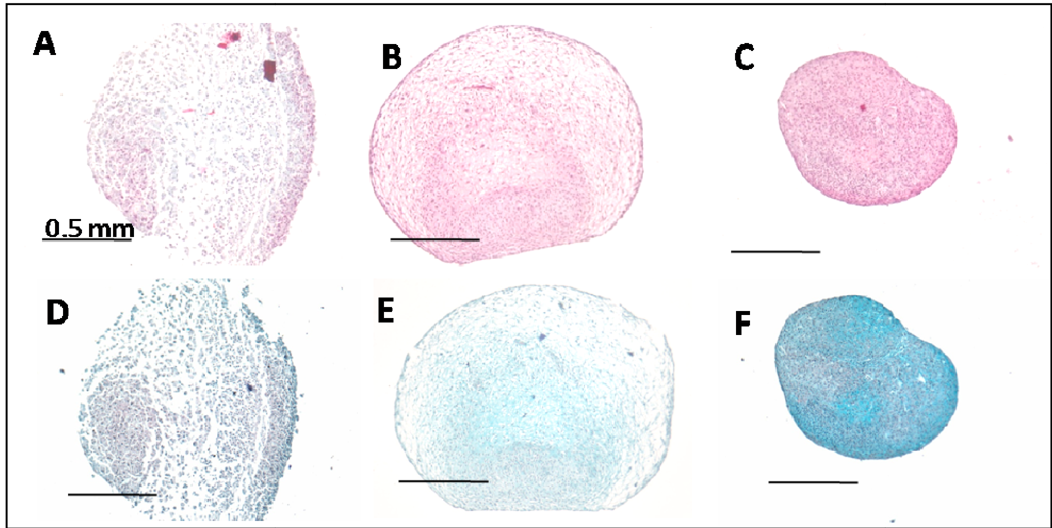

Figure 2.6: Safranin-O and HBQ staining. A-C Paraffin embedded sections of three week pellets stained with Safranin-O where red staining indicates the presence of proteoglycan. D-F Paraffin embedded sections of three week pellets stained with HBQ where blue staining indicates the presence of proteoglycan. A &D are of MSC pellets. B&E are of NPC pellets. C&F are of MSC in NPC out pellets. C & F are staining qualitatively more for red and blue respectively indicating higher GAG content.

The pellets stained using immunohistochemistry techniques with the human specific antibody enabled us to trace the location of the cells by their species with MSC

being human and the NPC being bovine. This technique enabled us to confirm that the desired configuration was made and maintained throughout the culture time as demonstrated in A-D of Figure 2.7.

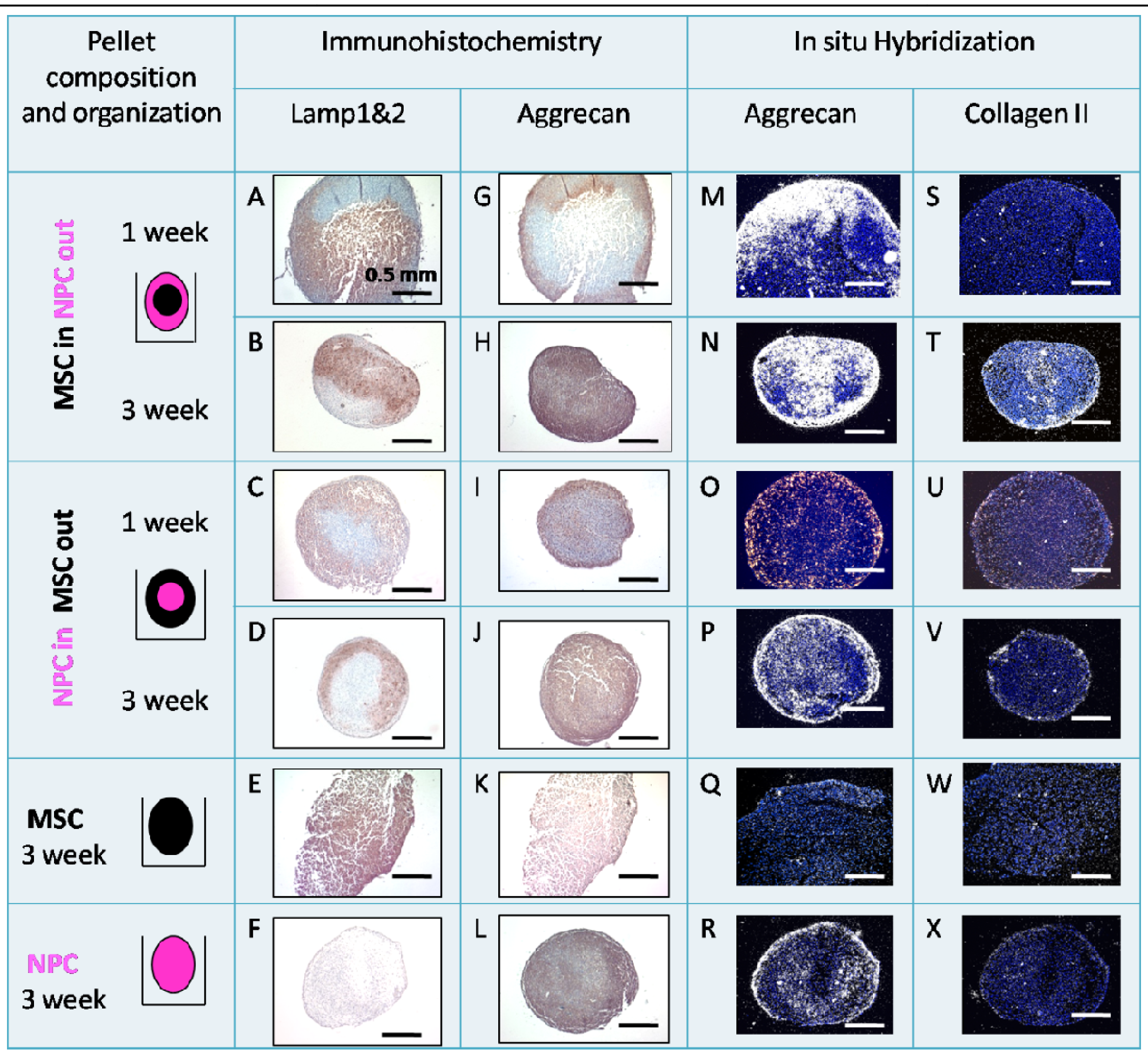

Figure 2.7: Immunohistochemistry and in situ hybridization. Images A-F are stained with Lamp1&2 antibody (in brown) which is a human species-specific antibody, counterstained with hematoxylin (in blue). They indicate the organizational structure of the pellet. Images G-L are stained with Aggrecan antibody (in brown), counterstained with hematoxylin (which stains cell nuclei blue). They indicate the location of the protein. Images M-R show the location of aggrecan gene expression (in white) in the pellet, counterstained with Hoechst dye (which stains cell nuclei blue). Images S-X show

the location of collagen II gene expression (in white) in the pellet, counterstained with Hoechst dye (which stains cell nuclei blue).

At the one week time point for the MSC in NPC out group, it is clear that the NPC on the outside are producing and expressing aggrecan, while the MSC do not seems to be producing the protein or expressing aggrecan RNA (figure 2.7 images A, G, M). At the three week time point for the same group, aggrecan protein and RNA is localized throughout the pellet (figure 2.7 images B,H, N, T). We also see that the collagen II expression has increased throughout the pellet with time.

At the one week time point for the NPC in MSC out group, aggrecan protein and RNA is located on the periphery of the pellet and in a central pocket where NPC are located (figure 2.7 images C, I , O, U). At the three week time point for this group, aggrecan is being made and expressed throughout the pellet however the center, where the NPC are located, does appear to have lower levels of RNA expression (figure 2.7 images D, J, P, V). There appears to be a slight increase in expression of collagen II on the periphery of the pellet at three weeks.

The control MSC group exhibited very faint staining of aggrecan protein and no staining of aggrecan RNA or collagen RNA (figure 2.7 images E, K, Q, W). The control NPC group exhibited aggrecan protein staining throughout the pellet (figure 2.7 images F, L, R, X). Aggrecan RNA was not uniform, with a pronounced ring of expression on the periphery of the pellet with some central staining. Collagen II RNA was very faintly expressed.

2.3.5 Quantitative RT-PCR

We were able to distinguish the gene expression levels of the MSC and NPC within the co-culture pellets by using specifies specific primers. The fold change was calculated with the species appropriate single cell type pellet (MSC for human and NPC for bovine). The MSC within the co-culture pellets had a 2-fold upregulation of aggrecan, a 675.5-fold upregulation of collagen II, a 190.3-fold upregulation of Sox9, a 2.7-fold downregulation of MMP13, and an 8.2-fold downregulation of collagen X, all of which were statistically significant (Figure 2.8). The levels of collagen I gene expression showed no significant differences.

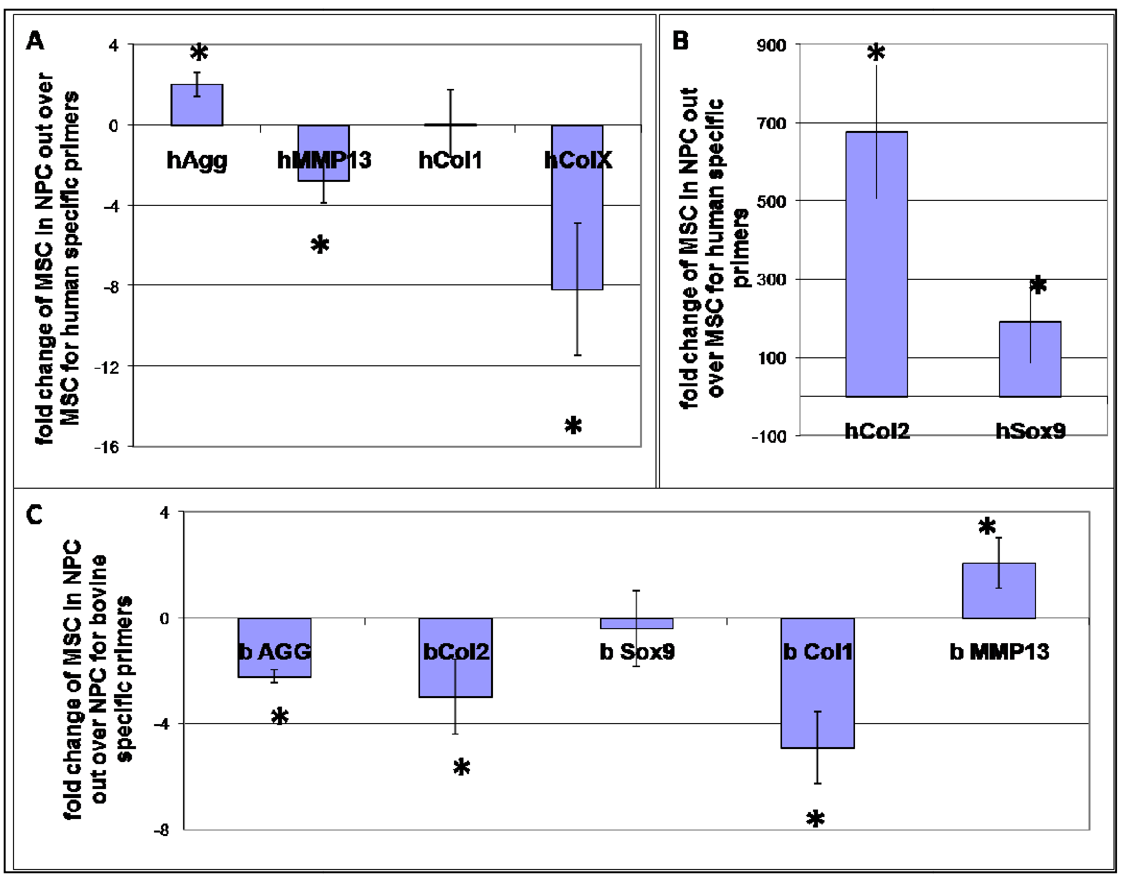

Figure 2.8: Quantitative RT-PCR with species specific Taqman primers was performed on three week pellets of three types: MSC, NPC, and 75% MSC in & 25% NPC out. For each reading, the gene expression was normalized to the housekeeping gene B2M and the fold change was calculated using the single cells type pellet of the appropriate species as the control. For the human genes measured in the co-culture pellets, the control was the MSC pellets. For the bovine genes measured in the co-culture pellets, the control was the NPC pellets.

* denotes samples with statistically significant difference $p<0.05$.

The NPC within the co-culture pellets had a 2.2-fold downregulation of aggrecan, a 2.9-fold downregulation of collagen II, a 4.9-fold downregulation of collagen I, and a 2-fold upregulation of MMP13. The levels of Sox9 gene expression showed no significant differences.

2.4 Discussion

A goal of this study was to ascertain the extent to which structured co-culture of MSCs and NPCs could enhance disc tissue regeneration in vitro. We tested the hypothesis that creating an organized pellet that allowed for structured homotypic and heterotypic interactions between MSCs and NPCs at various ratios would enhance proteoglycan production in a 3D pellet culture. He have shown that BCP with MSC's on the inside and NPCs on the outside were more synthetically active than the inverse conformation, a random mix conformation, or single-cell type pellets.

Whether these cell types enhanced each other's behavior in 3D culture has very important implications for cell–based, disc regeneration therapies. Presumably, a cell-based therapy will always elicit an interaction between the newly introduced cells and the host cells since NPCs would not be removed during a minimally invasive injection. Previous studies have shown that cell-cell contact between MSCs and NPCs was necessary to induce a stimulatory effect [77,79]. Our goal was to design a cell construct that could exploit those signaling interactions to enhance matrix production.

MSC/NPC interactions greatly affected the total number of cells in the pellet. After two weeks of culture, co-culture pellets resulted in much higher cell numbers than those observed in the single cell type pellets. Presumably, the different cell types were stimulating each other to proliferate and, at this time point, did not appear to require any specific structure or ratio to do so. By the third week of culture, pellet structure and cell ratios both became a statistically significant predictor of cell number. Based on the fact

that pellets with a ratio of 50/50 and 25%MSC / 75%NPC resulted in significantly more cells, we would conclude that the stimulatory effects occurring in co-culture pellets are originating from NPC signaling. One explanation as to why the ratio of 75%MSC / 25%NPC resulted in lower cell numbers may be that signals (e.g., secreted molecules) from NPC did not reach a sufficient concentration to stimulate the MSC. Further studies involving FACS sorting would help elucidate such issues. While the specific signaling pathways mediating interactions between MSC and NPC in our system remain unknown, and while our assays were not able to identify which cell types were proliferating within the same pellet, our results clearly show that over time the pellets benefited from more organization to fully exploit the trophic effects of co-culture.

For GAG production, both ratio and structure played an important role at different time points. At one week, the cells where not yet influenced by the interactions with the other cell type. At two weeks, co-culture pellets with a ratio of 50/50 showed significantly more matrix production suggesting that the cells began benefitting from co-culture signaling. Although the structure did not yet play a significant role, a trend emerged where having naïve cells at the center and mature cells on the outside was favorable. At three weeks, we showed both quantitatively (via DMMB) and qualitatively (via histology) that matrix production was significantly enhanced by structural organization. Only the pellets with an organized structure were able to sustain matrix production over three weeks while random pellets and single cell type pellets saw significant decreases.

Overall, our experiments led to the novel discovery that pellets produced the most matrix when MSCs were on the inside and NPCs were on the outside. This was likely due

to the fact that at the center of the pellet, MSCs presumably received two external stimuli that promoted differentiation. The first was the micro-environment at the center of the pellet, which had higher pressure and lower oxygen tension than on the outside of the pellets and may mimic the process of embryonic condensation. The microenvironment was therefore more conducive to initiating MSC differentiation into an NPC-like cell and subsequently synthesizing matrix [84]. The signals due to the environment likely propagated from the center of the pellet outwards. The second stimulus was the organized signaling interface with NPCs. This structure allowed the MSCs to interact with the NPCs only across one defined interface that may mimic the embryonic process of induction. MSCs may also propagate signals more readily amongst themselves than with other cell types [90]. This organized zone for interactions could have allowed the MSCs to transduce the signaling information from the NPCs to neighboring MSCs more quickly and efficiently than in a randomized organization, thereby propagating that signal from the outside of the pellet inwards. Most likely, the combination of the microenvironment, which produced a favorable signal propagating outwards, and the organized interaction interface, which produced a favorable signal propagating inwards, allowed the MSCs to differentiate and produce more matrix than any other configuration due to these ongoing external stimuli which mimic embryonic processes.

The in situ hybridization allowed us to localize gene expression within the pellet. We were able to demonstrate that the MSC within the MSC inside / NPC outside pellet differentiated over the three week culture time and began both expressing and secreting aggrecan. Interestingly, we saw early signs of aggrecan expression and secretion from MSC within the NPC inside / MSC outside pellets. The protein and gene expression

were limited to the outermost layer of the pellet. This is likely due to the epithelial-like morphology that cells have on the outer periphery of the pellet (Figure 2.9). Though this process is not well understood, it was observed in all pellets other than those that were 100% MSC. It is likely that this epithelial-like transformation causes the MSC to differentiate and secrete aggrecan. However, we found that those pellets with MSC on the outside consistently produced less aggrecan.

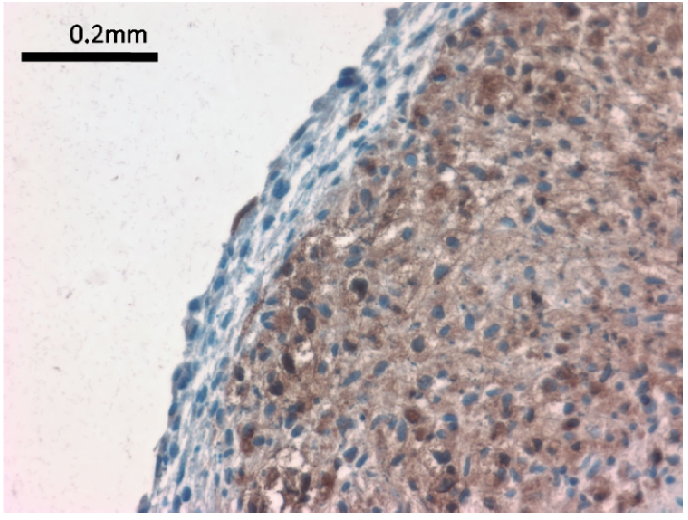

Figure 2.9: Epithelial-like morphology of cells in the outermost layer of the pellet. In this case the MSC are stained in brown on the inside and the NPC are unstained on the outside forming the layer. A hematoxylin counterstain was used to stain all cell nuclei blue. The NPC on the outside are elongated and finely layered over one another. MSC exhibit similar morphology when placed on the outside layer.

The gene expression data of the MSC inside and NPC outside pellets further demonstrated that MSC were differentiating due to the interaction with NPC with significant increases in expression of aggrecan, collagen II, and SOX9 all of which are positive markers for NPC-like cells. In addition, the downregulation of collagen X and

MMP13 were indicative of the advantage of using co-culture techniques in differentiating MSC.

The NPC within the MSC inside and NPC outside pellets showed a downregulation of aggrecan and collagen II. In addition, the SOX9 gene expression levels remained unchanged indicating that these cells were not in the process of de-differentiating. These results may be indicative of the NPC role as instructive cells as opposed to their purely secretory role in the 100% NPC pellets.

Further studies are required to assess the relevant signaling mechanisms though we believe the TGF-beta pathway is involved due to the fact that BCP made significantly more GAG when cultured in TGF-beta supplemented media. Le Visage and coworkers reported that MSC/NPC pellets produced more GAG when cultured in growth media than in chondrogenic media [79]. This is not the case for BCP pellets, indicating that the organizational structure is activating different signaling pathways than the randomized co-culture pellets.

Over the long term, we envision using such a bi-layer pellet system to form the basis for new cell-based therapies for disc degeneration. Several pellets could be injected using a minimally invasive procedure into the nucleus pulposus of patients with back disc disease [65,89]. These pellets would then serve to restore normal matrix composition in a sustainable way and ultimately restore tissue function.

CH3. Budding and satellite pellet formation

3.1 Introduction

Spinal intervertebral discs provide flexibility while supporting compressive forces. Each disc is composed of a peripheral, ligament-like annulus fibrosus and a central nucleus pulposus (NP). The NP contains chondrocyte-like cells embedded in a matrix of proteoglycan and type II collagen that is highly hydrophilic, which causes the tissue to swell and resist compression hydrostatically [91]. This high-pressure environment is incompatible with blood vessel maintenance, and consequently the disc is the largest avascular tissue in the body as well as a challenging environment for cell function and survival. For these reasons, disc degeneration is a common and an underlying cause of various spinal disorders [92].

Tissue engineering is a growing and dynamic field with the potential to provide patients with minimally-invasive treatments that repair or replace dysfunctional musculoskeletal tissues. A tissue engineering goal for the intervertebral disc is to re-establish pain-free motion by restoring the physical and biochemical properties of the matrix. This may be accomplished by stimulating host cells to resume matrix synthesis (particularly aggrecan) and/or by introducing new, more synthetically-active cells. Mesenchymal stem cells (MSC) are attractive for this purpose because they can differentiate into a variety of cell types, including chondrocytes, and are a ready source of undifferentiated autologous cells [93]. The feasibility of this approach has been demonstrated in several *in vivo* animal studies [94-96]. However, long-term functional regeneration of adult discs has not been achieved. Ultimately, the degenerative disc is a

hostile, often inflammatory environment, which experiences significant mechanical loading [97]. When immature MSC are suspended in a carrier and injected into a degenerative disc, they find it difficult to survive, stay in the desired location, and they may not receive the environmental cues that enable them to perform optimally to regenerate the tissue.

Pellet culture systems may have benefits in a tissue engineering setting as they can selectively recapitulate embryonic microenvironments for regenerative purposes [1.98-1.99]. During embryonic development, cartilage and disc formation begins with the aggregation of progenitor cells into a cell condensation. These condensations then progress towards differentiation via the process of tissue induction and begin to secrete matrix [98-106]. Several groups have looked at the concept of coculture systems of NPC and MSC because signaling between these cell types ultimately occurs in situ during MSC-mediated disc regeneration. Yamamoto and coworkers conducted a 4-day monolayer coculture study and reported significant increases in proteoglycan synthesis and cell proliferation when non-degenerative NPC and MSC were cultured with direct cell-cell contact [107]. They reasoned that MSC were acting as feeder cells that enhanced the ability of NPC to proliferate and secrete matrix. Richardson and colleagues also employed a similar two-dimensional coculture system and demonstrated that NPC cause MSC to differentiate into an NP-like phenotype as assessed by gene expression after FACS sorting [108]. They observed that a 75% NPC / 25%MSC ratio was optimum for MSC differentiation, as indicated by SOX9, collagen 2, and aggrecan gene expression [108]. However, using a three-dimensional coculture system, Le Visage and colleagues noted that a random mixture of MSC and degenerative NPC did not increase GAG

production beyond single cell type controls [109]. More recently, Vadala and colleagues have shown that 3D unstructured coculture of MSC and NPC upregulated key differentiation markers in MSC [110]. These papers have looked at the environmental triggers due to re-creating the condensation shape and the unique signaling due to coculturing.

We hypothesize that by mimicking cell condensations through the use of a pellet system, and by allowing for tissue induction via a bilaminar organization, disc regeneration can be enhanced. As a first step toward testing this hypothesis we generated bilaminar pellets of MSC and NP and analyzed their behavior in culture. We observe a unique phenomenon: the budding of coculture pellets and the formation of satellite pellets that separate from the main pellet. Interestingly, the satellite pellets are composed of both cell types and have a specific organization in which the MSC are on the inside and NPC on the outside. The occurrence of budding and the organization of satellite pellets may have important implications for the use of coculture pellets in cell-based therapies for disc regeneration.

3.2 Materials and Methods

3.2.1 Cell Culture

Bovine NPC were isolated from caudal discs of healthy adult cows within 48 hours of sacrifice. The NP tissue was carefully separated by gross dissection and digested in 0.5% collagenase/dispase and 2% antibiotic/antimycotic in low glucose Dulbecco's Modified Eagle Medium (DMEM) at 37°C for 4-6hrs with constant stirring. The cells were then plated in tissue culture flasks and expanded to the fourth passage in

NPC Media (DMEM with 1% antibiotic/antimycotic, 1.5% 400m Osmolarity, and 5% Fetal Bovine Serum (FBS)) at 37°C with 5% CO_2. Culture media was changed twice a week.

Commercially available human MSC were purchased (Lonza) and expanded to the sixth passage in monolayer culture using growth media (DMEM low glucose with 1% antibiotic/antimycotic and 10% FBS) at 37°C with 5% CO_2. Culture media was changed twice a week.

Human nucleus pulposus samples were obtained from a consenting 55 year-old female patient undergoing surgery for scoliosis. The tissue was digested and the cells expanded. In addition, bovine MSC were isolated from femur tissue and the cells were expanded.

3.2.2 Bilaminar Pellet formation

Human MSC and bovine NPC were used to make co-culture pellets. The cross-species human-MSC /bovine-NPC pellets enabled us to trace the location of the cells via their lineage. Three different types of pellets were formed, each consisting of 500,000 cells total: pellets of 100% one cell type, pellets of MSC and NPC with randomized organization, and pellets of MSC and NPC organized into a bilaminar. The pellets containing both MSC and NPC were formed with three different cell number ratios of 25/75, 50/50, and 75/25 respectively (Figure 3.1). To produce the 100% one cell type pellets, 500,000 cells were pipetted into a 15mL polypropylene tube and centrifuged at low speeds (300g) for 5min. To create the randomized pellets, both cell types were added to the same tube, pipetted to ensure thorough mixing, and centrifuged at low speed

for 5 min. In order to form the bilaminar organized pellets, the cell type that would form the inner sphere of the pellet was added to a 15mL polypropylene tube and centrifuged at low speed for five minutes. Subsequently, the second cell type that would form the outer shell was gently added to the same tube. The cells were then centrifuged again at low speed for 5 min. Organized pellets were formed for all three ratios with MSC on the inside and NPC on the outside and vice versa. All pellets were cultured in 2 mL of growth media for three days with caps loosened to allow for gas exchange. After three days, the pellets became spherical and were transferred to ultra-low attachment 24 well plates (Corning) for the remainder of their culture time. Media was changed three times a week.

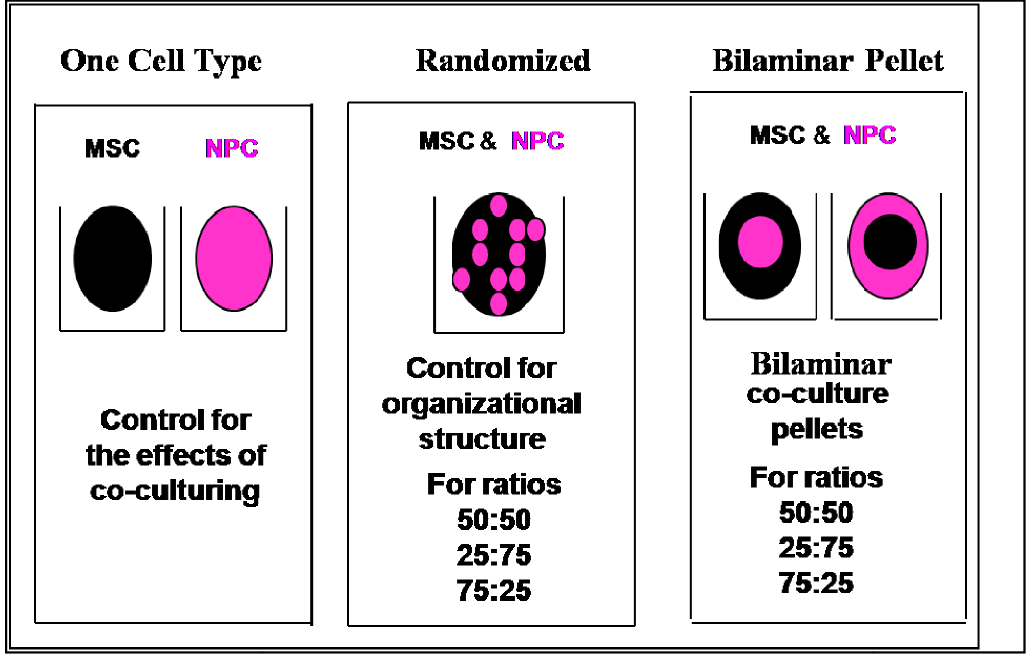

Figure 3.1: Depiction of the different groups composed on one cell type pellets, randomized pellets, and bilaminar pellets.

We also made same-species pellets as controls for species interactions. We made pellets with human MSC and human NPC and pellets with bovine MSC and bovine NPC.

3.2.3 Histology

3.2.3.1 Cell Lineage Tracing for Frozen Sections

Before being pelleted, cells were labeled with fluorescent cell membrane markers (either DiO or DiI, Invitrogen). After the pellets had reached their desired culture time, they were embedded in OCT Tissue-Tek (Sakura Finetek) and frozen sectioned at 7nm using a cryostat. Sections were then counterstained with the Hoescht dye, a fluorescent nuclear marker (Sigma). Images were taken using epi-fluorescent microscopy.

3.2.3.2 Immunohistochemistry with Paraffin Sections

At the end of the culture time, the pellets were fixed in 10% Buffered Formalin overnight and processed for paraffin sectioning at 7nm thickness. At this stage, the sections were immuno-stained using the human specific antibodies Lamp1 and Lamp2 (Abcam). The sections were then counterstained with hematoxylin.

3.3 Results

3.3.1 NPC culture

The NPC started as small rounded cells. They often required several days to attach to the tissue culture flask. Many of the cells never attached at all and were discarded. Though the cells were initially seeded in very small flasks (12.5 cm^2, Falcon), it took up to 2 weeks for the cells to reach confluence. Once the cells were confluent and passaged, their growth rate significantly increased, and they were easily expanded to the fourth passage.

3.3.2 Macroscopic observations

Immediately after centrifugation the pellets appeared flattened. Spheres formed within 48 hours and reached a maximum size of approximately 2mm in diameter. After one week of culture, co-culture pellets began to exhibit budding (Figure 3.2). During the course of the second week of culture, these buds separated from the main pellet entirely to form numerous independent satellite pellets of various sizes. As the satellite pellets budded off of the main pellet, the main pellet did not noticeably decrease in size. At the three week time point, several of the larger satellite pellets also began to exhibit budding.

The 100% MSC pellets did not exhibit budding nor did any satellite pellets form at any point. The 100% NPC pellets exhibited budding and satellite pellet formation on the same time frame as the co-culture pellets (obviously in this case the satellite pellets were composed entirely of NPC). The same-species pellets (e.g. human-MSC and human-NPC) exhibited the same behavior as the cross-species pellets (human-MSC and bovine-NPC). There was no difference in the budding or satellite formation rate between the bilaminar co-culture pellet and the random co-culture pellets.

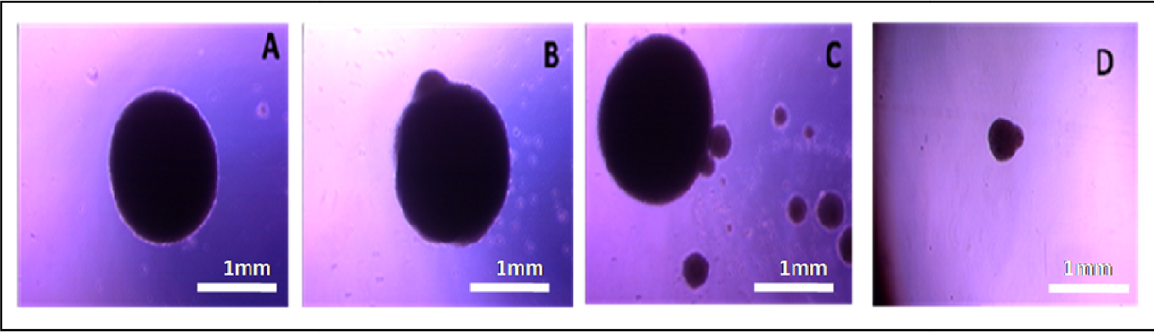

Figure 3.2: Budding and satellite pellet formation. (A) Coculture pellet after three days of culture. (B) A budding cocluture pellet at one week of culture. (C) Coculture pellet after three weeks, several satellite pellets have formed and separated. (D) A budding satellite pellet after three weeks of culture.

3.3.3 Histology

Both the frozen (Figure 3.3) and paraffin histological sections confirmed that the main pellets maintained their structure throughout the culture time even as satellite pellets budded off them (Figure 3.4).

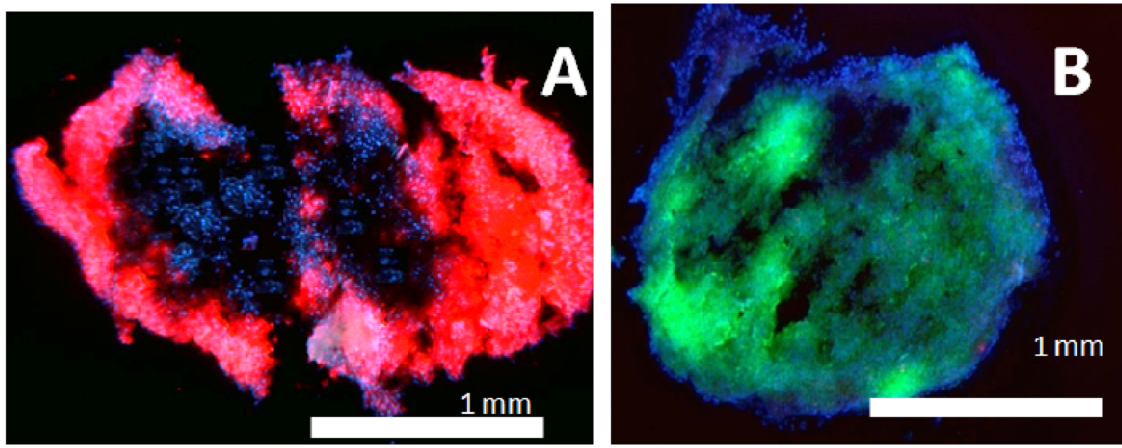

Figure 3.3: Frozen sections of bilaminar pellets after 3 weeks of culture. All cell nuclei are labeled with Hoescht dye (blue). A) Bilaminar pellet with MSC unstained and NPC dyed with DiI (red). The pellet is organized with 50% MCS on the inside and 50% NPC on the outside. B) Bilaminar pellet with MSC dyed with DiO (green) and NPC unstained. The pellet is organized with 75% MSC on the inside and 25% NPC on the outside.

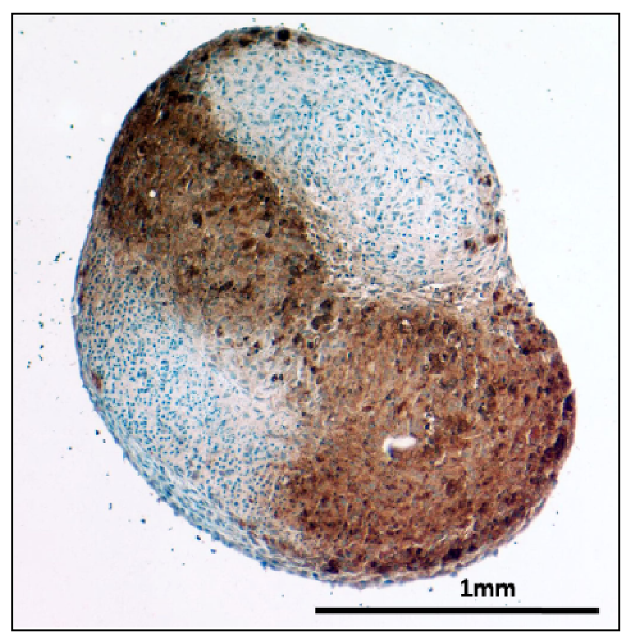

Figure 3.4: Three week bilaminar pellet with MSC on the inside (brown) and NPC on the outside after undergoing budding and satellite pellet formation. This is a paraffin section stained using immunohistochemistry with the human specific antibodies (brown), Lamp1 and Lamp2, and counterstained with hematoxylin (blue). Even after undergoing budding numerous times, the pellet conserved it's original structure.

The histology of the satellite-pellets confirmed that they were composed of both cell types. Most surprisingly, the satellite pellets appear to all have the same structure with MSC on the inside and NPC on the outside (Figure 3.5). This structural organization was independent of the structure and ratio of the main pellet that they stemmed from.

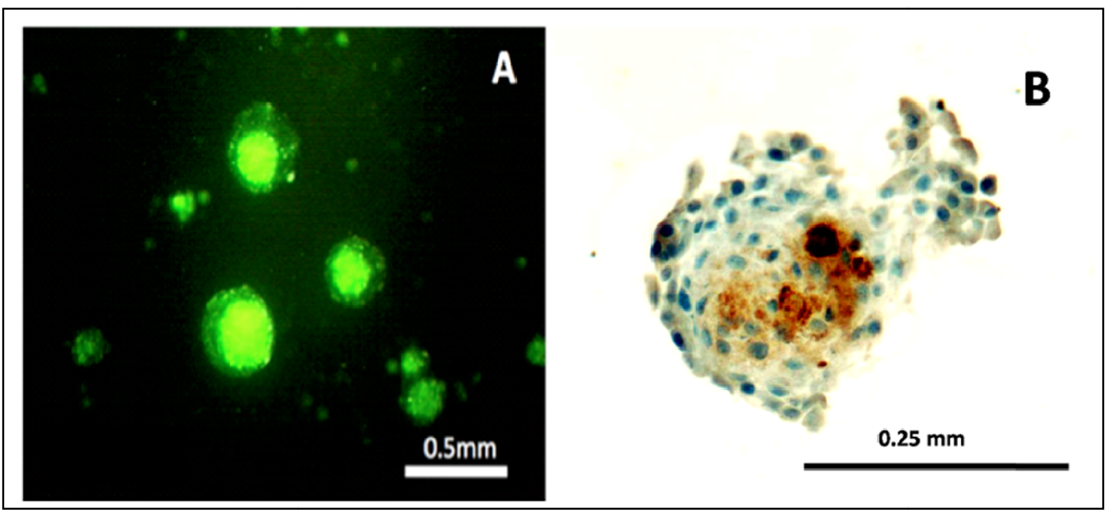

Figure 3.5: (A) Fluorescent microscopy image of a satellite pellet frozen section. MSC were pre-stained with DiO (green) before forming the main pellet. The center of the satellite pellet is stained with DiO while the exterior is free of dye indicating that it is composed of both cell types and has the bilaminar organization of MSC inside and NPC outside. (B) Immunohistochemistry performed on a paraffin section of a satellite pellet. The MSC at the center of the satellite pellet are stained with a human specific antibody (brown) and counterstained with hematoxylin. The satellite pellets have a bilaminar structure with MSC on the inside and NPC on the outside.

3.4 Discussion

How MSC and NPC influence each other's behavior in 3D culture has very important implications for cell–based, disc regeneration therapies. Presumably, a cell-based therapy will always elicit an interaction between the newly introduced cells and the host cells since NPC would not be removed during a minimally invasive injection. Previous studies have shown that cell-cell contact between MSC and NPC was necessary to induce a stimulatory effect [108-109].

Several groups have shown that unstructured coculture offers some advantage over using MSC or NPC alone in the search of a cell based strategy to regenerate the

nucleus [107-110]. It has been shown that these cell types have the ability to communicate with one another to increase MSC differentiation, cell proliferation, and matrix production [107-110]. In our system, we have observed a novel phenomenon whereby coculture pellets exhibit budding and the formation of satellite pellets. This occurrence may have important implications as to the behavior of constructs when administered in vivo during disc regeneration. What remains unclear as of yet is which underlying biological mechanisms are at work. However, we believe that such mechanisms will be rooted in the molecular and cellular processes underlying cartilage formation where similar behaviors exist [99-110].

In order to ensure that our results were not due to species incompatibility between human and bovine cells, we wanted to test the extent to which coculture pellets composed entirely of human cells and entirely of bovine cells would exhibit the same dynamic behaviors. Our results indicate that budding and satellite pellet formation are generalizable phenomena that arise in response to certain cell configurations rather than being due to species-incompatibility.

Overall, our experiments led to the novel and yet unexplained discovery that MSC and NPC, when cocultured in any arrangement and in any ratio, will form bilaminar satellite pellets with MSC on the inside and NPC on the outside. This implies that structural organization is intrinsic to these cell populations, can occur spontaneously in this cell culture system, and may be inherently favorable for cell-based tissue engineering strategies. We believe that bilaminar organization may accelerate MSC differentiation and the cells' ability to resist environmental factors. At the center of a pellet, cells experience higher pressure and lower oxygen tension than on the outside. This micro-

environment is therefore more conducive to MSC differentiation. In addition, the NPC readily form an epithelial-like layer on the outside of the tissue. This layer may ultimately protect the pellet from negative external signals, such as inflammatory cytokines.

From a therapeutic point of view, the phenomenon of budding and the generation of satellite pellets may be a beneficial feature that would serve to distribute donor cells throughout the host matrix. Over the long term, we envision using such a bilaminar pellet system to form the basis for new cell-based therapies for disc degeneration. Several pellets could be injected using a minimally invasive procedure into the nucleus pulposus of patients with back disc disease [92,111]. We anticipate that these satellite pellets will enhance the ability of this technique to repopulate and regenerate the disc nucleus. These pellets would then serve to restore normal matrix composition in a sustainable way and ultimately restore tissue function.

CH4. Structured co-culture outperforms stem cells and disc cells in a simulated degenerative disc environment

4.1 Introduction

Tissue engineering is a growing and dynamic field with the potential to provide patients with minimally-invasive treatments that repair or replace dysfunctional musculoskeletal tissues. For the intervertebral disc, the goal is to re-establish pain-free motion by restoring the disc's physical and biochemical properties [89-113]. This may be accomplished by stimulating host cells to resume matrix synthesis (particularly aggrecan) and/or by introducing new, more synthetically-active cells [66-69]. Our work focuses on the latter option.

Pellet culture systems may have benefits as part of a tissue engineering strategy as they can recapitulate embryonic microenvironments for regenerative purposes [70]. Furthermore, co-culture of stem cells and disc cells is a promising strategy for cell based tissue regeneration as makes use of signaling interaction between the two cell types to promote MSC differentiation [76-130]. We explored the benefits of co-culturing nucleus pulposus cells (NPC) and adult MSC using a 3D system that exploits embryonic processes such as tissue induction and condensation. We report here on a novel "bi-laminar cell pellet" approach that structures cell-cell signaling between MSCs and non-degenerative NPCs [131,132]. A spherical bi-laminar cell pellet (BCP) is used where one cell type forms an inner sphere enclosed within a shell of the other cell type (Figure 2). The BCP is composed of 75% MSC as the inner sphere and 25% NPC forming the outer

shell. We have previously shown in vitro that BCP produce 30-50% more matrix than the MSC and NPC controls indicating that the synergy between the two cell types in this spatially organized configuration is advantageous [131].

A key challenge to functional disc tissue engineering is that there are no established, validated pre-clinical models that simulate the condition of pathologic/painful disc degeneration. However, in order to accurately predict the effectiveness of any cell-based therapy it is crucial to simulate the key components of the harsh environment that the cells will experience in vivo since these environmental cues dramatically affect the therapeutic performance of the therapy [133-138]. A model that accurately reproduces the environment of a painful disc would enable optimization of tissue engineering approaches. In the degenerate disc these three key components are pressure, hypoxia, and inflammation [138].

The healthy disc serves to withstand substantial compressive loading while remaining pliant to facilitate motion. It is also the largest avascular organ of the body making the issues of nutrient and oxygen transport critical to cell survival [139]. Pressure and hypoxia are therefore fundamental components of the disc environment. As may be expected, mechanical load helps maintain matrix homeostasis in intervertebral discs, and mechanical and hypoxic stress can affect the cell viability and matrix synthesis of mesenchymal stem cells (MSCs) and nucleus pulposus cells (NPCs) [140-141]. Since loss of cellularity and reduced extracellular matrix production is a central feature of degenerative disc disease, the introduction of cells capable of surviving and producing normal extracellular matrix in a high pressure and low oxygen tension is critical for successful bioengineered disc regeneration.

Degeneration also coincides strongly with elevated levels of a variety of inflammatory and degenerative factors such as cytokines and MMPs. Specifically, there have been observed increases in the aggrecanase, ADAMTS-4,MMPs 1, 3, 7 and 13 (and perhaps others) and reductions in tissue inhibitors of metallopreteases, TIMPs –1and –3 (the latter being a known aggrecanase inhibitor), all or some of which appear linked to increases in IL-1 [142-143]. Other inflammatory cytokines, such as TNF-alpha are also elevated and likely inductive of degenerative effects [144-146].

In order to better anticipate the therapeutic effect of BCP in vivo, we mimicked key features of degenerate disc environment in vitro. The BCP were tested under three different culture conditions: in a bioreactor that provides pressure & hypoxia (mimicking human disc conditions at rest), with inflammatory cytokines (IL-1beta and TNF-alpha), and a bioreactor with inflammation (mimicking degenerative disc conditions). We have therefore employed the use of a bioreactor that mimics two important physiochemical aspects of the in vivo disc environment: pressure (0.1MPa) and oxygen tension (4.5%). The pressure is generated via osmotic swelling of hyaluronan gel and the low oxygen tension is a result of the diffusion across the dialysis membrane of a known pore size and through the gel. Our goal was to test our cell-based therapy in vitro under conditions representative of the eventual diseased tissue.

4.2 Materials & Methods

4.2.1 Bioreactor Design and Construction

The bioreactor is a simple design composed of easily available components. This permits easy fabrication of many replicate units that can be used simultaneously. Each bioreactor is composed of a commercially available dialysis cassette (Slide-A-Lyzer, Pierce) modified to enable pressurization. The cassette consists of a rigid frame, similar in size and shape to a 35mm slide, which holds two dialysis membranes (10 kDa pore size) in the center (Figure 4.1A). The membranes are separated by a silicone gasket. The cassette cavity (300 mL) is filled with a 15% hyaluronan gel (MW 80,000, Genzyme, Cambridge, MA) by inserting an 18-gauge needle attached to a syringe through the self-sealing gasket via guide ports in the frame. The hydrogel is entrapped within the device by the gasket and semi-permeable membranes in similar fashion to containment of the nucleus pulposus by the annulus and cartilaginous endplates.

A 15% hyaluronan (HA) gel was made by dissolving sodium hyaluronate powder in growth media. Three hundred microliters of this gel was injected through the self-sealing silicone port of a dialysis cassette and excess air was removed from the cassette. The cassette was then incubated in growth media (Dulbecco's modified eagle medium (DMEM) low glucose, 1% antibiotic/antimycotic, and 10% Fetal Bovine Serum (FBS)) for four hours to allow for gel volume expansion and osmotic pressurization (equilibration is based on osmotic pressure differences between the HA gel and the culture media). An autoclavable porous metal clip surrounds the device to permit fluid exchange but prevent membrane rupture due to internal pressurization (Figure 4.1B). Cassettes were then incubated in growth media at 37 degrees with 5% CO2.

4.2.2 Bioreactor Oxygen and Pressure Measurements

Bioreactor oxygen and pressure were performed as described previously [132]. The oxygen tension inside the bioreactor was 4.8% and the pressure was 0.12 MPa, which is consistent with physiologic levels within the human lumbar disc at rest.

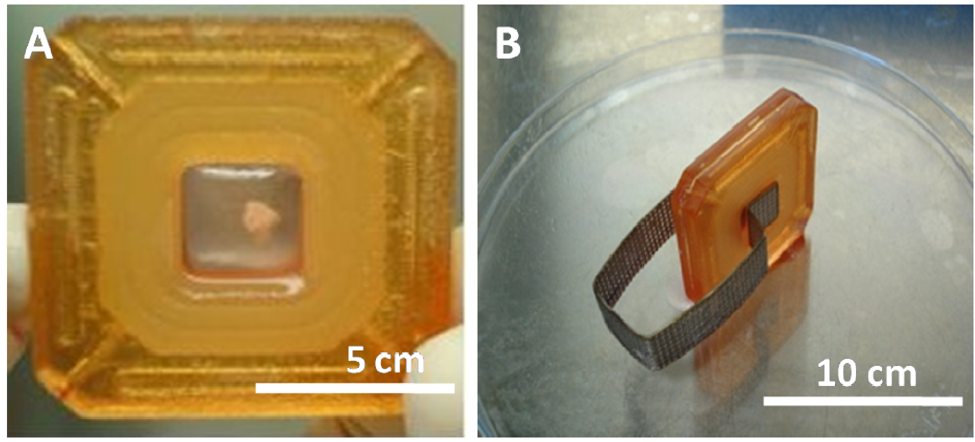

Figure 4.1: Bioreactor setup. A) The bioreactor is filled with HA gel which swells due to osmotic pressure. The pellet is then injected into the chamber. B) Once the bioreactor is swollen, an autoclaved porous metal clip is placed around the membrane so that it will not continue to swell and burst.

4.2.3 Cell Culture

Bovine NPC were isolated from caudal discs of healthy adult cows within 48 hours of sacrifice. The NP tissue was carefully separated by gross dissection and digested in 0.5% collagenase/dispase and 2% antibiotic/antimycotic in low glucose DMEM at 37°C for 4-6hrs with constant stirring. The cells were then plated in tissue culture flasks and expanded to the fourth passage in NPC Media (DMEM low glucose, 1% antibiotic/antimycotic, 1.5% 400m Osmolarity, and 5% FBS) at 37°C with 5% CO_2. Culture media was changed twice a week.

Commercially available human MSC were purchased (Lonza, Switzerland) and expanded to the sixth passage in monolayer culture using growth media at 37°C with 5% CO_2. Culture media was changed twice a week.

4.2.4 Pellet formation and culture

Human MSC and bovine NPC were used to make co-culture pellets. We have previously shown using controls of human MSC with human NPC pellets as well as bovine MSC with bovine NPC pellets that there are no significant cross-species interactions [131].

Two different types of pellets were formed, each consisting of 500,000 cells total: pellets of 100% one cell type and BCPs of 75% MSC and 25% NPC organized into a bilaminar (Figure 4.2). To produce the 100% one cell type pellets, 500,000 cells were pipetted into a 15mL polypropylene tube and centrifuged at low speeds (300g) for 5min.

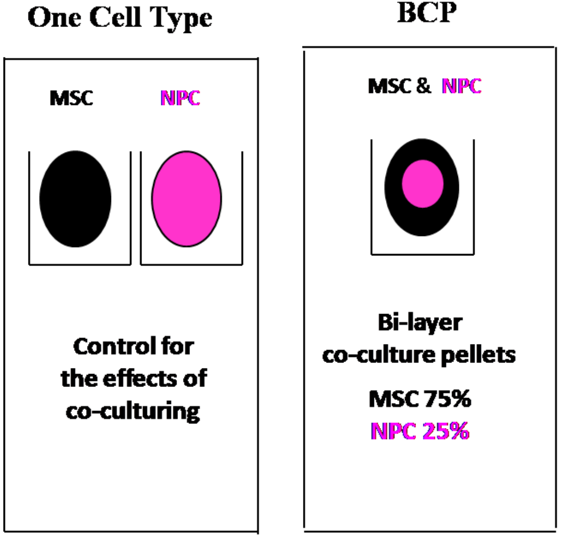

Figure 4.2: The two different types of pellets. The one cell type pellets are composed of 100% MSC or 100% NPC. The BCP pellet is composed of 75% MSC on the inside and 25% NPC on the outside.

In order to form the bilaminar organized pellets, 325,000 MSCs were added to a 15mL polypropylene tube and centrifuged at low speed for five minutes. Subsequently, 125 000 NPCs were gently added to the same tube. The cells were then centrifuged again at low speed for 5 min. All pellets were cultured in 2 mL of growth media for three days with caps loosened to allow for gas exchange. After three days, the pellets became spherical and were transferred to ultra-low attachment 24 well plates (Corning).

All pellets were cultured in growth media for a total of one week. At the end of the first week, some of the pellets were injected in a bioreactor using a Radiology Angiography needle (Beckton Dickinson, NJ) (Figure 4.3A). The pellets in the inflammatory group were cultured in growth media supplemented with IL-1beta and TNF-alpha at 10ng/mL each (Peprotech, NJ) (Figure 4.3B). The pellets in the bioreactor with inflammation group were injected into a bioreactor that contained the same cytokines as the inflammatory group (Figure 4.3C). There was an n=5 per pellet type per culture group.

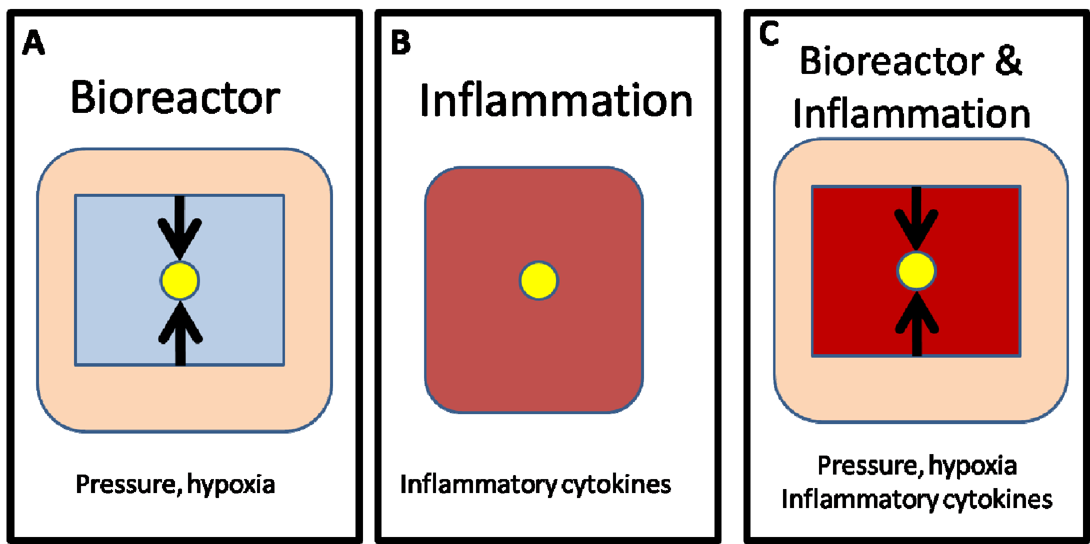

Figure 4.3: The three environmental conditions. A) In the bioreactor group, the pellets were exposed to hypoxia and pressure. B) In the inflammation group, the pellets were cultured with inflammatory cytokines. C) In the bioreactor and inflammation group which simulates the degenerative disc, the pellets were exposed to inflammatory cytokines, pressure, and hypoxia.

4.2.5 DNA and DMMB assays for proteoglycan quantification

At the end of two weeks, the pellets were separated for media or HA gel, depending on the culture conditions. The pellets were digested overnight at 60 degrees in a papain solution (20 U/mL in PBS). Digested pellets were assayed with a Quant-iT PicoGreen kit (Invitrogen, CA) to measure DNA content. Measurements were made using a spectrophotometer, with the excitation at 488nm and absorption at 525 nm.

Digested pellets were also assayed with a dimethylmethylene blue (DMMB) assay to quantify GAG content. A standard curve was made with chondroitin sulfate isolated from bovine trachea (Sigma, MO). Absorption was measured at 525nm using a spectrophotometer.

The results obtained from GAG quantification where normalized to the cell number as calculated from the DNA content. Statistical Analyses (ANOVA and Tukey post-hoc tests) were performed with JMP software.

4.2.6 In situ hybridization

In situ hybridization was performed [148]. Sections were hybridized with ^{35}S-labeled human riboprobes to aggrecan and collagen II-a1 (fibrillar collagen). Sections were counterstained with Hoechst dye (Sigma, MO). Hybridization signals were detected using darkfield and the nuclear stain with epifluorescence.

4.3 Results

4.3.1 DNA and DMMB assays for proteoglycan quantification

When cultured in the bioreactor, the NPC pellets produced significantly more GAG per cell than the other groups: 70-80% more than the BCP and MSC alone (Figure 4.4). The BCP showed a trend towards producing 53% more GAG per cell than MSC though this was not statistically significant.

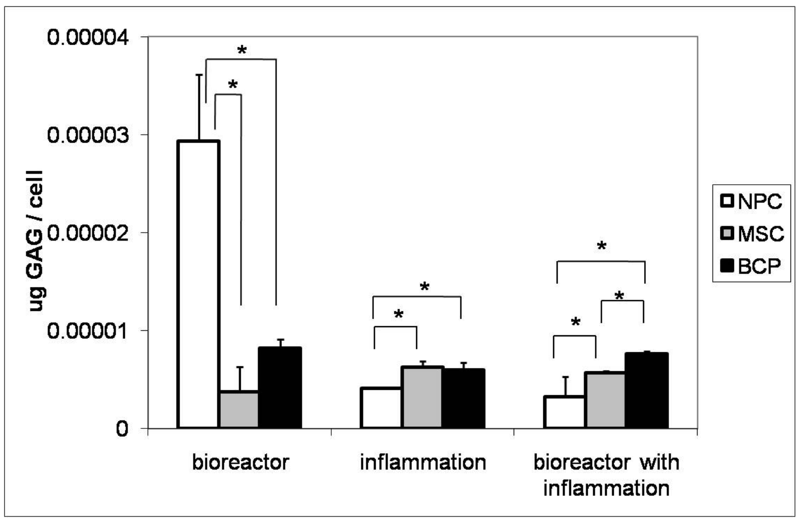

Figure 4.4: Graph of ugGAG produced per cell by each of the groups in the three different environmental conditions. In the bioreactor with inflammation group, the BCP produce significantly more ugGAG/cell than the controls.

When cultured in an inflammatory environment, the MSC and BCP groups produced 30-34% more GAG per cell than NPC ($p<0.05$). There was no statistically significant difference between the MSC and BCP.

When the pellets were cultured in a bioreactor with inflammation, the BCP made 25% more GAG per cell than MSC and 57% more than NPC ($p<0.05$). The MSC made 42% more GAG per cell than NPC ($p<0.05$).

4.3.2 In situ hybridization

The MSC pellets do not appear to have any significant levels of collagen II expression regardless of their environment (Figure 4.5 A,G,M). The levels of aggrecan expression do vary with the environmental conditions. There are low levels of expression in both the bioreactor and the bioreactor with inflammation group (Figure 4.5 B,N). The

highest levels of expression were in the inflammation group where expression does appear to be homogeneously spread (Figure 4.5H).

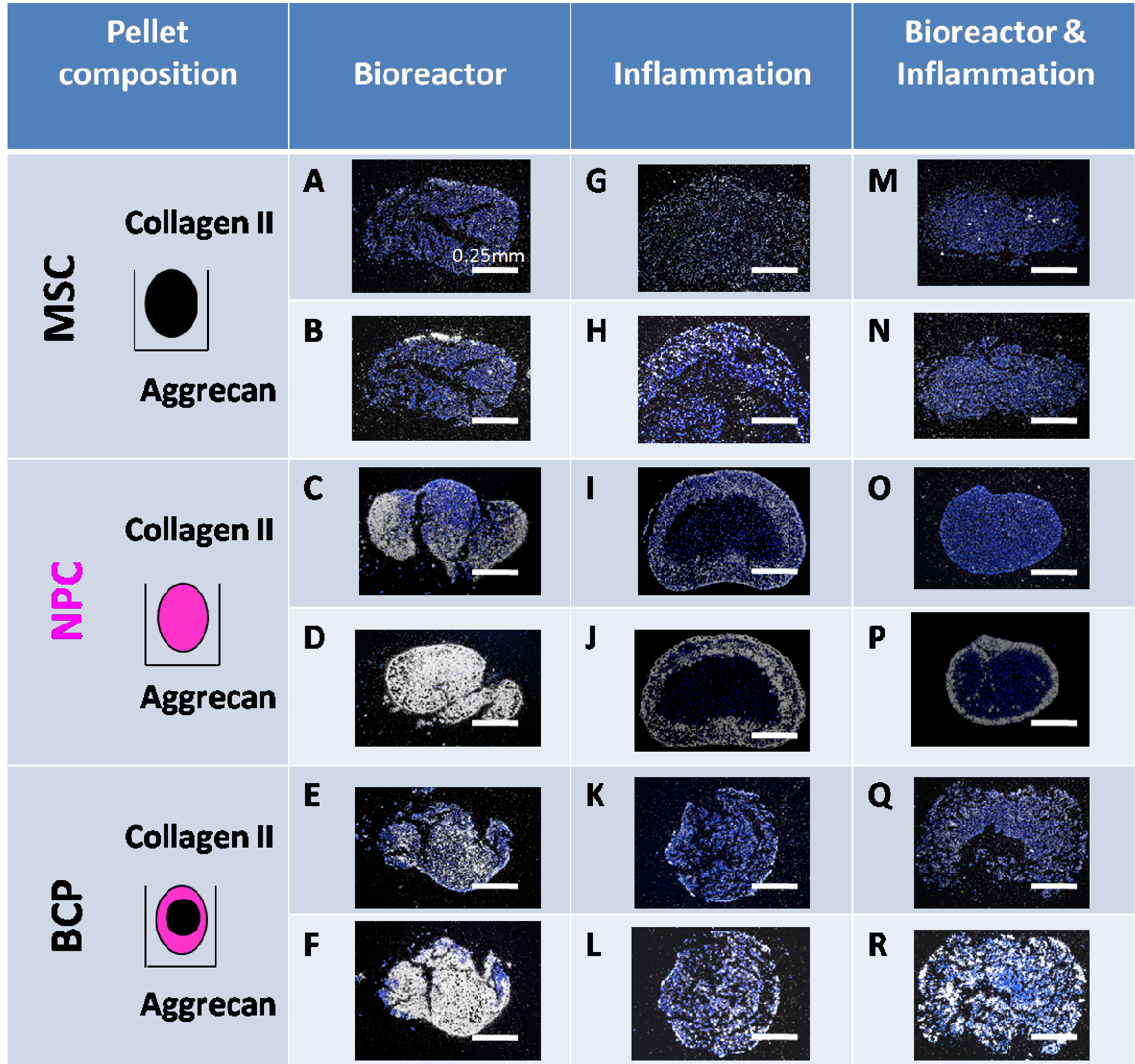

Figure 4.5: In situ hybridization. These images show the location of aggrecan and collagen II gene expression (in white) in the pellet, counterstained with Hoechst dye (which stains cell nuclei blue). Images A-F are of pellets cultured in a bioreactor. Images G-L are of pellets cultured in an inflammatory environment. Image M-R are of pellets culture in a bioreactor with inflammation.

For the NPC pellets, collagen and aggrecan expressing varied with environmental conditions. In the bioreactor group, the aggrecan and collagen II expression was very

strong and homogeneous (Figure 4.5C,D). In the inflammation group, aggrecan and collagen II expression both dropped significantly compared to the levels in the bioreactor (Figure 4.5 I,J). In addition, the expression was localized to the outer layer of the pellet. In the bioreactor with inflammation group, expression of aggrecan and collagen II dropped further below levels in the inflammatory group, particularly for collagen II expression which was extremely faint (Figure 4.5O). Aggrecan expression was again localized to the outer periphery of the pellet (Figure 4.5P).

For the BCP group, gene expression also varied with environmental conditions. In the bioreactor group, aggrecan and collagen II expression were high and homogeneous (Figure 4.5E,F). These levels decreased somewhat in the inflammatory group, but remained homogeneous localized as opposed to the NPC group (Figure 4.5K,L). Finally, in the bioreactor with inflammation group, aggrecan and collagen II expression both increased over inflammatory levels and remained homogeneously localized (Figure 4.5 Q,R).

4.4 Discussion

The goal of this study was to determine the environmental effects on pellet matrix synthesis and gene expression in order to predict how the pellets would react in a simulated in vivo environment. The in situ hybridization enables the qualitative visualization and localization of gene expression. In this case, we looked at both aggrecan and collagen II since they are the primary components of the matrix and targets of regenerative strategies. The GAG/cell serves as a primary quantitative indicator of the

amount of new matrix being produced.We cultured the pellets in all three environments in order to isolate the effects due to simulated inflammation as well as pressure & hypoxia.

Our data demonstrate that both GAG/celland gene expression vary significantly with culture conditions. We observed that NPC produced the most GAG/cell in the bioreactor that simulates the normal biomechanical pressure and oxygen tension of the disc. The in situ hybridization images also show that NPC expressed higher levels of aggrecan and collagen II than MSC and BCP. These results are expected since NPC are adapted to survive and produce matrix under these conditions. Interestingly, the BCP did show high levels of aggrecan and collagen II expression, indicating that the stem cells that make up 75% of the BCP had differentiated. This was not the case in the MSC group, where expression levels were very low. We suspect this behavior is due to MSC differentiation resulting from the unique BCP structure and organization.

The MSC and BCP groups produced more GAG/cell in the inflammatory environment than the NPC. The literature indicates that MSC may be immune-privileged cells and tolerant of inflammatory conditions [149]. Also, considering that the BCP are 75% MSC, it is not surprising that the BCP performed well in this environment as well. Importantly, NPC did not perform well in an inflammatory environment with GAG/cell dropping 86% from the bioreactor levels. Additionally, the gene expression of aggrecan and collagen II in the NPC group was lower than in the bioreactor conditions and localized to the periphery. These data indicate that NPC are very sensitive to inflammatory cytokines that have a strong debilitating effect on gene expression and aggrecan production.

Finally, we observed that in the bioreactor with cytokines the BCP produced the most matrix. The BCP group had the highest aggrecan expression levels. These data suggest that BCP would outperform NPC or MSC alone for disc tissue engineering. Interestingly, we also observed that MSC produced significantly more GAG than NPC, indicating that the inflammatory environment has a more dominant effect on these cells than pressure and hypoxia.

Ultimately, our goal is to investigate the benefits of BCP as a cell based therapies for disc degeneration. We envision that multiple pellets would be injected in a minimally invasive fashion into the nucleus pulposus of patients with black disc disease and discogenic pain. These results indicate that the BCP may be advantageous in the harsh, painful disc environment.

CH 5. Structured Co-culture of Stem Cells and Disc Cells Prevent Degeneration in a Rat Model

5.1 Introduction

Intervertebral disc tissue engineering with stem cells holds the promise of a minimally invasive low back pain treatment. Mesenchymal stem cells (MSCs) have the capacity to differentiate into disc-like cells and synthesize key matrix elements that may benefit patients with degenerate discs [66]. Yet, since previous studies have demonstrated the powerful influence of the local milieu on cell matrix synthesis, in vitro results may not predict in vivo behavior [53]. In order to test the plausibility of therapeutic efficacy as well as safety, these therapies should be tested and optimized with an in vivo model.

Animal models for disc degeneration should mimic human disc degeneration in order to accurately serve as predictors of the therapy's efficacy in vivo [152]. An ideal animal model would replicate the morphological, biochemical, and biomechanical aspects of human disc degeneration and provide a reproducible platform for analysis. There are other more practical issues that also need to be considered such as ethics, cost, and time that also affect the appropriateness of an animal model [152-154]. Rodents are desirable models for disc repair studies due to their low cost, ease of care, and fast healing times [155]. Tail discs are advantageous because they have an easy surgical approach and multiple discs can be analyzed in each animal, thus reducing the complexity of the surgery and the number of animals needed [156]. Rodent disc mechanical properties, when normalized to geometry, are comparable to those of human

lumbar discs [156]. Although adult rat discs contain notochordal cells, rodent disc morphology and cellularity are comparable to young human discs, and rats are among the few species that retain disc cell populations similar to those found in adult humans [157]. In addition, rat discs have similar innervation patterns to human discs that are important for replicating pathological processes [158].

Degenerative disc disease has been induced using an annular stab technique (where the incision is made in the annulus fibrosus – depressurizing the disc) in various animal models including the rat [159-160], rabbit [161-165,172], dog [166], sheep [167,168], goat [169], and pig [170,171]. The annular stab technique has consistently shown the desired morphological evidence of many human age-related degenerative disc disease features including [173]: radial and concentric annular tears, trabecular bone formation in the endplate, changes in cellularity and matrix composition. In addition to morphological signs of degeneration, several of these studies found other similarities to human disc degeneration such as high levels of pro-inflammatory cytokines [174], changes in disc mechanical properties [175], and loss of signal intensity on MRI images [172,176].

In addition to annular stab, several animal models also employ the use of a nucleotomy (where the nucleus pulposus, and therefore the cell content, is removed) in order to induce degeneration and as a method for creating some space in the disc for the therapeutic to be inserted [177-179]. These models have also successfully shown an induction of degenerative disc disease. In the case of the rat model, a nucleotomy will also result in the removal of notochordal cells that are not present in adult human disc making it more representative of the human disc environment at the cellular level.

The acute stage of disc injury is characterized by high levels of inflammatory cytokines, hypoxia, changes in pH, and alterations in mechanical loading [180-182]. During phase of wound healing, newly introduced cells are primarily challenged with surviving and staying in the desired location. Over longer-term, levels of inflammatory cytokines are diminished, nutrition and oxygen are limited, the environment becomes acidic, and morphology becomes disrupted [180-182]. The longer-term stage is a more challenging test of the treatment's ultimate therapeutic efficacy.

Previous attempts of injecting mesenchymal stem cells (MSCs) into the intervertebral disc have been met with varied results characterized by poor retention and limited therapeutic activity [183-187]. This is likely due to poor cell survival, undesirable differentiation, and ineffectual matrix synthesis [183-190]. Other approaches that rely on delivery of allograft nucleus pulposus cells (NPCs) have met with limited success, primarily because of limited NPC synthetic activity [191-193]. Combinations of cells and scaffolds have also been implanted in animal models of intervertebral disc degeneration but have yet to show long term repair [193-195]. Though progress is being made in the field of cell-based therapies for disc degeneration, there are still many obstacles to success due to the toxic environment of the degenerative disc where the pressure, ischemia, and inflammation limit the cells' abilities to mount a therapeutic response [151,159,189-191].

In order to address these issues, we have developed a novel spherical bi-laminar cell pellet (BCP) where MSCs form an inner-sphere enclosed within a shell of NPCs with a 75:25 ratio. In vitro, BCPs have a 48% increase in proteoglycan-synthesis as compared to MSC controls [52]. In a simulated degenerative disc environment in vitro, the BCP

produced more matrix per cell than MSC and NPC [53]. In order to further assess the BCP's potential as a therapeutic, we will now test it with an in vivo rat tail model of disc degeneration. The objective of this study is to compare the in vivo therapeutic efficacy of MSCs suspended in Fibrin, NPCs suspended in Fibrin, a combination and MSCs and NPCs suspended in Fibrin, and a BCP implanted with Fibrin in a degenerative intervertebral disc in both the acute and longer-term stages.

5.2 Methods

5.2.1 Rat selection and Anesthesia

Sixty 6-month old male Sprague Dawley Rats weighing approximately 250grams were used in this study with IACUC approval. Prior to surgery, each animal was anesthetized (by inhaling isofluorane) and medicated with buprenorphine (0.01 mg/kg body weight). While under anesthesia, the animal's paw pinch response and respiration pattern were monitored every 5 minutes.

5.2.2 Surgical Procedure

After placing the animal under general anesthesia, the rat was x-rayed in the area of interest and placed on a heating pad. The tail was cleaned with a surgical scrub and an occlusion cuff was placed on the proximal end of the tail. The cuff limited blood flow to the tail allowing the discs to be easily visualized while minimizing blood loss.

A longitudinal incision (approximately 1 inch long) was made to expose three proximal discs. Lateral stabs were performed in each of three exposed discs using a number 11 blade. The whole nucleus pulposus was removed using a small spatula and

the desired treatment was inserted into the disc space. The annulus was then sutured closed with two small suture points with 5.0 non-resorbable nylon sutures. The occlusion cuff was then removed. The cuff was on the tail for no longer than 15 minutes. Finally, the tail skin was closed using resorbable sutures.

During the recovery period from anesthesia, the rats were kept on a heating pad (also used during surgery) to maintain constant body temperature. The rats were allowed unrestricted activity and monitored daily for the seven days following surgery. After the tails healed, the rats were monitored three times weekly. Buprenorphine (0.01-0.02 mg/kg, SQ) was administered as needed for pain.

5.2.3 Treatment groups

Six groups were assessed at two time points (14 days and 35 days) in sixty rats (5 rats per group per time point): stab only (Stab group), fibrin-sealant (FS) only (Fibrin group), MSCs suspension in FS (MSC group), NPCs suspension in FS (NPC group), NPCs and MSCs suspension in FS (Mix group), BCP with FS (BCP group) (Figure 5.1). The Stab group serves as a positive control to assess the unobstructed acute inflammatory cycle. The FS only group serves as a negative control, while the other groups will characterize the treatment efficacy.

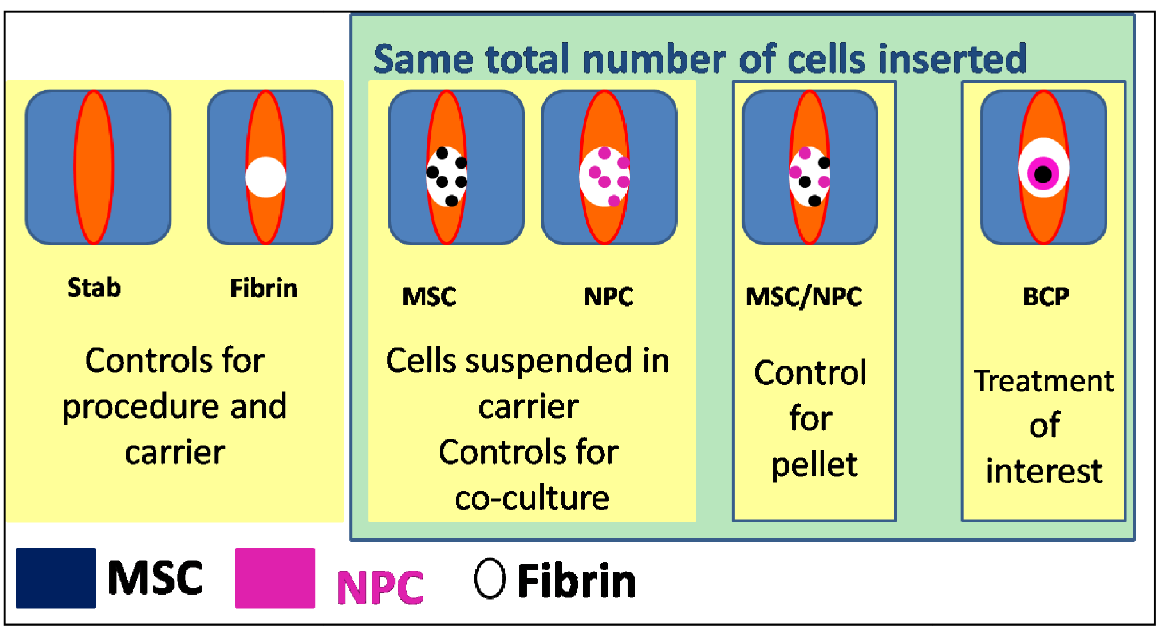

Figure 5.1: The six treatment groups for 14 and 35 day time points.

5.2.4 Cells and fibrin

MSCs were commercially obtained (Lonza, Switzerland), expanded to the 7th passage in growth media (DMEM low glucose, 1% antibiotic/antimycotic, and 10% FBS) at 37°C with 5% CO2. NPCs were isolated from adult caudal bovine tails and expanded to the 4th passage. Surgical grade fibrin was obtained (Baxter.

In each case of cell treatment, a total of 250,000 cells were inserted into the disc space with 4uL of FS. In the cases of cell suspensions, the cells were homogeneously suspended in the carrier prior to insertion.

In the case of BCP, the BCP was formed 7 days prior to insertion in order for it to become spherical using 75% MSC on the inside and 25% NPC on the outside with a total

of 250,000 cells. The BCP was first placed in the disc space and then covered with 4uL of fibrin (Figure 5.2).

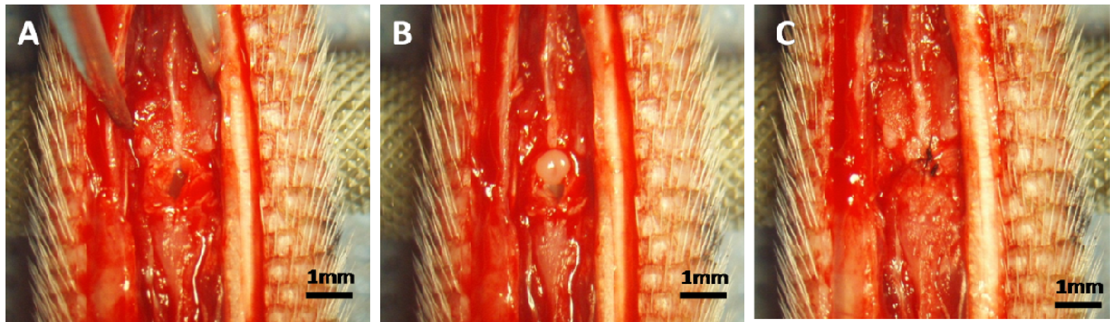

Figure 5.2: Surgical procedure. A) The hole is made by de-nucleating the disc. B) The BCP is inserted into the disc space. C) The annulus is closed with sutures.

5.2.5 Euthanasia

After the duration of interest (14 days, 35 days), the rats were euthanized by carbon dioxide asphyxiation followed by bilateral thoracotomy. The tail was x-rayed and the discs were harvested for analysis.

5.2.6 Histology, Disc height, Disc Grade

Some of the disc samples from both time points were processed for paraffin embedding, section at 7um thickness, and stained with Safranin-O and immunohistochemistry using the human specific antibody Lamp 1&2 (Abcam, MA).

Disc height measurements were taken from growth plate to growth plate on histologic samples (n=3 per group per time point). For each image, the height was

measured in three areas of the disc space: on the left side, center, and the right side. The ultimate disc height for that image was determined to be the average of the three.

Disc grade was measured by an orthopedic surgeon using blinded images according to the grading scheme in figure 5.3. This scheme has been adapted from other schemes of its type [165].

Category	Score of 1 (normal)	Score of 5 (degenerative)
Annulus Fibrosus Grade	Normal pattern of fibrocarilage lamellae without ruptured fibers and without a serpentine appearance	Moderate/severe interruption
Border between the Annulus Fibrosus and the Nucleus Pulposus	Normal	Moderate/severe disruption
Cellularity of the Nucleus Pulposus grade	Normal Cellularity	Moderate/severe decrease in the number of cells
Matrix of the Nucleus Pulposus	Normal gelatinous appearance	Moderate/severe condensation of the matrix
Endplate grade	Normal continuous and undisrupted	Complete disruption
Growthplate grade	Normal continuous and undisrupted	Complete disruption
Disc height grade	Normal disc height	Complete collapse

Figure 5.3: Disc grade scoring chart. The scoring was performed blindly according to the following catergories. Each category was given a score from 1, for normal discs, to 5, for degenerative discs.

5.2.7 ELISA, DMMB, PCR

Some of the disc samples from both time points were evaluated for cytokine contents. The entire discs were separated from the vertebrae, digested using TPER buffer (Pierce, IL), and quantified using enzyme linked immune-sorbent assay (ELISA) for rat IL-1, IL-4, and TNF-a (R&D Systems, MN) (n=5 per group per time point).

Digested samples used for ELISA were also assayed with a dimethylmethylene blue (DMMB) assay to quantify GAG content (n=5 per group per time point). Discs were homogenized and digested with solution. A standard curve was made with chondroitin sulfate isolated from bovine trachea (Sigma, MO). Absorption was measured at 525nm using a spectrophotometer.

In addition, some discs were allocated for real time quantitative PCR to identify the presence of human MSC and bovine NPC in the rat disc (n=5 per group per time point). The entire discs were separated from the vertebrae and RNA was extracted using a QIAshredder Fibrous Tissue mini kit (Qiagen, Germany). The RNA was reverse transcribed using iScript (Bio-Rad Laboratories, CA). The samples were analyzed using species specific primers (Figure 5.4) for human and bovine housekeeping genes and quantified using iQ Sybr Green Supermix (Bio-Rad Laboratories, CA).

Gene	Forward	Reverse
Human GAPDH	5'-gga ggt gaa ggt cgg agt-3'	5'-gaa gat ggt gat ggg att tc-3'
Bovine B2M	5'-tgg gtt cca tcc acc cca ga-3'	5'-tca gcg tgg gac agc agg ta-3'

Figure 5.4: Species specific primers used for PCR.

5.3 Results

5.3.1 Disc Height

At the two week time point, the NPC group had significantly higher discs than all other groups except the fibrin group ($p<0.05$). At the five week time point, the BCP group had the highest discs which was significantly higher than all groups except those of the NPC and MSC group ($p<0.05$) (Figure 5.5).

Group	2week	Std Dev	5week	Std Dev
Stab	40.08	7.68389369	31.43	4.4736289
Fibrin	47.62	10.8918015	22.84	6.00292429
MSC	45.84	10.5472477	45.64	7.70746391
NPC	58.00	2.9580593	47.74	8.31595677
Mix	45.22	2.81016607	38.72	3.78106442
BCP	46.30	4.76589971	49.94	5.37587512

Figure 5.5: Disc height measurements in mm arranged by group and time point. At the 2 week time point, NPC group is significantly higher than all groups except Fibrin ($p<0.05$). At the 5 week time point, BCP is significantly higher than all groups except NPC and MSC ($p<0.05$).

The change in disc height from the 2 week time point to the 5 week time point was decreasing for all groups except the BCP group which say an increase in disc height of 3.64 mm (Figure 5.6).

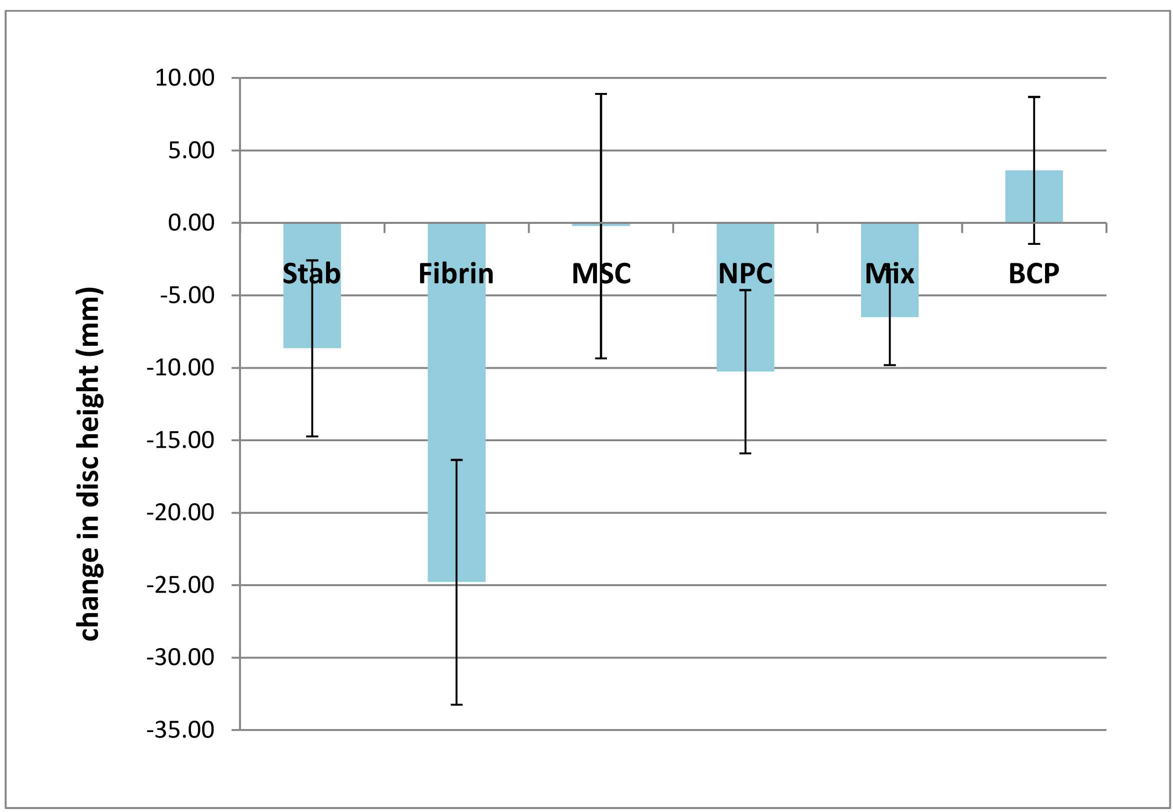

Figure 5.6: Change in disc height from the 2 week time point to the 5 week time point in mm. The BCP group is the only group that is seeing an increase in disc height.

5.3.2 Disc Grade

At the two week time point, there were no significant differences in disc grade between the groups. However, there was a trend of the NPC, Mix, and BCP groups having better disc quality than the MSC, Fibrin, and Stab groups (Figure 5.7). At the five week time point, the BCP group was significantly better than all of the other groups with a score of 2.14 ($p<0.05$). In addition, the BCP group was the only group to improve its disc grade between the 2 and 5 weeks time points.

	2wk	Std Dev	5wk	Std Dev	Δ 2-5 weeks
Stab	3.857143	1.616244	5	0	-1.142857
Fibrin	3.238095	0.808122	4.904762	0.164957	-1.666667
MSC	3.666667	0.978122	3.714286	0.202031	-0.047619
NPC	2.714286	0.714286	3.619048	0.412393	-0.904762
Mix	2.571429	0.877543	3.904762	0.704698	-1.333333
BCP	2.857143	0.404061	2.142857	0.285714	0.714286

Figure 5. 7: Disc grade was determined with blinded images using our grading scheme as described in

5.3.3 Histology

Untouched normal discs from other levels were isolated and processed for histology. These discs were used as the basis of comparison (Figure 5.8).

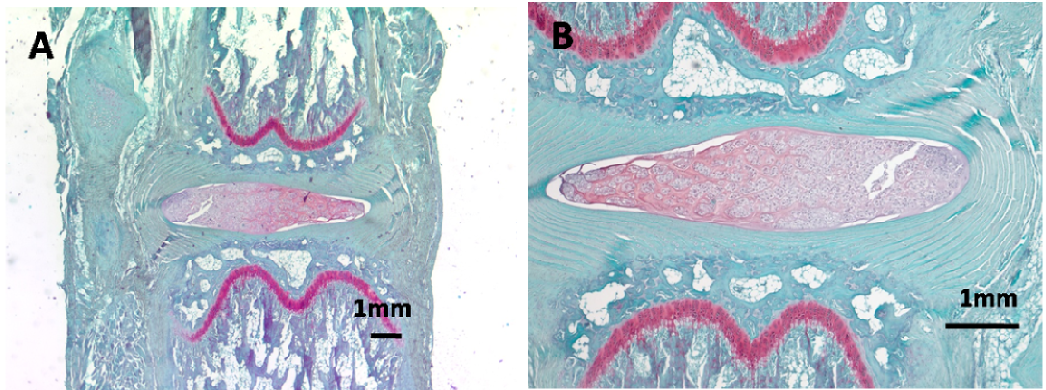

Figure 5.8: Normal disc where the endplate and growth plates are intact. The nucleus pulposus is cellular and stains brightly red due to its high proteoglycan content. The annulus forms uninterrupted concentric lamellae.

As early as the 2wk time point, the Stab group experiences a collapse in disc height and disruption to the endplate and growthplate (Figure 5.9A). In all other treatment groups, the disc height is well preserved though there is no apparent proteoglycan content or cellularity in the nucleus space. In the BCP treated group, a pellet is apparent in the nucleus space and a small amount of proteoglycan has begun to deposit (Figure 5.9F).

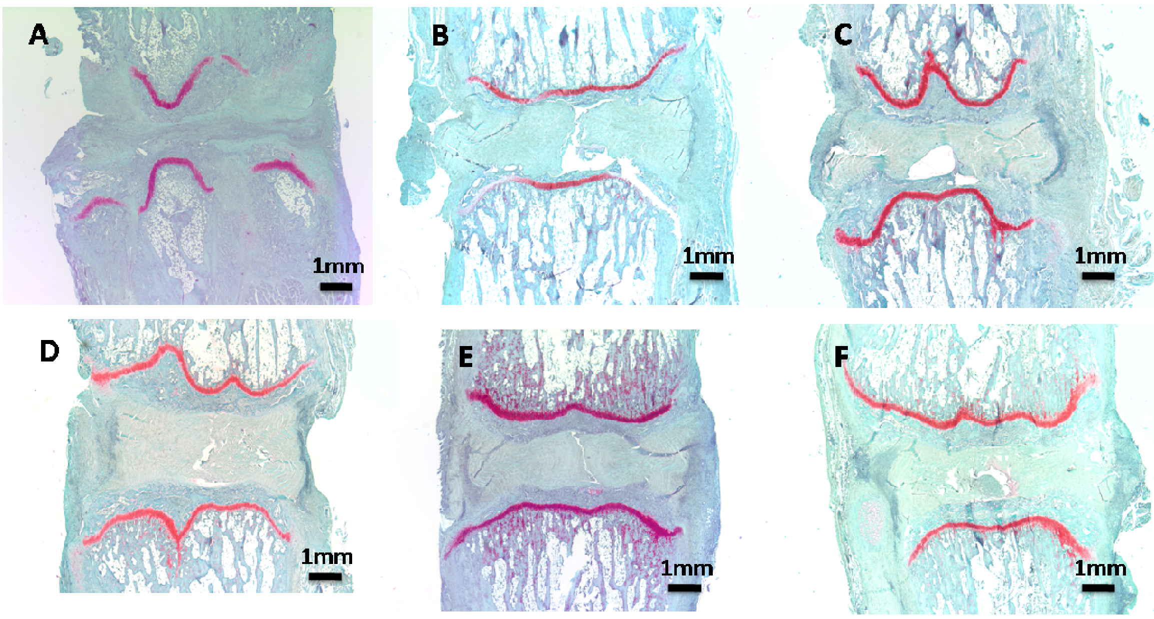

Figure 5.9: Histologic images with Safranin-O staining for the 2 wk time point. A)Stab, B) Fibrin, C) MSC, D) NPC, E) MSC & NPC, F) BCP.

At the 5 week time point, we see severe amounts of disruption to the endplate, growthplate, disc height is compromised, annulus disruption and significant loss of cellularity in all the groups except the BCP (Figure 5.10 A-E). In the BCP treated disc, disc health has been preserved (Figure 5.10F). The disc height is preserved as well as annulus integrity, endplate and growth plates. It is also the only treatment group to have any proteoglycan staining in the disc space.

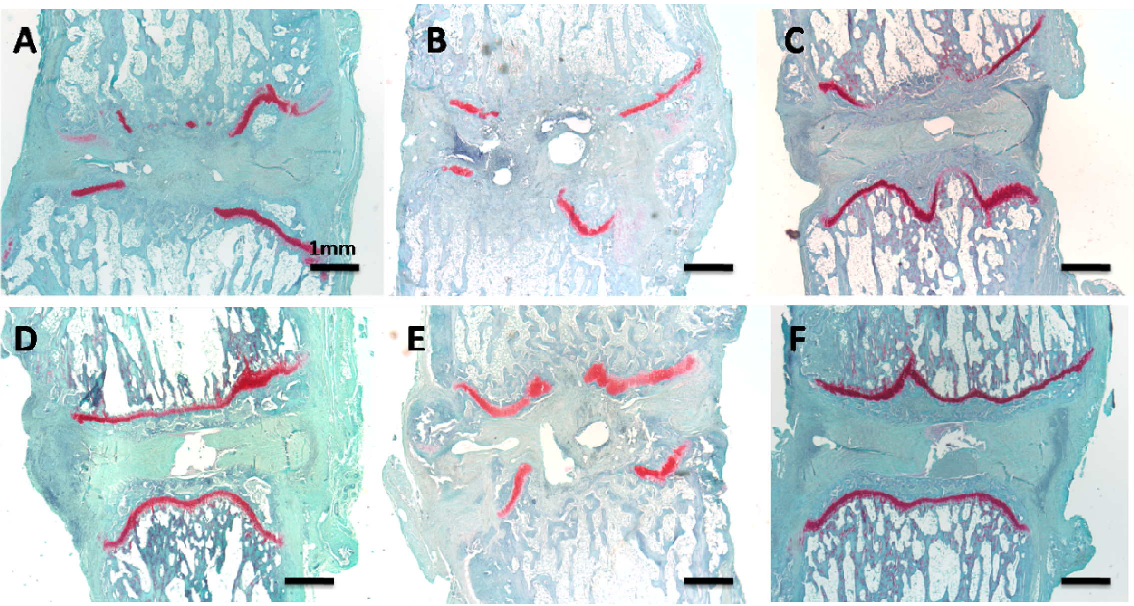

Figure 5.10: Histologic images with Safranin-O staining for the 5 wk time point. A)Stab, B) Fibrin, C) MSC, D) NPC, E) MSC & NPC, F) BCP.

We traced the cell lineage using immunohistochemistry techniques and were able to ascertain that the cell pellet is in fact composed in part of human cells even 5wks after implantation. The human cells were identified using a human specific antibody (LAMP 1&2) (Figure 5.11E). Furthermore, the new proteoglycan being deposited in the nucleus space (Figure 5.11D) is in fact being produced in the area populated by the human cells within the BCP (Figure 5.11E). Finally, in some instances, the BCP began integrating with the neighboring tissue. Figure 5.11F shows a 2wk BCP treated disc where the original pellet has maintained its centralized location but also begun to integrate with the surrounding tissue as evidenced by the location of human cells inside the disc.

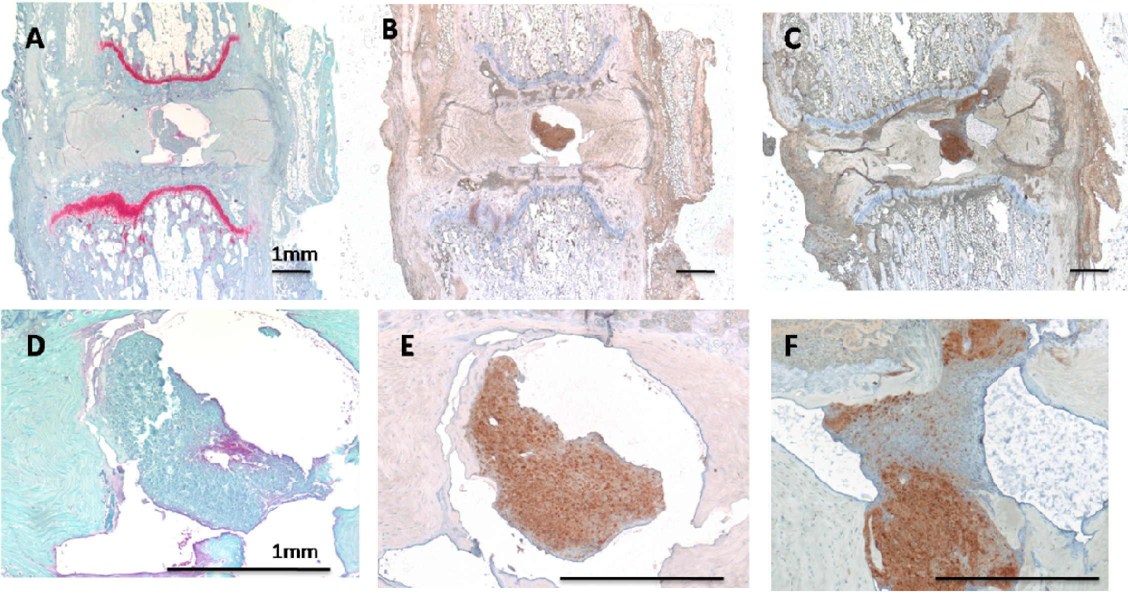

Figure 5.11: Cell lineage tracing in the rat disc. A) 5wk BCP treated disc with Safranin-O staining. B) This is an adjacent section to image A but stained using immunohistochemistry for a human specific antibody (LAMP1&2) in brown. The brown stained cells represent cells that are from the BCP. Images D & E are magnified images of the nucleus space respectively of A & B. It is apparent in image D that the BCP has positive staining for proteoglycan. C) 2wk BCP treated disc stained with LAMP1&2 showing human cells in brown. F) Magnified version of C demonstrating that the BCP has already begun integrating the host tissue and occupying the disc space.

5.3.4 DMMB

The DMMB results showed no significant difference among the groups or time points.

5.3.5 ELISA

The ELISAs for both IL-4 and TNF-a did not register readings for any of the samples. The ELISA reading for IL-1b did not show statistical differences among the

groups, however, they did show a downward trend between 2wk and 5wks (Figure 5.12). These results indicate no significant immunologic responses to the xenogenic transplants.

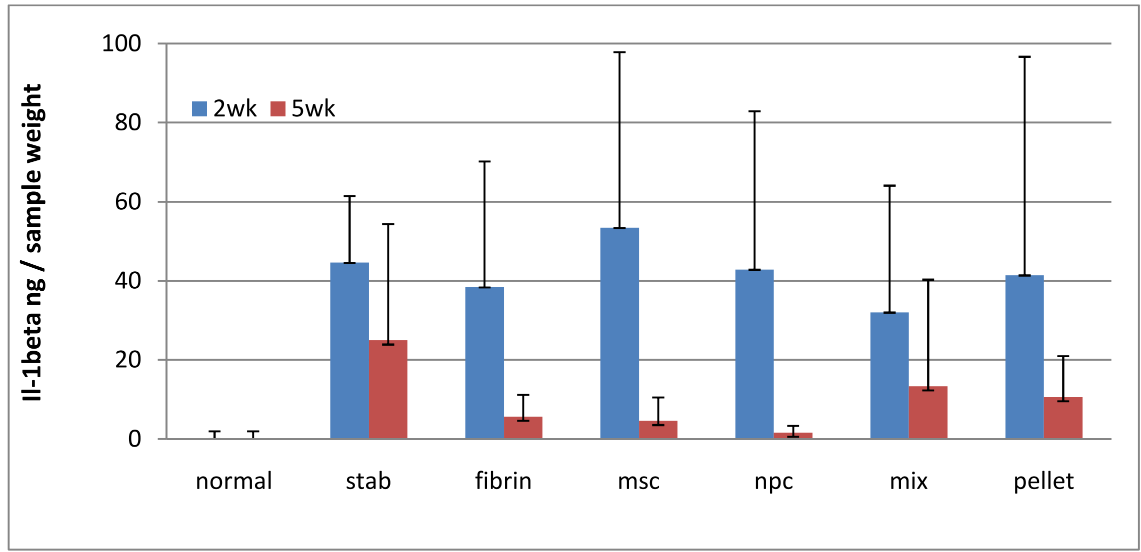

Figure 5.12: Il-1beta ELISA. The data did not have any statistically significant differences among the groups. However, there was a downward trend between the 2nd and 5th week. This is an indication that there is no significant immunologic response to the xenograph.

5.3.6 PCR

At the two week time point, the MSC had 50-70% lower retention rate than the Mix and BCP groups, which was statistically significant ($p<0.05$) (Figure 5.13). At five weeks, MSC had a trend of 20-40% lower retention rate than the Mix and BCP group. The NPC group had similar retention rates as the Mix and BCP groups for both time points.

	% retention of MSC		% retention of NPC	
	2 week	5 week	2 week	5 week
MSC	30	40	NA	NA
NPC	NA	NA	60	60
Mix	100	80	60	60
BCP	80	60	80	60

Figure 5.13: The retention of injected cells was measured using species specific primers with real time quantitative PCR. At two weeks , MSC had significantly lower retention rates than both the Mix and BCP groups ($p<0.05$).

5.4 Discussion

The goal of this study was to test the benefits of a BCP cell-based treatment in vivo in both the acute and chronic time points. We used several criteria to evaluate the treatment's performance including quantitative measures of disc height and quality, qualitative histologic evaluation, and measurements of cytokines, proteoglycan, and the retention of implanted cells. Our results suggest that the BCP offer advantages over single cell type approaches and cell-suspension approaches.

The disc height was well maintained for all groups except the Stab group at the 2 week time point. There was some variability in disc height within the group which may be due to surgical variability in annular suture tightness and placement. For all groups other than the Stab group, the disc height at 2 weeks appears to have been well preserved without any apparent presence of cells or matrix in the nucleus space, with the exception of the BCP group that did show both cell types and some new matrix deposition in the nucleus space. In fact, the Fibrin only group was statistically the same as the group with

the highest discs at 2weeks. Therefore, we believe that disc height preservation at the 2 week time point is primarily due to the presence of fibrin and in a smaller part to annular sutures.

By the five-week time point the fibrin carrier degraded leading to complete disc collapse in the Fibrin group. In addition, the annular sutures are no longer playing a significant role in disc preservation at this stage as evidenced by the further consistent collapse of the Stab group. The Mix group had the third lowest disc height. There was no significant difference amongst the top three groups of MSC, NPC, and BCP. However, only the BCP group had a mean increase in disc height while all other groups had a mean decrease. This suggests a synthetic activity by BCP group.

At the two-week time point, disc grade was not significantly different for any of the treatment groups. However, the BCP, Mix, and NPC groups exhibited a trend of being better than the MSC, Fibrin, and Stab groups. We attribute this to the presence of a greater number of annular disruptions and low cellularity. At the five-week time point, BCP had significantly improved disc quality than all other groups, and was the only group to improve from the two-week time point. The positive results of the BCP group are primarily due to the fact that it was the only group to show evidence of new matrix deposition and high cellularity.

The ELISA assays for IL-4 and TNF-alpha did not register any readings for any of the groups. This is likely due to the dissection method used while harvesting where the entire disc was collected but the ends of the vertebral bodies were not. It is possible that the immune reaction associated with those cytokines was localized in the vertebrae

and endplate and not in the disc. The ELISA for IL-1beta did detect varying levels of cytokine at the two week time point which dropped overall in at the five week time point. This is an indication that the immune response was associated with the surgical procedure and not with any of the treatment groups. Il-1beta levels dropped in at the five week time point, which further indicates that the cytokines are associated with the procedure. There was no difference between the Stab and Fibrin groups and the groups implanted with xenogenic cells indicating that there was immune response specific to the cell transplant that were mitigating our results.

The DMMB measurements were not able to discern any significant differences among the groups. We believe that this is due to the fact that the treatments were not given sufficient time to produce new proteins above the noise levels.

At the two week time point, MSC were significantly less well retained than the BCP and Mix group. This is likely due to the environmental factors which are particularly difficult for the MSC to sustain. Interestingly, when the MSC were combined with NPC in the Mix group, they were very well retained. This is probably because MSC in the Mix group were differentiating due to signaling interactions with the NPC. The more differentiated MSC in the Mix were therefore able to survive in the harsh environment while the MSC implanted by themselves were not. The cells in the other groups were retained at similar levels.

Our results are limited by several aspects of the chosen animal model. Annular incision and nucleotomy trigger rapid degeneration that isn't physiologically equivalent to human disc degeneration that occurs slowly over a lifetime. In addition, tail discs do

not have the same load bearing profile as those of the human lumbar spine. Most importantly, the ischemic stress in rat discs isn't comparable to that in much larger human discs. Future studies of BCPs in larger animal models that more closely mimic the human situation are warranted.

Despite these limitations, our data suggest improved BCP performance in vivo that parallel beneficial behaviors previously reported for in vitro models. Resilience of BCP to pressure, ischemia, and inflammation provide a functional advantage that appears to translate into clinically-relevant benefits.

CH6. Conclusions and future directions

6.1 Research Summary

The goal of this research was to create a new 3D co-culture system that sought to regenerate the intervertebral disc by mimicking the embryonic processes of condensation and induction. We measured the system's performance based on histology, protein synthesis, gene expression, and in situ hybridization. The work completed in this thesis has accomplished that goal while generating further ideas for future research.

6.1.1 The BCP and satellite pellets

The crucial first step was the creation of Bilaminar Cell Pellets (BCPs) and assessing whether their configuration and whether co-culture itself had any advantages over controls. We were able to establish that BCPs did show advantages over controls by increasing the amount of cell proliferation and the amount of GAG produced. Importantly, we developed a technique for mass producing BCPs using simple centrifugation steps in a reproducible manner. We also developed techniques for culturing BCPs using low attachment 24-well plates which enabled us to observe them under a microscope throughout their culture time.

At this stage, we became interested in determining which cell type within the BCP was responsible for these increases. In order to explore this question, we devised several methods of evaluation which relied on using the fact that the mesenchymal stem cells (MSCs) within the BCP were human cells and that the nucleus pulposus cells (NPCs) within the BCP were bovine cells. The first was to develop an immunohistochemistry

protocol for a human-specific antibody which would enable us to trace the MSC within the BCP. These images served as the basis for determining the location of the cell types in adjacent sections that were stained with other techniques. We were then able to stain adjacent sections with an antibody for aggrecan in order to determine the location of the protein within the BCP. We were also able to stain adjacent sections using in situ hybridization techniques in order to localize RNA expression of aggrecan and collagen II. We found that MSC were progressively differentiating throughout the culture time. At the one week time point, the MSC were not producing protein or expressing aggrecan and collagen II. However, at the three week time point, the MSC had differentiated and began producing and expressing these proteins.

In addition, we also designed quantitative real time PCR primers which were species specific. These data enables us to quantify the relative gene expression of both the MSC and NPC. These results further confirmed that MSC were differentiating due to the BCP configuration by upregulating several key genes such as sox9, aggrecan, and collagen II while downregulating collagen X and MMP13.

As our experiments progressed, we observed that the BCP exhibited budding behavior and began secreting numerous satellite pellets. Using both a cell labeling technique as well as the human specific antibody we were able to determine that the structure of the satellite pellets was similar of that found in the BCP. That is to say that the MSC were grouped at the center of the pellet and that the NPC formed a layer on their periphery. Most interestingly, the BCP-like configuration of the satellite pellets was occurring even when the satellite pellets were budding off of randomized co-culture pellets that did not have any structural organization amongst the cell types. These

findings imply that there is an underlying advantage to the BCP structural organization and that the cells are self-organizing due to its inherent benefits.

6.1.2 BCP culture in different environments

In order to further assess the relevance of the BCP for clinical use, we began investigating the effects of environmental factor on their performance. In particular, we were most interested in the effects of hypoxia, pressure, and inflammation which are all important in the degenerative disc. A bioreactor system was therefore designed to simulate the pressure and hypoxic levels in a human degenerative disc. In addition, the inflammatory cytokines, TNF-a and IL-1b, were used to supplement the culture media at concentration that are physiologically relevant. We were ultimately able to evaluate the pellets in three different environments: in the hypoxic and pressurized environment of the bioreactor (bioreactor), in the inflammatory environment in the presence of cytokines (inflammation), and in a simulated degenerative disc environment which combined all of these components (degenerative disc). We used a dimethylmethylene blue (DMMB) assay to quantify proteoglycan content.

The results indicated the NPC performed the best in the bioreactor which is due to the fact that these cells are the best adapted to this environment. However, in the inflammatory environment, the MSC and BCP groups outperformed the NPC which were severely hindered by the cytokines. Finally, in the degenerative disc environment, the BCP had a superior performance to both MSC and NPC controls. These data were used

as a predictor of BCP performance in vivo. Given these promising results, we decided to evaluate the BCP in an animal model.

6.1.3 In vivo evaluation of BCP

A rat tail model was used to evaluate whether the BCP could prevent disc degeneration following annular stab and nucleotomy. Multiple controls were used and fibrin sealant was selected as the carrier. Animals were sacrificed after 2 weeks in order to evaluate the treatment's acute performance and after 5 weeks to evaluate the longer term effects. Outcome measures for disc height and quality, inflammation, protein content, and cell content were all evaluated.

At the two week time point, the disc height was artificially maintained due to the fibrin and the annular sutures. The BCP treated group was the only one to have significant cell content and new proteoglycan deposition in the disc space. At the five week time point, the fibrin had degraded and the sutures were no longer maintaining disc height. Once again, the BCP group was the only treatment group able to synthesize any new matrix. In addition, the BCP group was the only group to show an increase in disc height and quality between the 2 and 5 week time points. These data demonstrated not only that the BCP was able to survive the harsh degenerative disc environment but also that it was able to begin mounting a therapeutic response.

6.2 Future directions

6.2.1 Further evaluation of BCP for disc degeneration

Further testing should be done in order to assess the BCP potential performance in a clinical application. In particular, in vitro models that contain further degrees of complexity should be used to simulate the human degenerative disc environment. These models should include simulating the acidic environment of the disc by incorporating a lower pH since pH is known to have an effect on cell behavior. In addition, a clinical application in humans would not involve a nucleotomy and the BCP would therefore be exposed to signaling from native degenerative NPC as well as annulus fibrosus cells. Incorporating these cells, which could be isolated from surgical patients, into an in vitro model would give further insight the BCP performance in a clinical setting.

Larger animal models will also be important in further evaluating BCP. The rat tail model fails to simulate the severity of the nutritional challenges that are present in a human degenerative disc. The primary reason is that rat disc are significantly smaller and therefore the diffusion distances are shorter. Using a pig, whose discs are similar in size to humans, will be indicative of the BCP ability to withstand the nutritional challenges of human discs. Furthermore, in the rat tail model, a nucleotomy was necessary in order to make sufficient space for the treatment. However, the procedure in a human disc would be minimally invasive and would not involve a nucleotomy. Again, using a larger animal model would eliminate the need for such an aggressive method and therefore be more indicative of procedure that would be done on human patients.

In addition, pairing the BCP with the appropriate scaffold and/or hydrogel will be an important step in preparing the therapeutic for a clinical application. The advantage of using scaffolds and/or hydrogels is that they are able to restore the mechanics of the tissue in the acute post-surgical period. In the case of the disc, they will be able to withstand significant loading and restore disc height if appropriately designed. Combining the BCP with a scaffold and/or hydrogel will further enhance the efficacy of this technology in the acute phase.

Finally, further work in finding a substitute for NPC within the BCP should be done. In particular, the use of pre-differentiated MSC as substitute for NPC would enable the entire construct to be autologous without incurring any donor site morbidity. In addition, since MSC are thought to be immune-privileged, creating a construct that is composed entirely of MSC (some that are naïve and some that are pre-differentiated) would mean that this technology could be made available off the shelf and would not require any donor cells from the patients. This would likely reduce the costs and wait time associated with the procedure and improve its adoption rate in the clinic.

6.2.2 Other applications for BCP

Further studies should be conducted in order to determine if the synergies observed in the BCP are a generalizable phenomenon that would occur with various mature cell types in order to regenerate other tissues such as bone, skin, and muscle amongst others. As first alternative application, we investigated the possibility of using BCP to treat focal defects in articular cartilage.

Preliminary studies were conducted by replacing the NPC outer layer of the BCP with juvenile articular chondrocytes (CH) provided by ISTO technologies, Inc. The CH cells are a rare and expensive resources, therefore using only 25% CH in a BCP represents a strategic and feasibility advantage. The pellets were culture for three weeks and evaluated for gene expression with quantitative real time PCR (Figure 6. 1).

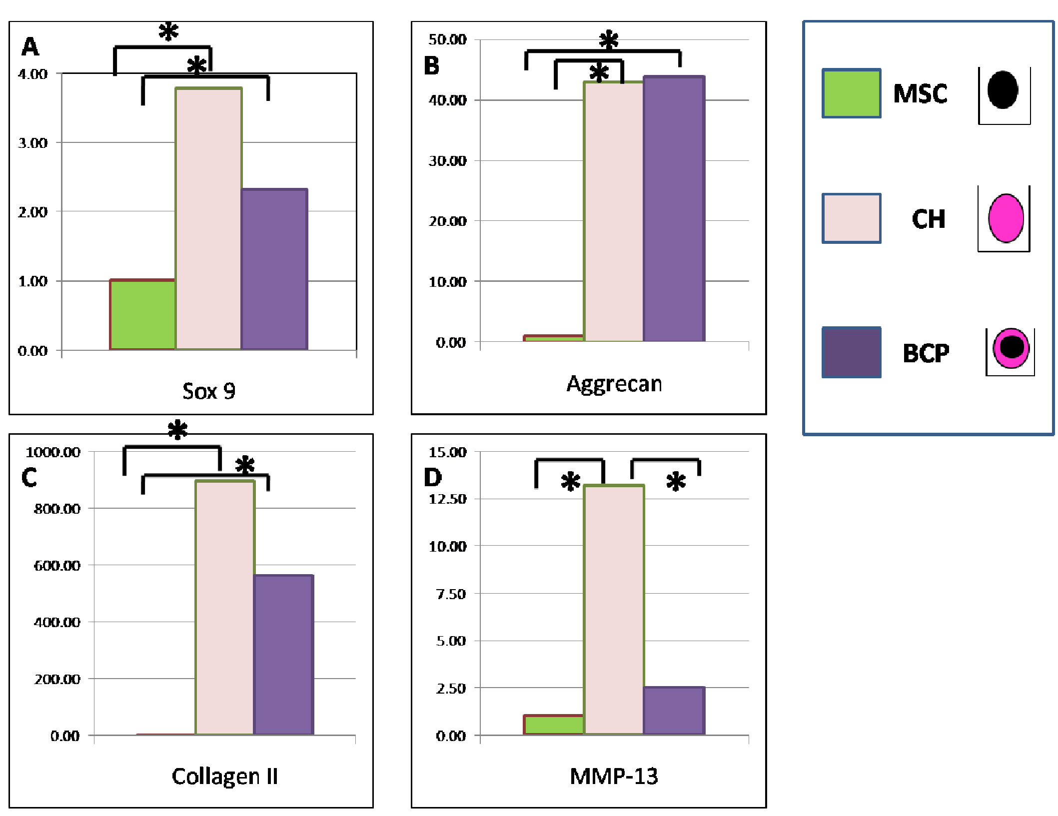

Figure 6. 1: Gene expression data for Sox9 (A), Aggrecan (B), Collagen II (C), and MMP13 (D) normalized to the housekeeping gene beta-2-microglobulin (n=5). The only significant difference between the BCP and CH group is the higher MMP13 expression of the CH group. MSC had significantly lower expression for all genes except MMP13 were it was the same as BCP.

Other three week pellets were analyzed for proteoglycan content using DMMB normalized to DNA content by Pico green (Figure 6.2).

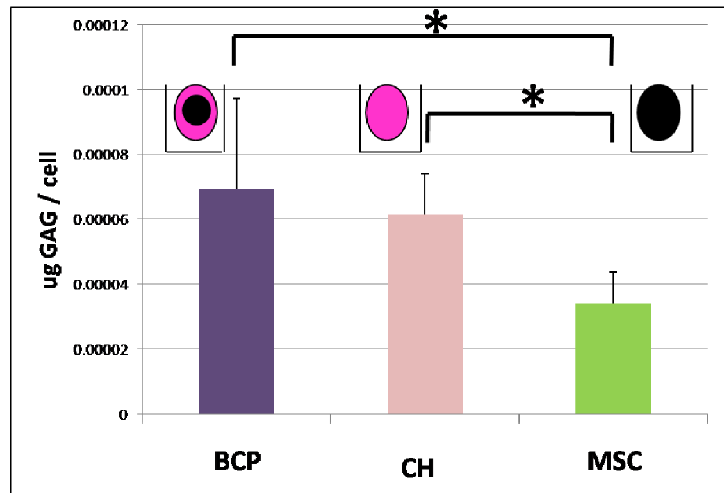

Figure 6.2: Results for ug GAG/cell after three weeks of culture (n=10). The BCP demonstrates a trend of being higher than all groups. However, the BCP and CH groups are statistically the same and both are higher than the MSC group.

The results indicate that the BCP is performing at levels equal to those of the CH group in both gene expression and protein synthesis. The CH group did produce significantly more MMP13 than the BCP group but that was the only significant difference between the two. The MSC performed significantly lower in gene expression and protein synthesis.

When evaluating these results it is important to note that the CH cells are juvenile cells which are very synthetically active and very difficult to procure in large numbers. In addition, the BCP is composed of 75% MSC and only 25% CH. Therefore, the similar levels of gene expression and protein synthesis produced by the BCP while using only 25% of the CH cells can be considered a promising result. This preliminary study is an

initial indication that the BCP concept may be a generalizable phenomenon that should be further investigated for use in an articular cartilage application as well as for the possible treatment of other tissues.

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Applendix

COMPOSITIONS AND METHODS FOR GENERATING MUSCULOSKELETAL TISSUE

CROSS-REFERENCE

[0001] This application claims the benefit of U.S. Provisional Patent Application No. 61/055,834, filed May 23, 2008, which application is incorporated herein by reference in its entirety.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

[0002] This invention was made with government support under AR049786 awarded by the National Institutes of Health. The government has certain rights in the invention.

BACKGROUND

[0003] The musculoskeletal system includes a variety of dense and soft connective tissues, including cartilage, bone, tendon, ligament, spinal intervertebral discs, and muscle. Musculoskeletal tissues differentiate embryonically from mesenchymal precursor cells.

[0004] An example of musculoskeletal tissue is cartilage. Three types of cartilage exist in mammals: hyaline cartilage, fibrocartilage, and elastic cartilage. Hyaline cartilage includes the articular cartilage of the joints, the cartilages of the trachea, bronchi, and larynx, and the nasal cartilages. Fibrocartilage is found in the intervertebral disc, tendinous and ligamentous insertions, menisci, the symphysis pubus, and insertions of joint capsules. Elastic cartilage is found in the pinna of the ears, in the epiglottis, and in the arytenoid cartilages of the larynx.

[0005] Musculoskeletal tissues play a variety of functional roles. For example, the spinal intervertebral discs serve as the shock absorbers of the axial body and also allow for considerable flexibility and motion. Each disc is composed of a peripheral, ligament-like annulus fibrosus and a central nucleus pulposus (NP). The NP contains chondrocyte-like cells embedded in a matrix of proteoglycan and type II collagen that is highly hydrophilic and allows the tissue to swell to resist compressive forces. The NP environment is one of high pressure, low pH, and low oxygen tension.

[0006] Musculoskeletal diseases are a major health concern. As an example, disc degeneration is a common feature among the aging population and is the underlying cause of various spinal disorders and disabilities. Disc degeneration is also believed to be the predominant cause of low back pain, which is the second most frequent reason for patients to visit their physicians and affects approximately 80% of the population at some point in their lives. When patients fail to respond to conservative care, a spinal fusion is typically performed even though it leads to immobility of the treated segments and predisposes adjacent discs to accelerated degeneration. A significant number of these patients do not benefit from fusion and require further treatments.

[0007] Another adverse musculoskeletal condition is arthritis, which affects millions of people in the United States alone. Although metallic joint replacements offer relief for some individuals with advanced disease, these devices have limited durability and are not suitable for young or highly active patients. Focal cartilage defects represent an early manifestation of arthritis. Sports injuries and trauma-induced focal cartilage defects are typically treated with microfracture (a procedure where small holes are drilled thru cartilage and into the underlying bone to stimulate healing), which has short-term benefits but also fails after 5-10 years. Despite its limitations, microfracture remains the gold standard for treatment. Recently, therapies that utilize autologous chondrocytes have been pursued; however, the use of a patient's own articular cartilage cells is critically limited by insufficient cell availability, donor site morbidity, cell heterogeneity, and inconsistent regenerative capacity.

[0008] There is a need in the art for compositions and methods of producing various musculoskeletal tissues.

Literature

[0009] Richardson et al. (2006) *Stem Cells* 24:707; Yamamoto et al. (2004) *Spine* 29:1508; Le Visage et al. (2006) *Spine* 31:2036; Vadalá et al. (2008) *Spine* 33:870; WO 2003/068149; U.S. Patent Nos. 6,355,239, 5,908,784, 5,486,359, 6,835,377.

SUMMARY OF THE INVENTION

[0010] The present disclosure provides compositions comprising musculoskeletal cells and mesenchymal stem cells in discrete regions. The present disclosure provides systems comprising a subject composition; and methods of using a subject composition to generate cartilage, bone, tendon, muscle, intervertebral disc, or other musculoskeletal tissues.

BRIEF DESCRIPTION OF THE DRAWINGS

[0011] Figure 1 is a schematic depiction of the experimental cell groups.

[0012] Figures 2A-D depict formation of satellite (“progeny”) cell compositions after various timesin *in vitro* culture.

[0013] Figures 3A and 3B depict frozen sections of bilaminar pellets after 3 weeks in *in vitro* culture.

[0014] Figure 4 depicts a three-week bilaminar pellet (cell composition) with mesenchymal stem cells (MSC) on the inside (inner layer) and nucleus pulposus cells (NPC) on the outside (outer layer) after undergoing budding and satellite pellet formation.

[0015] Figures 5A and 5B depict fluorescent microscopy image (Figure 5A) and immunohistochemistry staining (Figure 5B) of a satellite pellet.

[0016] Figure 6 is a graph depicting the number of cells per pellet after time in *in vitro* culture for cell compositions of various ratios of MSC to NPC, and various configurations.

[0017] Figure 7 is a graph depicting the amount of glycosaminoglycan (GAG) produced per cell pellet at 2 weeks in *in vitro* culture.

[0018] Figures 8A and 8B are graphs depicting the amount of GAG produced per cell pellet at 3 weeks in *in vitro* culture.

[0019] Figure 9 depicts the amount of GAG produced per cell pellet in serum-free culture medium, or in culture medium containing 10% fetal bovine serum.

[0020] Figures 10A and 10B depict histological analysis of a rat disc 2 weeks after introduction of a bilayer cell composition.

DEFINITIONS

[0021] The term "chondrocyte" refers to a cartilage-specific cell that gives rise to normal cartilage tissue growth *in vivo*; chondrocytes synthesize and deposit the supportive matrix (composed principally of collagen and proteoglycan) of cartilage.

[0022] As used herein, the term “mesenchymal stem cell” (“MSC”), both in singular and plural forms, refers to a stem cell that is capable of differentiating into more than one specific type of mesenchymal tissue cell. MSC can differentiate into the various mesenchymal lineages, for example, chondrocytes, myocytes, osteocytes, and tenocytes, as well as precursors of these cells, e.g., chondroblasts, myoblasts, and osteoblasts. MSC may express one or more cell surface markers, such as bone morphogenetic protein receptor

(BMPR), STRO-1, CD105, CD166, CD29, and CD44. MSC are generally CD34 negative ($CD34^-$). MSC can be positive for CD105, CD166, CD29, and CD44; and negative for CD14, CD34 and CD45.

[0023] The term “induced pluripotent stem cell” (or “iPS cell”), as used herein, refers to a pluripotent stem cell induced from a somatic cell, e.g., a differentiated somatic cell. iPS cells are capable of self-renewal and differentiation into cell fate-committed stem cells, including mesencymal stem cells, as well as various types of mature cells such as chondrocytes, osteocytes, etc.

[0024] The term "cross-linked" as used herein refers to a composition containing intermolecular cross-links and/or intramolecular cross-links arising from the formation of covalent bonds, ionic bonds, hydrogen bonding, or any combination thereof. "Cross-linkable" refers to a component or compound that is capable of undergoing reaction to form a cross-linked composition.

[0025] The terms "biocompatible polymer", "biocompatible cross-linked polymer matrix" and "biocompatibility" when used in relation to polymers are art-recognized terms. For example, biocompatible polymers include polymers that are neither themselves toxic to the host (e.g., a non-human animal or a human), nor degrade (if the polymer degrades) at a rate that produces monomeric or oligomeric subunits or other byproducts at toxic concentrations in the host. In certain embodiments, biodegradation generally involves degradation of the polymer in an organism, e.g., into its monomeric subunits, which may be known to be effectively non-toxic. Intermediate oligomeric products resulting from such degradation may have different toxicological properties, however, or biodegradation may involve oxidation or other biochemical reactions that generate molecules other than monomeric subunits of the polymer.

[0026] A “biocompatible” carrier or other component is a carrier or other component that does not substantially induce an undesirable response in an individual, e.g., the carrier or other component does not substantially induce an immune response in the host, does not substantially induce an inflammatory response in the host, etc.

[0027] "Injectable" as used herein means capable of being administered, delivered, or carried into the body via syringes, catheters, needles, and other means for injecting or infusing a composition in a liquid medium.

[0028] The terms “subject,” “individual,” “host,” and “patient” are used interchangeably herein to refer to a member or members of any mammalian species. Individuals thus include, without limitation, humans, non-human primates, canines, felines, ungulates (e.g., equine

(e.g., horses), bovine (e.g., cows), swine (e.g., pig), camels, etc.), rodents (e.g., rats, mice), and other mammalian subjects. Non-human animal models, particularly mammals, e.g. a non-human primate, a murine (e.g., a mouse, a rat), lagomorpha, etc. may be used for experimental investigations.

[0029] “Treating” or “treatment” of a condition, disorder, or disease includes: (1) preventing at least one symptom of the condition, disorder, or disease, e.g., causing a clinical symptom to not significantly develop in a mammal that may be exposed to or predisposed to a disease but does not yet experience or display symptoms of the disease, (2) inhibiting the disease, i.e., arresting or reducing the development of the disease or its symptoms, or (3) relieving the disease, i.e., causing regression of the disease or its clinical symptoms.

[0030] A "therapeutically effective amount" or "efficacious amount" means the amount of a compound that, when administered to a mammalian subject for the treatment of a condition, disorder, or disease, is sufficient, in combination with another agent, or alone in one or more doses, to effect such treatment for the condition, disorder, or disease. The "therapeutically effective amount" will vary depending on the compound, the condition, disorder, or disease and its severity and the age, weight, etc., of the subject to be treated.

[0031] Before the present invention is further described, it is to be understood that this invention is not limited to particular embodiments described, as such may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting, since the scope of the present invention will be limited only by the appended claims.

[0032] Where a range of values is provided, it is understood that each intervening value, to the tenth of the unit of the lower limit unless the context clearly dictates otherwise, between the upper and lower limit of that range and any other stated or intervening value in that stated range, is encompassed within the invention. The upper and lower limits of these smaller ranges may independently be included in the smaller ranges, and are also encompassed within the invention, subject to any specifically excluded limit in the stated range. Where the stated range includes one or both of the limits, ranges excluding either or both of those included limits are also included in the invention.

[0033] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can also be used in the practice or testing of the present invention, the

preferred methods and materials are now described. All publications mentioned herein are incorporated herein by reference to disclose and describe the methods and/or materials in connection with which the publications are cited.

[0034] It must be noted that as used herein and in the appended claims, the singular forms "a," "an," and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a mesenchymal stem cell" includes a plurality of such cells and reference to "the chondrogenic factor" includes reference to one or more chondrogenic factors and equivalents thereof known to those skilled in the art, and so forth. It is further noted that the claims may be drafted to exclude any optional element. As such, this statement is intended to serve as antecedent basis for use of such exclusive terminology as "solely," "only" and the like in connection with the recitation of claim elements, or use of a "negative" limitation.

[0035] The publications discussed herein are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the present invention is not entitled to antedate such publication by virtue of prior invention. Further, the dates of publication provided may be different from the actual publication dates which may need to be independently confirmed.

DETAILED DESCRIPTION

[0036] The present disclosure provides compositions comprising musculoskeletal cells and mesenchymal stem cells in discrete regions. The present disclosure provides systems comprising a subject composition; and methods of using a subject composition to generate cartilage, bone, tendon, muscle, intervertebral disc, or other musculoskeletal tissues.

[0037] The inventors have found that a structured, three-dimensional co-culture system that includes a dense population of immature, naïve cells such as mesenchymal stem cells within a layer of more mature, instructive cells (e.g., chondrocytes) provides a configuration that maximizes surface area to volume and enables important inductive cell-cell interactions that efficiently self-promote differentiation of the immature cells, thereby reducing or eliminating the need for exogenous differentiation factors.

[0038] The present disclosure provides a multi-layer three-dimensional cell composition comprising a less differentiated ("naïve" or "immature") cell and a more differentiated ("instructive" or "mature") cell. The present disclosure provides a multi-layer cell composition comprising a chondroblast, a chondrocyte, an osteoblast, an osteocyte, a

myoblast, a myocyte, a tenocyte, or a nucleus pulposus cell; and a stem cell or progenitor cell, e.g., a mesenchymal stem cell (MSC). The present disclosure further provides compositions comprising a subject multi-layer cell composition; and a biocompatible carrier. Depending on the cells included in the composition, a subject composition is useful for producing a musculoskeletal tissue such as cartilage, intervertebral disc tissue (e.g., nucleus pulposus tissue), muscle, tendon, or bone. Thus, the present disclosure provides cartilage production compositions, intervertebral disc production compositions, muscle production compositions, tendon production compositions, and bone production compositions.

[0039] A number of conditions and disorders can be treated by providing a cell composition that produces cartilage. Conditions and disorders that can be treated by providing a cell composition that produces cartilage are discussed in more detail below, and include disorders ranging from chronic degeneration brought about by disease, overuse, or trauma, to plastic or reconstructive surgery. As such, the present invention provides methods of generating cartilage, including *in vivo* methods of generating cartilage, where the methods generally involve introducing into an individual in need of cartilage replacement and/or cartilage regeneration an effective amount of a subject cell composition.

[0040] Various conditions and disorders can be treated by providing a cell composition that produces bone or bone components. Conditions and disorders that can be treated by providing a bone production composition include bone trauma (e.g., fractures, and the like); and degenerative bone disorders.

MULTI-LAYER CELL COMPOSITIONS

[0041] The present disclosure provides a multi-layer cell composition in which more differentiated cells and less differentiated cells are co-cultured in a multi-layer, three-dimensional configuration that mimics normal development.

[0042] A subject multi-layer three-dimensional cell composition includes: 1) a first layer comprising a first, more differentiated, cell type; and 2) a second layer comprising a second, less differentiated, cell type. The first and second layers form discrete regions in a composition, e.g., the first layer (e.g., first discrete region) includes cells in which the cells are at least 85% of a first, more differentiated, cell type, and the second layer (e.g., second discrete region) includes cells in which the cells are at least 85% of a second, less differentiated, cell type. The layers are not monolayers, e.g., the layers are at least two cells thick, e.g., at least 2-5 cells thick, at least 5-10 cells thick, at least 10-50 cells thick,

or more than 50 cells thick. In other words, a subject multi-layer cell composition has a three-dimensional configuration.

[0043] A subject multi-layer cell composition includes a first layer comprising a first, more differentiated ("instructive"), cell type. Suitable more differentiated cells include, e.g., osteoblasts, osteocytes, chondroblasts, chondrocytes, myoblasts, myocytes, tenocytes, and nucleus pulposus cells (NPC). A subject multi-layer cell composition includes a second layer comprising a second, less differentiated ("naïve"), cell type. Suitable less differentiated cell types include stem cells (e.g., adult stem cells, embryonic stem cells, and induced pluripotent stem (iPS) cells); and mesenchymal stem cells (MSC). In some instances, the layer comprising the less differentiated cell type is surrounded (partially or substantially completely) by the layer comprising the more differentiated cell type. In some instances, the layer comprising the more differentiated cell type is surrounded (partially or substantially completely) by the layer comprising the less differentiated cell type.

[0044] A "more differentiated" cell of a subject multi-cell layer does not include a fibroblast. Thus, e.g., the cells of a layer of a subject multi-layer cell composition generally comprise less than about 15%, less than about 10%, less than about 5%, less than about 2%, or less than about 1% fibroblasts.

[0045] In some instances, a subject cell composition has only two layers, e.g., a first layer comprising a first, more differentiated cell type and a second layer comprising a second, less differentiated cell type. Such a two-layer cell composition (also referred to herein as a "bilaminar cell composition") can include one or more additional components (e.g., a buffer, one or more chondrogenic factors, one or more osteogenic factors, a scaffold component, etc.), as described in detail below. In some instances, the layer comprising the less differentiated cell type is surrounded (partially or substantially completely) by the layer comprising the more differentiated cell type. In some instances, the layer comprising the more differentiated cell type is surrounded (partially or substantially completely) by the layer comprising the less differentiated cell type.

[0046] The more differentiated cells and the less differentiated cells are present in a subject cell composition at a ratio of 1:1 or greater than 1:1. For example, the ratio of more differentiated cells to less differentiated cells in a subject multi-layer cell composition is at least about 1:1, at least about 1.5:1, at least about 2:1, at least about 2.5:1, at least about 3:1, at least about 3.5:1, at least about 4:1, at least about 4.5:1, at least about 5:1, at least about 5.5:1, at least about 6:1, at least about 7:1, at least about 8:1, at least about 9:1, or

at least about 10:1. For example, the ratio of more differentiated cells to less differentiated cells in a subject multi-layer cell composition can range from about 1:1 to about 1.25:1, from about 1.25:1 to about 1.5:1, from about 1.5:1 to about 2:1, from about 2:1 to about 3:1, from about 3:1 to about 4:1, from about 4:1 to about 5:1, from about 5:1 to about 6:1, from about 6:1 to about 7:1, from about 7:1 to about 8:1, from about 8:1 to about 9:1, or from about 9:1 to about 10:1, or greater than 10:1.

[0047] As an example, a subject cell composition can include NPC and mesenchymal stem cells (MSC). NPC and MSC can be present in a subject cell composition at a ratio of 1:1 or greater than 1:1. For example, the ratio of NPC to MSC in a subject composition can be at least about 1:1, at least about 1.5:1, at least about 2:1, at least about 2.5:1, at least about 3:1, at least about 3.5:1, at least about 4:1, at least about 4.5:1, at least about 5:1, at least about 5.5:1, at least about 6:1, at least about 7:1, at least about 8:1, at least about 9:1, or at least about 10:1. For example, the ratio of NPC to MSC in a subject composition can range from about 1:1 to about 1.25:1, from about 1.25:1 to about 1.5:1, from about 1.5:1 to about 2:1, from about 2:1 to about 3:1, from about 3:1 to about 4:1, from about 4:1 to about 5:1, from about 5:1 to about 6:1, from about 6:1 to about 7:1, from about 7:1 to about 8:1, from about 8:1 to about 9:1, or from about 9:1 to about 10:1, or greater than 10:1.

[0048] As another example, a subject cell composition can include osteocytes (or osteoblasts, or a mixture of osteocytes and osteoblasts) and MSC. Osteocytes (or osteoblasts, or a mixture of osteocytes and osteoblasts) and MSC can be present in a subject cell composition at a ratio of 1:1 or greater than 1:1. For example, the ratio of osteocytes (or osteoblasts, or a mixture of osteocytes and osteoblasts) to MSC in a subject composition can be at least about 1:1, at least about 1.5:1, at least about 2:1, at least about 2.5:1, at least about 3:1, at least about 3.5:1, at least about 4:1, at least about 4.5:1, at least about 5:1, at least about 5.5:1, at least about 6:1, at least about 7:1, at least about 8:1, at least about 9:1, or at least about 10:1. For example, the ratio of osteocytes (or osteoblasts, or a mixture of osteocytes and osteoblasts) to MSC in a subject composition can range from about 1:1 to about 1.25:1, from about 1.25:1 to about 1.5:1, from about 1.5:1 to about 2:1, from about 2:1 to about 3:1, from about 3:1 to about 4:1, from about 4:1 to about 5:1, from about 5:1 to about 6:1, from about 6:1 to about 7:1, from about 7:1 to about 8:1, from about 8:1 to about 9:1, or from about 9:1 to about 10:1, or greater than 10:1.

[0049] As an example, a subject cell composition can include chondrocytes (or chondroblasts, or a mixture of chondrocytes and chondroblasts) and MSC. Chondrocytes (or chondroblasts,

or a mixture of chondrocytes and chondroblasts) and MSC can be present in a subject cell composition at a ratio of 1:1 or greater than 1:1. For example, the ratio of chondrocytes (or chondroblasts, or a mixture of chondrocytes and chondroblasts) to MSC in a subject composition can be at least about 1:1, at least about 1.5:1, at least about 2:1, at least about 2.5:1, at least about 3:1, at least about 3.5:1, at least about 4:1, at least about 4.5:1, at least about 5:1, at least about 5.5:1, at least about 6:1, at least about 7:1, at least about 8:1, at least about 9:1, or at least about 10:1. For example, the ratio of chondrocytes (or chondroblasts, or a mixture of chondrocytes and chondroblasts) to MSC in a subject composition can range from about 1:1 to about 1.25:1, from about 1.25:1 to about 1.5:1, from about 1.5:1 to about 2:1, from about 2:1 to about 3:1, from about 3:1 to about 4:1, from about 4:1 to about 5:1, from about 5:1 to about 6:1, from about 6:1 to about 7:1, from about 7:1 to about 8:1, from about 8:1 to about 9:1, or from about 9:1 to about 10:1, or greater than 10:1.

[0050] As an example, a subject cell composition can include myocytes (or myoblasts, or a mixture of myocytes and myoblasts) and MSC. Myocytes (or myoblasts, or a mixture of myocytes and myoblasts) and MSC can be present in a subject cell composition at a ratio of 1:1 or greater than 1:1. For example, the ratio of myocytes (or myoblasts, or a mixture of myocytes and myoblasts) to MSC in a subject composition can be at least about 1:1, at least about 1.5:1, at least about 2:1, at least about 2.5:1, at least about 3:1, at least about 3.5:1, at least about 4:1, at least about 4.5:1, at least about 5:1, at least about 5.5:1, at least about 6:1, at least about 7:1, at least about 8:1, at least about 9:1, or at least about 10:1. For example, the ratio of myocytes (or myoblasts) to MSC in a subject composition can range from about 1:1 to about 1.25:1, from about 1.25:1 to about 1.5:1, from about 1.5:1 to about 2:1, from about 2:1 to about 3:1, from about 3:1 to about 4:1, from about 4:1 to about 5:1, from about 5:1 to about 6:1, from about 6:1 to about 7:1, from about 7:1 to about 8:1, from about 8:1 to about 9:1, or from about 9:1 to about 10:1, or greater than 10:1.

[0051] In some instances, the less differentiated cells and the more differentiated cells are present in a subject cell composition at a ratio of 1:1 or greater than 1:1. For example, the ratio of less differentiated cells to more differentiated cells in a subject multi-layer cell composition is at least about 1:1, at least about 1.5:1, at least about 2:1, at least about 2.5:1, at least about 3:1, at least about 3.5:1, at least about 4:1, at least about 4.5:1, at least about 5:1, at least about 5.5:1, at least about 6:1, at least about 7:1, at least about 8:1, at least about 9:1, or at least about 10:1. For example, the ratio of less differentiated

cells to more differentiated cells in a subject multi-layer cell composition can range from about 1:1 to about 1.25:1, from about 1.25:1 to about 1.5:1, from about 1.5:1 to about 2:1, from about 2:1 to about 3:1, from about 3:1 to about 4:1, from about 4:1 to about 5:1, from about 5:1 to about 6:1, from about 6:1 to about 7:1, from about 7:1 to about 8:1, from about 8:1 to about 9:1, or from about 9:1 to about 10:1, or greater than 10:1.

[0052] As an example, a subject cell composition can include MSC and NPC, where MSC and NPC are present in a subject cell composition at a ratio of 1:1 or greater than 1:1. For example, the ratio of MSC to NPC in a subject composition is at least about 1:1, at least about 1.5:1, at least about 2:1, at least about 2.5:1, at least about 3:1, at least about 3.5:1, at least about 4:1, at least about 4.5:1, at least about 5:1, at least about 5.5:1, at least about 6:1, at least about 7:1, at least about 8:1, at least about 9:1, or at least about 10:1. For example, the ratio of MSC to NPC in a subject composition can range from about 1:1 to about 1.25:1, from about 1.25:1 to about 1.5:1, from about 1.5:1 to about 2:1, from about 2:1 to about 3:1, from about 3:1 to about 4:1, from about 4:1 to about 5:1, from about 5:1 to about 6:1, from about 6:1 to about 7:1, from about 7:1 to about 8:1, from about 8:1 to about 9:1, or from about 9:1 to about 10:1, or greater than 10:1.

[0053] As another example, a subject cell composition can include MSC and osteocytes or osteoblasts, where the MSC and the osteocytes (or osteoblasts, or a mixture of osteocytes and osteoblasts) are present in a subject cell composition at a ratio of 1:1 or greater than 1:1. For example, the ratio of MSC to osteocytes (or osteoblasts, or a mixture of osteocytes and osteoblasts) in a subject composition is at least about 1:1, at least about 1.5:1, at least about 2:1, at least about 2.5:1, at least about 3:1, at least about 3.5:1, at least about 4:1, at least about 4.5:1, at least about 5:1, at least about 5.5:1, at least about 6:1, at least about 7:1, at least about 8:1, at least about 9:1, or at least about 10:1. For example, the ratio of MSC to osteocytes (or osteoblasts, or a mixture of osteocytes and osteoblasts) in a subject composition can range from about 1:1 to about 1.25:1, from about 1.25:1 to about 1.5:1, from about 1.5:1 to about 2:1, from about 2:1 to about 3:1, from about 3:1 to about 4:1, from about 4:1 to about 5:1, from about 5:1 to about 6:1, from about 6:1 to about 7:1, from about 7:1 to about 8:1, from about 8:1 to about 9:1, or from about 9:1 to about 10:1, or greater than 10:1.

[0054] As another example, a subject cell composition can include MSC and chondrocytes (or chondroblasts, or a mixture of chondrocytes and chondroblasts) where the MSC and the chondrocytes (or chondroblasts, or a mixture of chondrocytes and chondroblasts) are present in a subject cell composition at a ratio of 1:1 or greater than 1:1. For example, the

ratio of MSC to chondrocytes (or chondroblasts, or a mixture of chondrocytes and chondroblasts) in a subject composition is at least about 1:1, at least about 1.5:1, at least about 2:1, at least about 2.5:1, at least about 3:1, at least about 3.5:1, at least about 4:1, at least about 4.5:1, at least about 5:1, at least about 5.5:1, at least about 6:1, at least about 7:1, at least about 8:1, at least about 9:1, or at least about 10:1. For example, the ratio of MSC to chondrocytes (or chondroblasts, or a mixture of chondrocytes and chondroblasts) in a subject composition can range from about 1:1 to about 1.25:1, from about 1.25:1 to about 1.5:1, from about 1.5:1 to about 2:1, from about 2:1 to about 3:1, from about 3:1 to about 4:1, from about 4:1 to about 5:1, from about 5:1 to about 6:1, from about 6:1 to about 7:1, from about 7:1 to about 8:1, from about 8:1 to about 9:1, or from about 9:1 to about 10:1, or greater than 10:1.

[0055] As another example, a subject cell composition can include MSC and myocytes (or myoblasts, or a mixture of myocytes and myoblasts) where the MSC and the myocytes (or myoblasts, or a mixture of myocytes and myoblasts) are present in a subject cell composition at a ratio of 1:1 or greater than 1:1. For example, the ratio of MSC to myocytes (or myoblasts, or a mixture of myocytes and myoblasts) in a subject composition is at least about 1:1, at least about 1.5:1, at least about 2:1, at least about 2.5:1, at least about 3:1, at least about 3.5:1, at least about 4:1, at least about 4.5:1, at least about 5:1, at least about 5.5:1, at least about 6:1, at least about 7:1, at least about 8:1, at least about 9:1, or at least about 10:1. For example, the ratio of MSC to myocytes (or myoblasts, or a mixture of myocytes and myoblasts) in a subject composition can range from about 1:1 to about 1.25:1, from about 1.25:1 to about 1.5:1, from about 1.5:1 to about 2:1, from about 2:1 to about 3:1, from about 3:1 to about 4:1, from about 4:1 to about 5:1, from about 5:1 to about 6:1, from about 6:1 to about 7:1, from about 7:1 to about 8:1, from about 8:1 to about 9:1, or from about 9:1 to about 10:1, or greater than 10:1.

[0056] The spatial relationship between the more differentiated cells (e.g., chondroblasts, chondrocytes, osteoblasts, osteocytes, myoblasts, myocytes, tenocytes, or NPC) and the less differentiated cells (e.g., adult stem cells, embryonic stem cells, iPS cells, MSC, etc.) is non-random, e.g., a subject composition is not a randomly distributed mixture of more differentiated cells and less differentiated cells. Instead, a subject composition comprises a first layer (e.g., a first discrete region) comprising a plurality of more differentiated cells (e.g., chondroblasts, chondrocytes, osteoblasts, osteocytes, myoblasts, myocytes, tenocytes, or NPC); and a second layer (e.g., a second discrete region) comprising a

plurality of less differentiated cells (e.g., adult stem cells, embryonic stem cells, iPS cells, MSC, etc.).

[0057] The cells in the first layer are at least about 85%, at least about 90%, at least about 95%, or greater than 95%, of a single, more differentiated, cell type (e.g., chondroblasts, chondrocytes, osteoblasts, osteocytes, myoblasts, myocytes, tenocytes, or NPC). The cells in the second layer are at least about 85%, at least about 90%, at least about 95%, or greater than 95%, of a single, less differentiated, cell type (e.g., adult stem cells, embryonic stem cells, iPS cells, MSC, etc.).

[0058] As an example, the cells in the first layer are at least about 85% NPC, at least about 90% NPC, at least about 95% NPC, or greater than 95% NPC; and the cells in the second layer are at least about 85% MSC, at least about 90% MSC, at least about 95% MSC, or greater than 95% MSC. As another example, the cells in the first layer are at least about 85% osteocytes or osteoblasts, at least about 90% osteocytes (or osteoblasts), at least about 95% osteocytes (or osteoblasts), or greater than 95% osteocytes (or osteoblasts); and the cells in the second layer are at least about 85% MSC, at least about 90% MSC, at least about 95% MSC, or greater than 95% MSC. As another example, the cells in the first layer are at least about 85% chondrocytes or chondroblasts, at least about 90% chondrocytes (or chondroblasts), at least about 95% chondrocytes (or chondroblasts), or greater than 95% chondrocytes (or chondroblasts); and the cells in the second layer are at least about 85% MSC, at least about 90% MSC, at least about 95% MSC, or greater than 95% MSC. As another example, the cells in the first layer are at least about 85% myocytes or myoblasts, at least about 90% myocytes (or myoblasts), at least about 95% myocytes (or myoblasts), or greater than 95% myocytes (or myoblasts); and the cells in the second layer are at least about 85% MSC, at least about 90% MSC, at least about 95% MSC, or greater than 95% MSC. As another example, the cells in the first layer are at least about 85% tenocytes, at least about 90% tenocytes, at least about 95% tenocytes, or greater than 95% tenocytes; and the cells in the second layer are at least about 85% MSC, at least about 90% MSC, at least about 95% MSC, or greater than 95% MSC.

[0059] The first layer and the second layer can have any of a variety of spatial relationships. For example, the first layer can completely surround the second layer. As another example, the first layer can incompletely (e.g., partially) surround the second layer. As another example, the first layer and the second layer can have a side-by-side spatial relationship. Other spatial relationships are possible. In some embodiments, a subject multi-layer cell composition has a roughly spherical form, in which the more differentiated cells are

present in an inner layer and the less differentiated cells are present in an outer layer. In other embodiments, a subject multi-layer cell composition has a roughly spherical form, in which the less differentiated cells are present in an inner layer and the more differentiated cells are present in an outer layer.

[0060] As an example, a subject composition can comprise NPC and MSC in a substantially spherical form, in which the NPC are in a first layer, the MSC are in a second layer, and in which the second layer surrounds the first layer, e.g., the first layer is an inner layer and the second layer is an outer layer. As an example, a subject composition can comprise NPC and MSC in a spherical form, in which the NPC are in a first layer, the MSC are in a second layer, and in which the second layer surrounds the first layer, e.g., the second layer is an inner layer and the first layer is an outer layer. As other examples, a subject cell composition can comprise NPC and MSC in a cuboidal form, in a cylindrical form, in a rectangular prism form, or in an irregular form.

[0061] As another example, a subject composition can comprise NPC and MSC in a substantially spherical form, in which the MSC are in a first layer, the NPC are in a second layer, and in which the second layer surrounds the first layer, e.g., the first layer is an inner layer and the second layer is an outer layer. As other examples, a subject cell composition can comprise MSC and NPC in a cuboidal form, in a cylindrical form, in a rectangular prism form, or in an irregular form; in some instances, the MSC will be in a first, inner region or layer, and the NPC will be in a second, outer layer or region, and the second layer or region will substantially surround the first layer or region.

[0062] As another example, a subject composition can comprise chondrocytes (or chondroblasts) and MSC in a substantially spherical form, in which the MSC are in a first layer, the chondrocytes (or chondroblasts) are in a second layer, and in which the second layer surrounds the first layer, e.g., the first layer is an inner layer and the second layer is an outer layer. As other examples, a subject cell composition can comprise MSC and chondrocytes (or chondroblasts) in a cuboidal form, in a cylindrical form, in a rectangular prism form, or in an irregular form; in some instances, the MSC will be in a first, inner region or layer, and the chondrocytes (or chondroblasts) will be in a second, outer layer or region, and the second layer or region will substantially surround the first layer or region.

[0063] As another example, a subject composition can comprise osteocytes (or osteoblasts) and MSC in a substantially spherical form, in which the MSC are in a first layer, the osteocytes (or osteoblasts) are in a second layer, and in which the second layer surrounds

the first layer, e.g., the first layer is an inner layer and the second layer is an outer layer. As other examples, a subject cell composition can comprise MSC and osteocytes (or osteoblasts) in a cuboidal form, in a cylindrical form, in a rectangular prism form, or in an irregular form; in some instances, the MSC will be in a first, inner region or layer, and the osteocytes (or osteoblasts) will be in a second, outer layer or region, and the second layer or region will substantially surround the first layer or region.

[0064] As another example, a subject composition can comprise myocytes (or myoblasts) and MSC in a substantially spherical form, in which the MSC are in a first layer, the myocytes (or myoblasts) are in a second layer, and in which the second layer surrounds the first layer, e.g., the first layer is an inner layer and the second layer is an outer layer. As other examples, a subject cell composition can comprise MSC and myocytes (or myoblasts) in a cuboidal form, in a cylindrical form, in a rectangular prism form, or in an irregular form; in some instances, the MSC will be in a first, inner region or layer, and the myocytes (or myoblasts) will be in a second, outer layer or region, and the second layer or region will substantially surround the first layer or region.

[0065] The dimensions of a subject multi-layer cell composition can vary. For example, where a subject cell composition is substantially spherical, the average diameter of the cell composition can range from about 0.1 mm to about 5 mm, e.g., from about 0.1 mm to about 0.5 mm, from about 0.5 mm to about 0.75 mm, from about 0.75 mm to about 1.0 mm, from about 1.0 mm to about 1.5 mm, from about 1.5 mm to about 2 mm, from about 2 mm to about 3 mm, from about 3 mm to about 4 mm, or from about 4 mm to about 5 mm . Suitable dimensions can depend on a variety of factors, including, e.g., the site of *in vivo* use.

[0066] In some embodiments, a subject multi-layer cell composition has a unit volume of from about 0.05 mm^3 to about 0.5 cm^3, or more, e.g., from about 0.05 mm^3 to about 0.1 mm^3, from about 0.1 mm^3 to about 0.5 mm^3, from about 0.5 mm^3 to about 0.75 mm^3, from about 0.75 mm^3 to about 1.0 mm^3, from about 1.0 mm^3 to 1.5 mm^3, from about 1.5 mm^3 to about 2 mm^3, from about 2 mm^3 to about 3 mm^3, from about 3 mm^3 to about 4 mm^3, from about 4 mm^3 to about 5 mm^3, from about 5 mm^3 to about 7 mm^3, from about 7 mm^3 to about 8 mm^3, or from about 8 mm^3 to about 10 mm^3, from about 10 mm^3 to about 12 mm^3, from about 12 mm^3 to about 14 mm^3, or from about 14 mm^3 to about 15 mm^3, from about 15 mm^3 to about 25 mm^3, from about 25 mm^3 to about 50 mm^3, from about 50 mm^3 to about 100 mm^3, from about 0.1 cm^3 to about 0.2 cm^3, from about 0.2 cm^3 to about 0.3 cm^3, from about 0.3 cm^3 to about 0.4 cm^3, or from about 0.4 cm^3 to about 0.5 cm^3.

[0067] In some embodiments, a subject multi-layer cell composition has a unit volume of from about 0.1 mm^3 to about 5 mm^3, or more, e.g., from about 0.1 mm^3 to about 0.5 mm^3, from about 0.5 mm^3 to about 0.75 mm^3, from about 0.75 mm^3 to about 1.0 mm^3, from about 1.0 mm^3 to 1.5 mm^3, from about 1.5 mm^3 to about 2 mm^3, from about 2 mm^3 to about 3 mm^3, from about 3 mm^3 to about 4 mm^3, or from about 4 mm^3 to about 5 mm^3.

[0068] The number of cells in the first layer can range from about 10^2 to about 10^9, e.g., the first layer can comprise from about 10^2 cells to about 5 x 10^2 cells, from about 5 x 10^2 cells to about 10^3 cells, from about 10^3 cells to about 5 x 10^3 cells, from about 5 x 10^3 cells to about 10^4 cells, from about 10^4 cells to about 5 x 10^4 cells, from about 5 x 10^4 cells to about 10^5 cells, from about 10^5 cells to about 5 x 10^5 cells from about 5 x 10^5 cells to about 10^6 cells, from about 10^6 cells to about 5 x 10^6 cells, from about 5 x 10^6 cells to about 10^7 cells, from about 10^7 cells to about 5 x 10^7 cells, from about 5 x 10^7 cells to about 10^8 cells, or from about 5 x 10^8 cells to about 10^9 cells.

[0069] The number of cells in the second layer can range from about 10^2 to about 10^9, e.g., the second layer can comprise from about 10^2 cells to about 5 x 10^2 cells, from about 5 x 10^2 cells to about 10^3 cells, from about 10^3 cells to about 5 x 10^3 cells, from about 5 x 10^3 cells to about 10^4 cells, from about 10^4 cells to about 5 x 10^4 cells, from about 5 x 10^4 cells to about 10^5 cells, from about 10^5 cells to about 5 x 10^5 cells from about 5 x 10^5 cells to about 10^6 cells, from about 10^6 cells to about 5 x 10^6 cells, from about 5 x 10^6 cells to about 10^7 cells, from about 10^7 cells to about 5 x 10^7 cells, from about 5 x 10^7 cells to about 10^8 cells, or from about 5 x 10^8 cells to about 10^9 cells.

[0070] The density of cells (including less differentiated cells and more differentiated cells) in a subject multi-layer cell composition can range from about 10^4 cells/mm^3 to about 10^9 cells/mm^3, e.g., from about 10^4 cells/mm^3 to about 10^5 cells/mm^3, from about 10^5 cells/mm^3 to about 10^6 cells/mm^3, from about 10^6 cells/mm^3 to about 10^7 cells/mm^3, from about 10^7 cells/mm^3 to about 10^8 cells/mm^3, or from about 10^8 cells/mm^3 to about 10^9 cells/mm^3, or greater than 10^9 cells/mm^3.

[0071] In some embodiments, a subject multi-layer cell composition is referred to as a "multi-layer cell unit," where a unit can have a volume of from about 0.05 mm^3 to about 0.5 cm^3, or more, e.g., from about 0.05 mm^3 to about 0.1 mm^3, from about 0.1 mm^3 to about 0.5 mm^3, from about 0.5 mm^3 to about 0.75 mm^3, from about 0.75 mm^3 to about 1.0 mm^3, from about 1.0 mm^3 to 1.5 mm^3, from about 1.5 mm^3 to about 2 mm^3, from about 2 mm^3 to about 3 mm^3, from about 3 mm^3 to about 4 mm^3, from about 4 mm^3 to about 5 mm^3, from about 5 mm^3 to about 7 mm^3, from about 7 mm^3 to about 8 mm^3, or from about 8

mm^3 to about 10 mm^3, from about 10 mm^3 to about 12 mm^3, from about 12 mm^3 to about 14 mm^3, or from about 14 mm^3 to about 15 mm^3, from about 15 mm^3 to about 25 mm^3, from about 25 mm^3 to about 50 mm^3, from about 50 mm^3 to about 100 mm^3, from about 0.1 cm^3 to about 0.2 cm^3, from about 0.2 cm^3 to about 0.3 cm^3, from about 0.3 cm^3 to about 0.4 cm^3, or from about 0.4 cm^3 to about 0.5 cm^3; and where a unit can include from 10^2 to about 10^9 cells (e.g., total of less differentiated cells plus more differentiated cells), e.g., from about 10^2 cells to about 5 x 10^2 cells, from about 5 x 10^2 cells to about 10^3 cells, from about 10^3 cells to about 5 x 10^3 cells, from about 5 x 10^3 cells to about 10^4 cells, from about 10^4 cells to about 5 x 10^4 cells, from about 5 x 10^4 cells to about 10^5 cells, from about 10^5 cells to about 5 x 10^5 cells from about 5 x 10^5 cells to about 10^6 cells, from about 10^6 cells to about 5 x 10^6 cells, from about 5 x 10^6 cells to about 10^7 cells, from about 10^7 cells to about 5 x 10^7 cells, from about 5 x 10^7 cells to about 10^8 cells, or from about 5 x 10^8 cells to about 10^9 cells. Where a subject multi-layer cell unit is roughly spherical, a subject multi-layer cell unit can also be referred to as a “multi-layer cell pellet” or a “multi-layer cell sphere” or a “multi-layer cell spheroid.”

[0072] The less differentiated cells within a subject multi-layer cell composition are stimulated to differentiate into a more differentiated cell type. For example, depending on the cell type of the more differentiated cell, an MSC can differentiate into a chondrocyte, an osteocyte, a myocyte, a tenocyte, etc.

[0073] For example, where a subject multi-layer cell composition comprises NPC and MSC, the MSC within a subject composition are stimulated to differentiate into chondrocytes. For example, after maintaining a subject multi-layer cell composition for a suitable time and under suitable conditions, MSC in the composition differentiate into chondrocytes, e.g., after culturing a subject cell composition for a suitable time and under suitable conditions at least about 2%, at least about 5%, at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 60%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or more than 95% of the MSC differentiate into chondrocytes.

[0074] For example, where a subject multi-layer cell composition comprises osteocytes (or osteoblasts) and MSC, the MSC within a subject composition are stimulated to differentiate into osteocytes (or osteoblasts). For example, after maintaining a subject multi-layer cell composition for a suitable time and under suitable conditions, MSC in the composition differentiate into osteocytes (or osteoblasts), e.g., after culturing a subject

cell composition for a suitable time and under suitable conditions at least about 2%, at least about 5%, at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 60%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or more than 95% of the MSC differentiate into osteocytes (or osteoblasts).

[0075] As another example, where a subject multi-later cell composition comprises myocytes (or myoblasts) and MSC, the MSC within a subject composition are stimulated to differentiate into myocytes (or myoblasts). For example, after maintaining a subject multi-layer cell composition for a suitable time and under suitable conditions, MSC in the composition differentiate into myocytes (or myoblasts), e.g., after culturing a subject cell composition for a suitable time and under suitable conditions at least about 2%, at least about 5%, at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 60%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or more than 95% of the MSC differentiate into myocytes (or myoblasts).

[0076] Chondrocytes express one or more of the following markers: 11-fibrau; aggrecan; annexin VI; beta-1 integrin (CD29); cartilage oligomeric matrix protein (COMP); cathepsin B; CD44, CD151, and CD49c; chondrocyte expressed protein-68 (CEP-68); cartilage matrix protein (CMP; matrilin-1); collagen II (type II collagen); collagen IX; Sox9; and collagen X (type X collagen). Chondrocytes can be identified as, e.g., $CD29^+$, $CD90^+$, $CD166^+$, $CD49^+$, $CD44^+$, $CD54^+$, $CD14^-$, $CD34^-$, $CD24^-$, and $CD31^-$. Expression of such markers can be detected using a quantitative polymerase chain reaction (qPCR) assay (e.g., to detect an mRNA marker, or an mRNA encoding a polypeptide marker); an immunoassay using an antibody specific for a polypeptide marker; and the like.

[0077] Chondrocytes can be characterized by secretion of one or more of the following: type II collagen; type X collagen; and a proteoglycan such as aggrecan. A subject multi-layer cell composition can induce differentiation of an MSC to a chondrocyte that secretes type II collagen and aggrecan. Aggrecan is a proteoglycan comprising a protein core that is modified with glycosaminoglycans (GAG) such as chondroitin sulfate and keratan sulfate. Whether a chondrocyte secretes aggrecan can be determined by detecting the presence of GAG. GAG can be detected using any known assay, including, e.g., a 1,9-dimethylmethylene blue (DMMB) assay (see, e.g., Oke et al. (2003) *Am. J. Vet. Res.*

64:894); and a safranin-O staining method (see, e.g., Rosenberg (1971) *J. Bone Joint Surg.* 53:69).

[0078] Osteocytes express one or more of the following markers: alpha 1(I) procollagen; bone Gla protein (BGP); bone sialoprotein (BSP); Cbfa1/Osf2; collagen type I; E11; osteocalcin; osteopontin; Phex; and RP59. Expression of such markers can be detected using a quantitative polymerase chain reaction (qPCR) assay (e.g., to detect an mRNA marker, or an mRNA encoding a polypeptide marker); an immunoassay using an antibody specific for a polypeptide marker; and the like.

[0079] Myocytes include skeletal myocytes. Myocyte-specific markers are known in the art. Skeletal myocyte markers include, e.g., Arpp, Caveolin-3, myosin, nestin, and troponin I.

[0080] In some embodiments, e.g., where a subject cell composition comprises chondrocytes, a subject cell composition can produce GAG. For example, a subject cell composition can produce from about 4 μg GAG to about 100 μg GAG per cell composition, e.g., from about 4 μg to about 5 μg, from about 5 μg to about 25 μg, from about 25 μg to about 50 μg, or from about 50 μg to about 100 μg, or more, per cell pellet.

[0081] A subject cell composition retains cell viability when cultured *in vitro* or when introduced into an individual, e.g., when maintained *in vivo*. For example, at least about 50%, at least about 60%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, or at least about 95%, or more, of the cells in a given cell layer remain viable after at least 2 weeks, at least 4 weeks, or at least 8 weeks in *in vitro* cell culture or after at least 1 week, at least 2 weeks, at least 4 weeks, or at least 8 weeks, *in vivo*.

[0082] In some instances, a subject cell composition can produce discrete progeny compositions that retain the multi-layer configuration of the original ("parent") cell composition. For example, a subject parent cell composition can include two layers (a bilaminar cell composition), where the composition is roughly spherical, and where a first, inner layer comprises at least about 85% naïve cells (e.g., MSC or other stem cell, as described above), where a second, outer layer comprises at least about 85% "instructive" cells (e.g., NPC, chondroblasts, chondrocytes, osteoblasts, osteocytes, myoblasts, myocytes, or tenocytes), and where the second layer substantially surrounds the first layer. After a period of time in culture *in vitro* or after a period of time *in vivo*, the parent cell composition produces a progeny cell composition (referred to in the Examples as a "satellite"), where the progeny cell composition physically separates from the parent cell composition, where the progeny cell composition retains the bilaminar configuration (e.g., discrete first and second layers) of the parent cell composition, and where at least

about 50% of the cells in the progeny cell composition remain viable for a period of time of at least two weeks *in vitro* or *in vivo*. In some embodiments, a subject cell composition comprises a parent cell composition and one or more progeny cell compositions.

Cells

[0083] The less differentiated cells and the more differentiated cells can be obtained from any of a variety of sources. Alternatively, as discussed below, cells can be obtained by inducing a stem cell to differentiate. For example, a stem cell can be induced to differentiate into an NPC, an osteocyte (or an osteoblast), a chondrocyte (or a chondroblasts), a myocyte (or a myoblast), a tenocyte, or an MSC. As noted above, a more differentiated cell includes an NPC, an osteocyte (or an osteoblast), a chondrocyte (or a chondroblasts), a myocyte (or a myoblast), and a tenocyte.

[0084] Cells used in a subject cell composition can be obtained from a variety of sources, e.g., from a cadaver, from a living individual, from a post-natal individual, from a juvenile, from an adult individual, from fetal tissue, from a healthy individual, from healthy tissue, from a tissue bank, etc. Cells can be obtained from any form of muscle, from any form of bone, from any form of connective tissue, etc. Cells can be obtained from two or more such sources.

[0085] The individual from whom a cell is obtained is generally a mammal, including, e.g., a human, a non-human primate, an ungulate (e.g., a porcine, an ovine, a bovine, etc.), a lagomorph, a rodent (e.g., a murine such as a rat or a mouse), etc. For example, cells (e.g., osteoblasts, osteocytes, chondroblasts, chondrocytes, myoblasts, myocytes, tenocytes, NPC, MSC, etc.) can be isolated from a human, or from a non-human mammal.

[0086] Cells can be obtained from any of a variety of sources, as described above. Such cells can be isolated from a source, e.g., isolated such that the desired cell type (e.g., chondrocyte, osteocyte, myocyte, tenocyte, NPC, etc.) is present at from about 75% to about 80%, from about 85% to about 90%, from about 90% to about 95%, or more than 95% purity, e.g., other (non-desired) cell types are present at less than 25%, less than 20%, less than 15%, or less than 5%.

[0087] Cells obtained from a source can be expanded *in vitro*. Cells expanded *in vitro* will thus include progeny of parent cells isolated from a source, which progeny cells may or may not be genetically identical to the parent cells. However, cells expanded *in vitro* will substantially retain a phenotype associated with the desired cell type.

[0088] Relative to an intended recipient of a subject cell composition, the cells of a subject cell composition can be autologous, allogeneic, or xenogeneic. For example, where the intended or prospective recipient of a subject cell composition is a human, the cells present in the cell composition can be human cells, e.g., isolated from a human or are obtained by inducing human stem cell(s) to differentiate. Where the intended or prospective recipient of a subject cell composition is a human, the cells present in a subject cell composition can be autologous or allogeneic. Where the intended or prospective recipient of a subject cell composition is a human, the cells present in a subject cell composition can in some cases by xenogeneic.

[0089] Mesenchymal stem cells (MSC) can be isolated from embryonal mesoderm, placenta, cord blood, from bone marrow (e.g., adult bone marrow), or from fat. Methods of isolating and culturing MSC are known in the art; and any known method can be used to obtain MSC. See, e.g., U.S. Pat. No. 5,736,396, which describes isolation and culture of human MSC; and U.S. Patent Publication No. 2007/0292872. MSC can be identified as Stro-1^{+}, $CD106^{+}$, $CD73^{+}$, $CD11b^{-}$, glycophorin-A^{-}, $CD45^{-}$, $CD34^{-}$, $CD31^{-}$, and $CD117^{-}$.

[0090] NPC can be isolated from the nucleus pulposus of intervertebral discs. Isolation of NPC from intervertebral discs can be carried out using any known method, e.g., a method as described in U.S. Patent Publication No. 2003/0220692. NPC express one or more of the following markers: hypoxia-inducing factor-1alpha (HIF-1α); hypoxia-inducing factor-1beta (HIF-1β), glucose transporter-1; matrix metalloprotease-2; lactate dehydrogenase-A; and thrombospondin-1.

[0091] Osteocytes (or osteoblasts) can be isolated from bone marrow (e.g., human bone marrow), bone marrow stromal cell cultures, human osteoblast explant cultures, or osteocyte (or osteoblast) explant cultures from collagenase-treated bone. See, e.g., Jonsson et al. (1999) *Acta Orthop. Scand*. 70:365. Osteocyte precursor cells can be isolated from bone marrow, e.g., human bone marrow. Osteocytes (or osteoblasts) can be isolated using any known method; see, e.g., U.S. Patent No. 6,811,776 for methods of isolating and culturing osteocytes.

[0092] Chondrocytes can be isolated from bone marrow (e.g., human bone marrow), human bone marrow mesenchymal stromal cells, cartilage (e.g., hyaline cartilage, fibrocartilage, or elastic cartilage), and the like. Additional sources of chondroprogenitor cells include, without limitation, mesenchymal stem cells, cartilage cells, umbilical cord stem cells, bone marrow stromal cells, adipose stromal cells or chondrogenic progenitor cells derived from periosteum or synovium. Chondrocytes can also be isolated and expanded

as described in U.S. Patent No. 7,273,756. Chondrocytes include, but are not limited to, juvenile articular chondrocytes, adult articular chondrocytes, synovial capsule chondrocytes, and periosteum chondrocytes.

[0093] Chondroblasts, chondrocytes, osteoblasts, osteocytes, myoblasts, myocytes, and tenocytes can also be generated from MSC using any of various well-known methods, where such methods include, e.g., culturing MSC in the presence of growth factors that promote differentiation of MSC into a more differentiated cell; bioreactor differentiation; and the like. For example, culturing MSC *in vitro* in a culture medium comprising TGFβ can induce differentiation of chondrocytes; thus chondrocytes can be generated from MSC by culturing the MSC *in vitro* in a culture medium comprising TGFβ. As another example, osteocytes can be generated from MSC by culturing the MSC *in vitro* in a culture medium comprising bone morphogenic protein-4 (BMP4). Other methods of inducing osteogenic, tendonogenic, chondrogenic, or myogenic differentiation of an MSC in *in vitro* culture are known in the art, and any known method can be used; see, e.g., U.S. Patent Publication No. 2007/0292872 and U.S. Patent No. 5,736,396.

[0094] NPC, osteoblasts, osteocytes, chondroblasts, chondrocytes, myoblasts, myocytes, tenocytes, and MSC can be induced from embryonic stem (ES) cells, e.g., an ES cell can be induced to differentiate into an NPC, an osteoblast, an osteocyte, a chondroblast, a chondrocyte, a myoblast, a myocyte, a tenocyte, or an MSC. Methods for inducing differentiation of ES cells *in vitro* are known in the art. See, e.g., U.S. Patent Publication No. 2006/0057720.

[0095] Suitable ES cells include, but are not limited to, any of a variety of available human ES lines, e.g., BG01(hESBGN-01), BG02 (hESBGN-02), BG03 (hESBGN-03) (BresaGen, Inc.; Athens, GA); SA01 (Sahlgrenska 1), SA02 (Sahlgrenska 2) (Cellartis AB; Goeteborg, Sweden); ES01 (HES-1), ES01 (HES-2), ES03 (HES-3), ES04 (HES-4), ES05 (HES-5), ES06 (HES-6) (ES Cell International; Singapore); UC01 (HSF-1), UC06 (HSF-6) (University of California, San Francisco; San Francisco, CA); WA01 (H1), WA07 (H7), WA09 (H9), WA13 (H13), WA14 (H14) (Wisconsin Alumni Research Foundation; WARF; Madison, WI). Cell line designations are given as the National Institutes of Health (NIH) code, followed in parentheses by the provider code. See, e.g., U.S. Patent No. 6,875,607.

[0096] Methods of culturing human ES cells are known in the art. See, e.g., U.S. Patent No. 6,875,607. Human ES cells can be cultured *in vitro* using any known method. Suitable human ES cell lines can be positive for one, two, three, four, five, six, or all seven of the

following markers: stage-specific embryonic antigen-3 (SSEA-3); SSEA-4; TRA 1-60; TRA 1-81; Oct-4; GCTM-2; and alkaline phosphatase. Human ES cell lines can be negative for SSEA-1.

[0097] NPC, osteoblasts, osteocytes, chondroblasts, chondrocytes, myoblasts, myocytes, tenocytes, and MSC can be induced from induced pluripotent stem (iPS) cells e.g., an iPS cell can be induced to differentiate into an NPC, an osteoblast, an osteocyte, a chondroblast, a chondrocyte, a myoblast, a myocyte, a tenocyte, or an MSC. For example, an iPS can be cultured in the presence of one or more osteogenic factors, one or more myogenic factors, one or more tendenogenic factors, or one or more chondrogenic factors.

[0098] iPS cells can be generated from mammalian cells (including mammalian somatic cells, such as human somatic cells) using, e.g., known methods. Examples of suitable mammalian cells include, but are not limited to: fibroblasts, skin fibroblasts, dermal fibroblasts, bone marrow-derived mononuclear cells, skeletal muscle cells, adipose cells, peripheral blood mononuclear cells, macrophages, hepatocytes, keratinocytes, oral keratinocytes, hair follicle dermal cells, epithelial cells, gastric epithelial cells, lung epithelial cells, synovial cells, kidney cells, skin epithelial cells, pancreatic beta cells, and osteoblasts.

[0099] Mammalian cells used to generate iPS cells can originate from a variety of types of tissue including but not limited to: bone marrow, skin (e.g., dermis, epidermis), muscle, adipose tissue, peripheral blood, foreskin, skeletal muscle, and smooth muscle. The cells used to generate iPS cells can also be derived from neonatal tissue, including, but not limited to: umbilical cord tissues (e.g., the umbilical cord, cord blood, cord blood vessels), the amnion, the placenta, and various other neonatal tissues (e.g., bone marrow fluid, muscle, adipose tissue, peripheral blood, skin, skeletal muscle etc.).

[00100] Cells used to generate iPS cells can be derived from tissue of a non-embryonic subject, a neonatal infant, a child, or an adult. In some embodiments, cells used to generate iPS cells are obtained from a post-natal human. Cells used to generate iPS cells can be derived from neonatal or post-natal tissue collected from a subject within the period from birth, including cesarean birth, to death. For example, the tissue source of cells used to generate iPS cells can be from a subject who is greater than about 10 minutes old, greater than about 1 hour old, greater than about 1 day old, greater than about 1 month old, greater than about 2 months old, greater than about 6 months old, greater than about 1 year old, greater than about 2 years old, greater than about 5 years

old, greater than about 10 years old, greater than about 15 years old, greater than about 18 years old, greater than about 25 years old, greater than about 35 years old, >45 years old, >55 years old, >65 years old, >80 years old, <80 years old, <70 years old, <60 years old, <50 years old, <40 years old, <30 years old, <20 years old or <10 years old.

[00101] In general, cells used to generate iPS cells are substantially genetically identical to a somatic cell from a post-natal human, e.g., are substantially genetically identical to a somatic cell of the post-natal human from which the cell used to generate the iPS cell is derived.

[00102] iPS cells produce and express on their cell surface one or more of the following cell surface antigens: SSEA-3, SSEA-4, TRA-1-60, TRA-1-81, TRA-2-49/6E, and Nanog. In some embodiments, iPS cells produce and express on their cell surface SSEA-3, SSEA-4, TRA-1-60, TRA-1-81, TRA-2-49/6E, and Nanog. iPS cells express one or more of the following genes: Oct-3/4, Sox2, Nanog, GDF3, REX1, FGF4, ESG1, DPPA2, DPPA4, and hTERT. In some embodiments, an iPS cell expresses Oct-3/4, Sox2, Nanog, GDF3, REX1, FGF4, ESG1, DPPA2, DPPA4, and hTERT.

[00103] Methods of generating iPS cells are known in the art, and a wide range of methods can be used to generate iPS cells. See, e.g., Takahashi and Yamanaka (2006) Cell 126:663–676; Yamanaka et al. (2007) Nature 448:313-7; Wernig et al. (2007) Nature 448:318-24; Maherali (2007) Cell Stem Cell 1:55–70; Maherali and Hochedlinger (2008) Cell Stem Cell 3:595-605; Park et al. (2008) Cell 134:1-10; Dimos et al. (2008) Science 321:1218-1221; Blelloch et al. (2007) Cell Stem Cell 1:245-247; Stadtfeld et al. (2008) Science 322:945-949; Stadtfeld et al. (2008) 2:230-240; Okita et al. (2008) Science 322:949-953.

[00104] In some embodiments, iPS cells are generated from somatic cells by forcing expression of Oct-3/4 and Sox2 polypeptides. In some embodiments, iPS cells are generated from somatic cells by forcing expression of Oct-3/4, Sox2 and Klf4 polypeptides. In some embodiments, iPS cells are generated from somatic cells by forcing expression of Oct-3/4, Sox2, Klf4 and c-Myc polypeptides. In some embodiments, iPS cells are generated from somatic cells by forcing expression of Oct-4, Sox2, Nanog, and LIN28 polypeptides.

[00105] As an example, iPS cells can be generated from somatic cells (e.g., skin fibroblasts) by genetically modifying the somatic cells with one or more expression constructs encoding Oct-3/4 and Sox2. As one example, somatic cells are genetically modified with one or more expression constructs comprising nucleotide sequences

encoding Oct-3/4, Sox2, c-myc, and Klf4. As another example, somatic cells are genetically modified with one or more expression constructs comprising nucleotide sequences encoding Oct-4, Sox2, Nanog, and LIN28.

[00106] Cells undergoing induction of pluripotency as described above, to generate iPS cells, can be contacted with additional factors which can be added to the culture system, *e.g.,* included as additives in the culture medium. Examples of such additional factors include, but are not limited to: histone deacetylase (HDAC) inhibitors, see, e.g. Huangfu et al. (2008) Nature Biotechnol. 26:795-797; Huangfu et al. (2008) Nature Biotechnol. 26: 1269-1275; DNA demethylating agents, see, e.g., Mikkelson et al (2008) Nature 454, 49-55; histone methyltransferase inhibitors, see, e.g., Shi et al. (2008) Cell Stem Cell 2:525-528; L-type calcium channel agonists, see, e.g., Shi et al. (2008) 3:568-574; Wnt3a, see, e.g., Marson et al. (2008) Cell 134:521-533; and siRNA, see, e.g., Zhao et al. (2008) Cell Stem Cell 3: 475-479.

Additional components

[00107] A subject multi-layer cell composition can include one or more additional components, where suitable additional components include: a) a buffer; b) an osteogenic factor; c) a chondrogenic factor; d) a myogenic factor; e) a tendonogenic factor; f) a cell culture medium component; g) a scaffold component. Suitable buffers, osteogenic factors, chondrogenic factors, and scaffold components that can be included in a subject multi-layer cell composition are those described below in the context of a cartilage or a bone production composition.

[00108] Suitable cell culture medium components are known to those skilled in the art. For example, cell culture medium components as found in any of a variety of standard culture media (e.g., Dulbecco's modified Eagle's medium (DMEM), Roswell Park Memorial Institute 1640 (RPMI 1640), and the like), and can include, e.g., amino acids, glucose, vitamins, salts, sodium pyruvate, and the like. The culture medium can further include albumin.

Method of making a multi-layer cell composition

[00109] The present disclosure provides methods of making a subject multi-layer cell compositions. The methods generally involve: a) forming a pellet of the first layer cells (e.g., cells of a more differentiated cell type, such as NPC, osteoblasts, osteocytes, chondroblasts, chondrocytes, myoblasts, myocytes, tendocytes, etc.) in a liquid medium in a tube having an inner surface that is substantially non-adherent for the cells; and b) adding the second layer cells (e.g., cells of a less differentiated cell type, such as adult

stem cells, embryonic stem cells, iPS cells, MSC, etc.) to the pellet. The pellet becomes suspended in the liquid medium, and the second layer cells adhere to and surround the pellet.

[00110] The tube has an inner surface that is substantially non-adherent for the cells, e.g., at least the inner surface of the tube is polypropylene or some other material to which the cells do not readily adhere.

MUSCULOSKELETAL TISSUE PRODUCTION COMPOSITIONS

[00111] A subject multi-layer, three-dimensional cell composition is useful for repairing and/or regenerating a musculoskeletal tissue such as an intervertebral disc, bone, skeletal muscle, tendon, or cartilage. For repairing and/or regenerating a musculoskeletal tissue, a subject multi-layer, three-dimensional cell composition can be provided with a biocompatible carrier. For example, one, two, three, four, five, or more (as described elsewhere herein) multi-layer cell composition units are in a composition comprising a biocompatible carrier. A subject musculoskeletal tissue production composition can be used to repair a musculoskeletal tissue, to generate a musculoskeletal tissue, or to fill in missing musculoskeletal tissue. Thus, e.g., a subject cartilage production composition can be used to fill in cartilage, and can also be used to fill in an area in an intervertebral disc.

[00112] The present disclosure thus provides compositions comprising: a) a subject multi-layer cells composition; and b) a biocompatible carrier. Depending on the cell types contained within the multi-layer cell compositions, a subject composition will produce cartilage, intervertebral disc tissue, muscle, tendon, or bone. Thus, the present disclosure provides a cartilage production composition comprising: a) a subject multi-layer cells composition, where the first layer includes chondrocytes (and/or chondroblasts) or NPC; and b) a biocompatible carrier. Thus, the present disclosure provides a cartilage production composition comprising: a) a subject multi-layer cells composition, where the first layer includes chondrocytes (and/or chondroblasts) or NPC; and b) a biocompatible carrier. The present disclosure also provides a bone production composition comprising: a) a subject multi-layer cells composition, where the first layer includes osteocytes (and/or osteoblasts); and b) a biocompatible carrier. The present disclosure also provides an intervertebral disc tissue production composition comprising: a) a subject multi-layer cells composition, where the first layer includes NPC; and b) a biocompatible carrier. The present disclosure also provides a muscle production composition comprising: a) a subject multi-layer cells composition, where the first layer includes myoblasts and/or myocytes; and b) a biocompatible carrier. The present disclosure also provides a tendon

production composition comprising: a) a subject multi-layer cells composition, where the first layer includes tenocytes; and b) a biocompatible carrier.

[00113] A subject cartilage production composition, bone production composition, muscle production composition, tendon production composition, or intervertebral disc tissue production composition can be a liquid at a first temperature, and a solid or a gel at a second temperature. For example, a subject cartilage production composition, bone production composition, muscle production composition, tendon production composition, or intervertebral disc tissue production composition can be a liquid at a first temperature of from about 19°C to about 30° (e.g., from about 19°C to about 25°C, or from about 25°C to about 30°C); and a solid or a gel at a second temperature of from about 30°C to about 40°C (e.g., from about 30°C to about 35°C, from about 35°C to about 38°C, or from about 38°C to about 40°C).

[00114] A subject cartilage production composition, bone production composition, muscle production composition, tendon production composition, or intervertebral disc tissue production composition can be liquid at a first temperature, as noted above, e.g., the composition is injectable through a needle of about 18 gauge, or other conduit (e.g., a tube, a catheter, etc.) having a bore of a similar gauge.

[00115] A subject cartilage production composition, bone production composition, muscle production composition, tendon production composition, or intervertebral disc tissue production composition can comprise from about 1 to about 5000 multi-layer cell composition units, e.g., a subject cartilage production composition, bone production composition, muscle production composition, tendon production composition, or intervertebral disc tissue production composition can comprise from about 1 to about 5, from about 5 to about 10, from about 10 to about 50, from about 50 to about 100, from about 100 to about 250, from about 250 to about 500, from about 500 to about 750, from about 750 to about 1000, from about 1000 to about 2000, from about 2000 to about 3000, from about 3000 to about 4000, or from about 4000 to about 5000, multi-layer cell composition units.

[00116] A subject cartilage production composition, bone production composition, muscle production composition, tendon production composition, or intervertebral disc tissue production composition will have dimensions, a shape, and a volume that can vary, depending on factors such as the intended treatment site, the intended use, etc. For example, a subject cartilage production composition, bone production composition, muscle production composition, tendon production composition, or intervertebral disc

tissue production composition can have a volume of from about 2 mm^3 to about 10 cm^3, e.g., a subject cartilage production or bone production composition can have a volume in a range of from about 2 mm^3 to about 5 mm^3, from about 5 mm^3 to about 7.5 mm^3, from about 7.5 mm^3 to about 10 mm^3, from about 10 mm^3 to about 15 mm^3, from about 15 mm^3 to about 20 mm^3, from about 20 mm^3 to about 25 mm^3, from about 25 mm^3 to about 50 mm^3, from about 50 mm^3 to about 100 mm^3, from about 100 mm^3 to about 500 mm^3, from about 1 cm^3, from about 1 cm^3 to about 2 cm^3, from about 2 cm^3 to about 3 cm^3, from about 3 cm^3 to about 4 cm^3, from about 4 cm^3 to about 5 cm^3, from about 5 cm^3 to about 7.5 cm^3, or from about cm^3 to about 10 cm^3.

[00117] A subject cartilage production composition, bone production composition, muscle production composition, tendon production composition, or intervertebral disc tissue production composition can have any of a variety of shapes, or can be amorphous. For example, a subject cartilage production composition, bone production composition, muscle production composition, tendon production composition, or intervertebral disc tissue production composition can be in the shape of a disc, or in the shape of a body part to be replaced or repaired. A subject cartilage production composition, bone production composition, muscle production composition, tendon production composition, or intervertebral disc tissue production composition can be amorphous at a first temperature, e.g., before implantation into a treatment site in an individual; and can assume a shape at a second temperature, e.g., at the body temperature of an individual into whom the composition is implanted.

[00118] A subject cartilage production composition, bone production composition, muscle production composition, tendon production composition, or intervertebral disc tissue production composition can include one or more scaffold components, as described in more detail below. Where a subject cartilage production composition, bone production composition, muscle production composition, tendon production composition, or intervertebral disc tissue production composition includes one or more scaffold components, the composition can have a certain stiffness, appropriate to the intended use and treatment site. For example, a subject cartilage production composition can have a Young's modulus (or elastic modulus, or modulus of elasticity) in a range of from about 1 megapascal (MPa) to about 50 MPa, e.g., a subject cartilage production composition can have an elastic modulus of from about 1 MPa to about 2 MPa, from about 2 MPa to about 2.5 MPa, from about 2.5 MPa to about 3 MPa, from about 3 MPa to about 4 MPa to about 5 MPa, from about 5 MPa to about 10 MPa, from about 10 MPa to about 20

MPa, from about 20 MPa to about 30 MPa, from about 30 MPa to about 40 MPa, or from about 40 MPa to about 50 MPa. A subject cartilage production composition can have the stated elastic modulus before introduction (e.g., implantation) into a treatment site of an individual and/or after introduction (e.g., implantation) into a treatment site of an individual.

Scaffold components

[00119] A subject cartilage production composition, bone production composition, muscle production composition, tendon production composition, or intervertebral disc tissue production composition can include a scaffold component, e.g., one or more macromolecules that provide support and/or structure and/or chondrogenic or osteogenic conditions to cells within the composition. Macromolecules included in the scaffold can include polypeptides, proteoglycans, polysaccharides, glycosaminoglycans, synthetic polymers, and the like. In certain embodiments, the scaffold is a hydrogel. In certain embodiments, the scaffold is a semi-interpenetrating network hydrogel.

[00120] Suitable scaffolds and scaffold components include those described in U.S. Patent Publication Nos. 2006/0293751; 2007/0048291; 2007/0276509; and 2007/098675; and in U.S. Patent No. 7,241,736.

[00121] A scaffold can be in a variety of shapes including sheets, cylinders, tubes, spheres, or beads. A scaffold can also be provided in a shape that provides natural contours of a body part, e.g., a nose or nose part, an ear or ear part, a meniscus, etc.

[00122] Suitable scaffolds include, but are not limited to, scaffolds comprising photopolymerizable components; scaffold comprising fibrin glue components (e.g., thrombin and fibrinogen); alginates, including modified alginates; agarose; and collagen scaffolds.

[00123] Suitable scaffolds include those that form a hydrogel. The term "hydrogel" as used herein refers to a hydrophilic cross-linked polymer capable of containing a large volume fraction of water. For example, a hydrogel can contain more than about 70%, more than about 75%, more than about 80%, more than about 85%, or more than about 90% water on a volume/volume basis. When a hydrophilic polymer is formed *in situ* (e.g., *in vivo*), it can acquire water from its environment or from solutions used to create the hydrogel.

[00124] A scaffold can include a glycosaminoglycan (e.g., a polysaccharide comprising a basal structure containing an amino sugar and uronic acid or galactose). Suitable

glycosaminoglycans include, but are not limited to, hyaluronic acid, chondroitin, chondroitin sulfate, dermatan sulfate, keratan sulfate, heparin, and heparan sulfate.

[00125] Suitable hydrophilic polymers include synthetic polymers such as poly(ethylene glycol), poly(ethylene oxide), partially or fully hydrolyzed poly(vinyl alcohol), poly(vinylpyrrolidone), poly(ethyloxazoline), poly(ethylene oxide)-co-poly(propylene oxide) block copolymers (poloxamers and meroxapols), poloxamines, carboxymethyl cellulose, and hydroxyalkylated celluloses such as hydroxyethyl cellulose and methylhydroxypropyl cellulose, and natural polymers such as polypeptides, polysaccharides or carbohydrates such as Ficoll™, polysucrose, hyaluronic acid, dextran, heparan sulfate, chondroitin sulfate, heparin, or alginate, and proteins such as gelatin, collagen, albumin, or ovalbumin or copolymers or blends thereof. As used herein, “cellulose” includes cellulose and cellulose derivatives; similarly, “dextran” includes dextran and dextran derivatives thereof. In certain embodiments, the hydrophilic polymer is a poly(ethylene glycol).

[00126] Examples of materials that can be used to form a hydrogel include modified alginates. Alginate is a carbohydrate polymer isolated from seaweed, which can be crosslinked to form a hydrogel by exposure to a divalent cation such as calcium. Alginate is ionically crosslinked in the presence of divalent cations, in water, at room temperature, to form a hydrogel matrix. Modified alginate derivatives may be synthesized which have an improved ability to form hydrogels. The use of alginate as the starting material is advantageous because it is available from more than one source, and is available in good purity and characterization. As used herein, the term "modified alginates" refers to chemically modified alginates with modified hydrogel properties. Naturally occurring alginate may be chemically modified to produce alginate polymer derivatives that degrade more quickly. For example, alginate may be chemically cleaved to produce smaller blocks of gellable oligosaccharide blocks and a linear copolymer may be formed with another preselected moiety, e.g. lactic acid or epsilon-caprolactone. The resulting polymer includes alginate blocks which permit ionically catalyzed gelling, and oligoester blocks which produce more rapid degradation depending on the synthetic design. Alternatively, alginate polymers may be used wherein the ratio of mannuronic acid to guluronic acid does not produce a film gel, which are derivatized with hydrophobic, water-labile chains, e.g., oligomers of epsilon-caprolactone. The hydrophobic interactions induce gelation.

[00127] In some embodiments, a scaffold component comprises a moiety comprising an arginine-glycine-aspartic acid (RGD) peptide covalently linked to another component. For example, an alginate can comprise a covalently linked moiety comprising an RGD peptide.

[00128] Additionally, polysaccharides which gel by exposure to monovalent cations, including bacterial polysaccharides, such as gellan gum, and plant polysaccharides, such as carrageenans, may be crosslinked to form a hydrogel using methods analogous to those available for the crosslinking of alginates described above. Polysaccharides which gel in the presence of monovalent cations form hydrogels upon exposure, for example, to a solution comprising physiological levels of sodium. Hydrogel precursor solutions also may be osmotically adjusted with a nonionic compound, such as mannitol, and then injected to form a gel.

[00129] Polysaccharides that are very viscous liquids or are thixotropic, and form a gel over time by the slow evolution of structure, are also useful. For example, hyaluronic acid, which forms an injectable gel with a consistency like a hair gel, may be utilized. Modified hyaluronic acid derivatives can also be used. As used herein, the term "hyaluronic acids" refers to natural and chemically modified hyaluronic acids. Modified hyaluronic acids may be designed and synthesized with preselected chemical modifications to adjust the rate and degree of crosslinking and biodegradation. For example, modified hyaluronic acids may be designed and synthesized which are esterified with a relatively hydrophobic group such as propionic acid or benzylic acid to render the polymer more hydrophobic and gel-forming, or which are grafted with amines to promote electrostatic self-assembly. Modified hyaluronic acids thus may be synthesized which are injectable, in that they flow under stress, but maintain a gel-like structure when not under stress. Hyaluronic acid and hyaluronic derivatives are available from commercial sources.

[00130] Other polymeric hydrogel precursors include polyethylene oxide-polypropylene glycol block copolymers such as Pluronics™ or Tetronics™, which are crosslinked by hydrogen bonding and/or by a temperature change, as described in Steinleitner et al., Obstetrics & Gynecology, vol. 77, pp. 48-52 (1991); and Steinleitner et al., Fertility and Sterility, vol. 57, pp. 305-308 (1992). Other materials which may be utilized include proteins such as fibrin, collagen, and gelatin. Polymer mixtures also may be utilized. For example, a mixture of polyethylene oxide and polyacrylic acid which gels by hydrogen bonding upon mixing may be utilized. In one embodiment, a mixture of a 5% w/w

solution of polyacrylic acid with a 5% w/w polyethylene oxide (polyethylene glycol, polyoxyethylene) 100,000 can be combined to form a gel over the course of time, e.g., as within a few seconds.

[00131] Water soluble polymers with charged side groups may be crosslinked by reacting the polymer with an aqueous solution containing ions of the opposite charge, either cations if the polymer has acidic side groups or anions if the polymer has basic side groups. Examples of cations for cross-linking of the polymers with acidic side groups to form a hydrogel are monovalent cations such as sodium, divalent cations such as calcium, and multivalent cations such as copper, calcium, aluminum, magnesium, strontium, barium, and tin, and di-, tri- or tetra-functional organic cations such as alkylammonium salts. Aqueous solutions of the salts of these cations are added to the polymers to form soft, highly swollen hydrogels and membranes. The higher the concentration of cation, or the higher the valence, the greater the degree of cross-linking of the polymer. Additionally, the polymers may be crosslinked enzymatically, e.g., fibrin with thrombin.

[00132] Suitable ionically crosslinkable groups include phenols, amines, imines, amides, carboxylic acids, sulfonic acids and phosphate groups. Negatively charged groups, such as carboxylate, sulfonate and phosphate ions, can be crosslinked with cations such as calcium ions. The crosslinking of alginate with calcium ions is an example of this type of ionic crosslinking. Positively charged groups, such as ammonium ions, can be crosslinked with negatively charged ions such as carboxylate, sulfonate and phosphate ions. In some cases, the negatively charged ions contain more than one carboxylate, sulfonate or phosphate group.

[00133] Exemplary anions for cross-linking of the polymers to form a hydrogel are monovalent, divalent or trivalent anions such as low molecular weight dicarboxylic acids, for example, terephthalic acid, sulfate ions and carbonate ions. Aqueous solutions of the salts of these anions are added to the polymers to form soft, highly swollen hydrogels and membranes, as described with respect to cations.

[00134] A variety of polycations can be used to complex and thereby stabilize the polymer hydrogel into a semi-permeable membrane. Examples of materials that can be used include polymers having basic reactive groups such as amine or imine groups, e.g., having a molecular weight of between 3,000 daltons and 100,000 daltons, where exemplary polymers include polyethylenimine and polylysine. These are commercially available. An exemplary polycation is poly(L-lysine); examples of synthetic polyamines

include polyethyleneimine, poly(vinylamine), and poly(allyl amine). Also suitable for use are naturally-occurring polycations such as chitosan.

[00135] Polyanions that can be used to form a semi-permeable membrane by reaction with basic surface groups on the polymer hydrogel include polymers and copolymers of acrylic acid, methacrylic acid, and other derivatives of acrylic acid, polymers with pendant SO_3H groups such as sulfonated polystyrene, and polystyrene with carboxylic acid groups. These polymers can be modified to contain active species polymerizable groups and/or ionically crosslinkable groups. Methods for modifying hydrophilic polymers to include these groups are well known to those of skill in the art.

[00136] Suitable polymers include natural polymers, semisynthetic polymers, and synthetic polymers. Suitable synthetic polymers include, but are not limited to, polymers or copolymers derived from polydioxane, polyphosphazene, polysulphone resins, poly(acrylic acid), poly(acrylic acid) butyl ester, poly(ethylene glycol), poly(propylene), polyurethane resins, poly(methacrylic acid), poly(methacrylic acid)-methyl ester, poly(methacrylic acid)-n butyl ester, poly(methacrylic acid)-t butyl ester, polytetrafluoroethylene, polyperfluoropropylene, poly N-vinyl carbazole, poly(methyl isopropenyl ketone), poly alphamethyl styrene, polyvinylacetate, poly(oxymethylene), poly(ethylene-co-vinyl acetate), a polyurethane, a poly(vinyl alcohol), and polyethylene terephthalate; ethylene vinyl alcohol copolymer (commonly known by the generic name EVOH or by the trade name EVAL); polybutylmethacrylate; poly(hydroxyvalerate); poly(L-lactic acid) or poly(L-lactide); poly(e-caprolactone); poly(ethylene glycol) (PEG); a derivatized PEG, poly(ethylene glycol) dimethacrylate (PEGDA); poly(lactide-co-glycolide); poly(hydroxybutyrate); poly(hydroxybutyrate-co-valerate); polydioxanone; polyorthoester; polyanhydride; polyethylene terephthalate (PET); polyethylene oxide (PEO), e.g., crosslinkable PEO, non-crosslinkable PEO; poly(glycolic acid) (PGA); poly(D,L-lactide) (PDLL); poly(L-Lactide)(PLL); copolymers of PGA, PDLA, and/or PLA; poly(glycolic acid-co-trimethylene carbonate); polyphosphoester; polyphosphoester urethane; poly(amino acids); cyanoacrylates; poly(trimethylene carbonate); poly(iminocarbonate); copoly(ether-esters) (e.g., PEO/PLA); polyalkylene oxalates; polyphosphazenes; polyurethanes; silicones; polyesters; polyolefins; polyisobutylene and ethylene-alphaolefin copolymers; acrylic polymers and copolymers; vinyl halide polymers and copolymers, such as polyvinyl chloride; polyvinyl ethers, such as polyvinyl methyl ether; polyvinylidene halides, such as polyvinylidene fluoride and polyvinylidene chloride; polyacrylonitrile; polyvinyl ketones; polyvinyl aromatics, such as polystyrene;

polyvinyl esters, such as polyvinyl acetate; copolymers of vinyl monomers with each other and olefins, such as ethylene-methyl methacrylate copolymers, acrylonitrile-styrene copolymers, ABS resins, and ethylene-vinyl acetate copolymers; polyamides, such as Nylon 66 and polycaprolactam; alkyd resins; polycarbonates; polyoxymethylenes; polyimides; polyethers; epoxy resins; polyurethanes; rayon; rayon-triacetate; cellulose; cellulose acetate; cellulose butyrate; cellulose acetate butyrate; cellophane; cellulose nitrate; cellulose propionate; cellulose ethers; amorphous Teflon; and carboxymethyl cellulose.

[00137] Suitable hydrogel monomers include the following: lactic acid, glycolic acid, acrylic acid, 1-hydroxyethyl methacrylate (HEMA), ethyl methacrylate (EMA), propylene glycol methacrylate (PEMA), acrylamide (AAM), N-vinylpyrrolidone, methyl methacrylate (MMA), glycidyl methacrylate (GDMA), glycol methacrylate (GMA), ethylene glycol, fumaric acid, and the like. Common cross linking agents include tetraethylene glycol dimethacrylate (TEGDMA) and N,N'-methylenebisacrylamide. The hydrogel can be homopolymeric, or can comprise co-polymers of two or more of the aforementioned polymers.

[00138] Suitable polymers for inclusion in a hydrogel include, but are not limited to, poly(N-isopropylacrylamide); poly(N-isopropylacrylamide-co-acrylic acid); hyaluronic acid or hyaluronate; crosslinked hyaluronic acid or hyaluronate; PHEMA; or copolymers p(NIPAAm)-based sIPNs and other hydrogel sIPNs (semi-interpenetrating networks).

[00139] In some embodiments, the hydrogel is a temperature-sensitive hydrogel. In some embodiments, a temperature-sensitive hydrogel is a polyacrylic acid or derivative thereof, e.g., poly (N-isopropylacrylamide) gel, and the increase in temperature causes the hydrogel to contract, thereby forcing the active agent out of the hydrogel. Alternatively, the temperature-sensitive hydrogel is an interpenetrating hydrogel network of poly(acrylamide) and poly(acrylic acid), and the increase in temperature causes the hydrogel to swell. The temperature required for triggering release of an active agent from the hydrogel is generally about normal body temperature, e.g., about 37°C.

[00140] Non-limiting examples of suitable scaffold materials are PEGDA and PET, e.g., a scaffold that includes PEGDA and PET; and a sIPN network hydrogel, e.g., a sIPS network hydrogel comprising a non-crosslinkable PEO. For example, a scaffold comprising PEGDA and PET at a ratio of 30:70 provides for good chondrocyte matrix synthesis with sufficient mechanical properties and cell viability.

Collagen

[00141] Suitable scaffold components include collagen; a collagen derivative; a methylated collagen; a combination of a collagen or a derivative thereof and a fibrinogen; a combination of a collagen or a derivative thereof and a thrombin; a combination of (a) a collagen or a derivative thereof; (b) a fibrinogen; and (c) a thrombin; a combination of a methylated collagen and a poly(ethylene glycol) or a derivative thereof; atelopeptidic collagen telopeptide collagen crosslinked collagen; and the like.

Fibrin glue

[00142] Suitable scaffold components include fibrin glue components such as fibrinogen and thrombin. For example, a scaffold component can include a fibrinogen component comprising fibrinogen; and a thrombin component comprising thrombin. The fibrinogen component can further include aprotinin, a fibrinolysis inhibitor. The thrombin component can further include $CaCl_2$. The ratio of fibrinogen to thrombin can range from about 0.5:1 to about 2:1, e.g., from about 0.5:1 to about 1:1, from about 1:1 to about 1.5:1, or from about 1.5:1 to about 2:1.

PEG-PEG polymers

[00143] Suitable scaffold components include co-polymers of poly(ethylene glycol) of different molecular weights. For example, a scaffold component can include a first PEG polymer of an average molecular weight in the range of from about 2,000 daltons (Da) to about 10,000 Da; and a second PEG polymer of an average molecular weight in the range of from about 10,000 Da to about 50,000 Da. The first and/or the second PEG polymer can be modified with a glycosaminoglycan, e.g., chondroitin sulfate, heparan sulfate, hyaluronic acid, etc.

[00144] An exemplary PEG gel comprises a nucleophilic "8-arm" octomer (PEG-NH_2, MW 20 kDa) and a "2-arm" amine-specific electrophilic dimer (SPA-PEG-SPA, MW 3.4 kDa), and is available from Shearwater Corporation, Huntsville, Ala. The addition-elimination polymerization reaction results in a nitrogen-carbon peptide-like linkage, resulting in a stable polymer whose rate of polymerization increases with pH and gel concentration.

Photopolymerizable polymers

[00145] Suitable polymers include synthetic polymers that comprise a photopolymerizable moiety. Suitable polymers include, e.g., water-soluble synthetic polymers including, but not limited to, poly(ethylene oxide) (PEO), poly(ethylene glycol) (PEG), poly(vinyl alcohol) (PVA), poly(vinylpyrrolidone) (PVP), poly(ethyloxazoline)

(PEOX) polyaminoacids, pseudopolyamino acids, and polyethyloxazoline, as well as copolymers of these with each other or other water soluble polymers or water insoluble polymers, provided that the conjugate is water soluble. Exemplary photopolymerizable moieties are acrylates, diacrylates, oligoacrylates, methacrylates, dimethacrylates, oligomethoacrylates, or other biologically acceptable photopolymerizable groups.

[00146] A synthetic polymer comprising one or more photopolymerizable moieties can be crosslinked via photopolymerization to one or more polysaccharides that are modified with one or more suitable photopolymerization moieties. Suitable polysaccharides include, e.g., alginate, hyaluronic acid, chondroitin sulfate, dextran, dextran sulfate, heparin, heparin sulfate, heparan sulfate, chitosan, gellan gum, xanthan gum, guar gum, water soluble cellulose derivatives, and carrageenan. For example, a polysaccharide can be modified by the addition of carbon-carbon double or triple bond-containing moieties, including acrylate, diacrylate, methacrylate, ethacrylate, 2-phenyl acrylate, 2-chloro acrylate, 2-bromo acrylate, itaconate, oliogoacrylate, dimethacrylate, oligomethacrylate, acrylamide, methacrylamide, styrene groups, and other biologically acceptable photopolymerizable groups.

[00147] Initiation of polymerization is accomplished by irradiation with light at a wavelength of between about 200 nm-700 nm, e.g., in the long wavelength ultraviolet range or visible range, e.g., 320 nm or higher, or from about 376 nm to about 514 nm. This light can be provided by any appropriate source able to generate the desired radiation, such as a mercury lamp, longwave ultraviolet (UV) lamp, He-Ne laser, or an argon ion laser, or through the use of fiber optics.

[00148] An example of a water soluble conjugate is a block copolymer of polyethylene glycol and polypropylene oxide, e.g., poly(ethylene glycol) (PEG) polymers that include one or more photopolymerizable moieties that are polymerizable by photoinitiation. For example, a suitable polymer is a PEG polymer that includes one or more polymerizable moieties that are polymerizable by free radical generation, e.g., using visible or long wavelength ultraviolet radiation. One exemplary photopolymerizable PEG polymer is PEG-diacrylate. A suitable PEG polymer has an average molecular weight in a range of from about 2000 daltons (Da) to about 20,000 Da, e.g., from about 2,000 Da to about 4,000 Da, from about 4,000 Da to about 7,000 Da, from about 7,000 Da to about 10,000 Da, from about 10,000 Da to about 20,000 Da, from about 20,000 Da to about 30,000 Da, or from about 30,000 Da to about 40,000 Da. The PEG polymer comprises one or more photopolymerizable moieties, as described above.

[00149] A non-limiting example of a suitable polysaccharide is a glycosaminoglycan (e.g., a chondroitin sulfate, a heparan sulfate, a hyaluronic acid, etc.). An example of a chondroitin sulfate is chondroitin-4-sulfate (CS-4) and chondroitin-6-sulfate (CS-6). In some cases, a combination of CS-4 and CS-6 is used. The CS-4/CS-6 mixture can include 10%-90% CS-4 and 10%-90% CS6, e.g., a CS-4/CS-6 mixture can comprise 10%-20% CS-4 and 80%-90% CS-6; 20%-30% CS-4 and 70%-80% CS-6; 30%-40% CS4 and 60%-70% CS-6; 40%-60%CS4 and 40%-60% CS-6; 70%-80% CS-4 and 20%-30% CS-6; or 80%-90% CS4 and 10%-20% CS-6. The chondroitin sulfate is modified with a moiety such as acrylate, diacrylate, methacrylate, ethacrylate, 2-phenyl acrylate, 2-chloro acrylate, 2-bromo acrylate, itaconate, oliogoacrylate, dimethacrylate, oligomethacrylate, acrylamide, methacrylamide, styrene groups, and other biologically acceptable photopolymerizable groups. For example, the CS-4 and the CS-6 can comprise a methacrylate moiety.

[00150] Exemplary photopolymerizable polymers includes chondroitin sulfate and a poly(ethylene glycol) as described in, e.g., Varghese et al. (2008) *Matrix Biol.* 27:12-21; Wang et al. (2007) *Nat. Mater*. 6:385; Elisseeff (2008) *Nat. Mater*. 7:271; Hwang et al. (2007) *Methods Mol. Biol.* 407:351.

Buffers

[00151] A subject cartilage production composition, bone production composition, muscle production composition, tendon production composition, or intervertebral disc tissue production composition can include, in addition to the above-mentioned cells, a buffer. Suitable buffers include, but are not limited to, (such as N,N-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES), bis(2-hydroxyethyl)amino-tris(hydroxymethyl)methane (BIS-Tris), N-(2-hydroxyethyl)piperazine-N'3-propanesulfonic acid (EPPS or HEPPS), glycylglycine, N-2-hydroxyehtylpiperazine-N'-2-ethanesulfonic acid (HEPES), 3-(N-morpholino)propane sulfonic acid (MOPS), piperazine-N,N'-bis(2-ethane-sulfonic acid) (PIPES), sodium bicarbonate, 3-(N-tris(hydroxymethyl)-methyl-amino)-2-hydroxy-propanesulfonic acid) TAPSO, (N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES), N-tris(hydroxymethyl)methyl-glycine (Tricine), tris(hydroxymethyl)-aminomethane (Tris), etc.).

Chondrogenic factors

[00152] A subject cartilage production composition can include one or more chondrogenic factors, where suitable chondrogenic factors include, but are not limited to,

transforming growth factor-beta (TGF-β), e.g., TGF-β1 and/or TGF-β3; inhibin A; chondrogenic stimulating activity factor; bone morphogenic protein-4 (BMP-4); a vitamin A analog, e.g., retinoic acid; a fibroblast growth factor (FGF), e.g., FGF-2; growth and differentiation factor-5 (GDF-5; see, e.g., U.S. Patent No. 7,198,790); and the like. In some embodiments, a subject cell composition does not include any of the above-listed chondrogenic factors. If present, a chondrogenic factor can be present in a concentration of from about 1 ng/ml to about 100 μg/ml. As an example, TGF-β can be present in a concentration of from about 1 ng/ml to about 10 ng/ml.

Osteogenic factors

[00153] A subject bone production composition can include one or more osteogenic factors, where suitable osteogenic factors include, but are not limited to, TGF-β1, TGF-β2, TGF-β1.2, vascular endothelial growth factor (VEGF), insulin-like growth factor I or II, bone morphogenic protein (BMP)-2 (BMP2), BMP4, BMP6, and BMP7. If present, an osteogenic factor can be present in a concentration of from about 1 ng/ml to about 100 μg/ml.

Osmolality

[00154] In some embodiments, the osmolality of a subject composition is in a range of from about 100 mOsmols/kg to about 1000 mOsmols/kg, e.g., from about 100 mOsmols/kg to about 200 mOsmols/kg, from about 200 mOsmols/kg to about 300 mOsmols/kg, from about 300 mOsmols/kg to about 400 mOsmols/kg, from about 400 mOsmols/kg to about 500 mOsmols/kg, from about 500 mOsmols/kg to about 750 mOsmols/kg, or from about 750 mOsmols/kg to about 1000 mOsmols/kg.

METHODS OF PRODUCING CARTILAGE

[00155] The present disclosure provides methods of producing cartilage *in vitro* and/or *in vivo*, the methods generally involving maintaining a subject cartilage production composition *in vitro* and/or *in vivo* under suitable conditions and for a suitable period of time to induce chondrocyte differentiation of an MSC in the cell composition, such that cartilage is produced by the chondrocyte(s). Maintaining a subject cartilage production composition *in vitro* or *in vivo* under suitable conditions and for a suitable period of time results in production of cartilage. Cartilage that can be produced and/or repaired using a subject method includes hyaline cartilage, fibrocartilage, and elastic cartilage.

[00156] A subject cartilage production composition can be maintained at a temperature of from about 32°C to about 39°C, e.g., from about 32°C to about 35°C, from about 35°C to about 37°C, or from about 37°C to about 39°C.

[00157] A suitable period of time can be a period of time required for at least about 2%, at least about 5%, at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 60%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or more than 95% of the MSC to differentiate into chondrocytes.

[00158] A suitable period of time can be a period of time required for production of from about 5 µg GAG (e.g., a GAG associated with aggrecan) to about 10 µg GAG per 0.5 x 10^6 cells in the composition.

[00159] A suitable period of time can range from about 2 days to about 3 weeks, e.g., from about 2 days to about 5 days, from about 5 days to about 7 days, from about 1 week to about 2 weeks, or from about 2 weeks to about 3 weeks.

[00160] A subject cartilage production composition can be maintained *in vitro* for a first period of time, then maintained *in vivo* (e.g., maintained *in vivo* indefinitely). For example, subject cartilage production composition can be maintained *in vitro* for a first period of time, then introduced into a treatment site in an individual. For example, a subject cartilage production composition can be maintained *in vitro* for a first period of time of from about 2 days to about 3 weeks, e.g., from about 2 days to about 5 days, from about 5 days to about 7 days, from about 1 week to about 2 weeks, or from about 2 weeks to about 3 weeks; then introduced into a treatment site in an individual.

[00161] Alternatively, a subject multi-layer cell composition can be maintained *in vitro* for a first period of time of from about 2 days to about 3 weeks, e.g., from about 2 days to about 5 days, from about 5 days to about 7 days, from about 1 week to about 2 weeks, or from about 2 weeks to about 3 weeks; and, after the first period of time *in vitro*, the individual multi-layer cell compositions (e.g., multi-layer cell units) can be used to form a subject cartilage production composition *in vitro*; then the cartilage production composition can be introduced into a treatment site in an individual.

[00162] In some embodiments, a subject multi-layer cell composition is maintained at a pressure that is above atmospheric pressure (e.g., above the average atmospheric pressure at sea level). For example, a subject multi-layer cell composition is maintained *in vitro* at a pressure of from about 0.5 MPa to about 1 MPa, from about 1 MPa to about 2 MPa, from about 2 MPa to about 3 MPa, from about 3 MPa to about 4 MPa, or from about 4 MPa to about 5 MPa.

[00163] As noted above, maintaining a subject cartilage production composition *in vitro* and/or *in vivo* for a period of time results in the production of cartilage. Production of cartilage can be measured in various ways, including, e.g., production of proteoglycans (e.g., as measured by GAG); production of collagen (e.g., type II collagen); etc. In some embodiments, a subject method provides for production of from about 5 µg GAG (e.g., GAG associated with aggrecan) to about 10 µg GAG per 0.5 x 10^6 cells over a period of time of from about 2 days to about 3 weeks.

[00164] A subject cartilage production composition can be introduced into a treatment site, where treatment sites include, e.g., a diarthroidal joint, an intervertebral disc, or any site of cartilage degeneration, cartilage damage, or missing cartilage.

METHODS OF PRODUCING INTERVERTEBRAL DISC TISSUE

[00165] The present disclosure provides methods of producing an intervertebral disc tissue *in vitro* and/or *in vivo*, the methods generally involving maintaining a subject intervertebral disc production composition *in vitro* and/or *in vivo* under suitable conditions and for a suitable period of time. Maintaining a subject cartilage production composition *in vitro* or *in vivo* under suitable conditions and for a suitable period of time results in production of an intervertebral disc tissue. Intervertebral disc tissues include endplate tissue, annulus tissue, and nucleus pulposus tissue. In some embodiments, a subject intervertebral disc composition provides for repair and/or generation of nucleus pulposus tissue.

[00166] A subject intervertebral disc production composition can be maintained at a temperature of from about 32°C to about 39°C, e.g., from about 32°C to about 35°C, from about 35°C to about 37°C, or from about 37°C to about 39°C.

[00167] A suitable period of time can be a period of time required for at least about 2%, at least about 5%, at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 60%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or more than 95% of the MSC to differentiate.

[00168] A suitable period of time can range from about 2 days to about 3 weeks, e.g., from about 2 days to about 5 days, from about 5 days to about 7 days, from about 1 week to about 2 weeks, or from about 2 weeks to about 3 weeks.

[00169] A subject intervertebral disc production composition can be maintained *in vitro* for a first period of time, then maintained *in vivo* (e.g., maintained *in vivo* indefinitely).

For example, subject intervertebral disc production composition can be maintained *in vitro* for a first period of time, then introduced into a treatment site in an individual. For example, a subject intervertebral disc production composition can be maintained *in vitro* for a first period of time of from about 2 days to about 3 weeks, e.g., from about 2 days to about 5 days, from about 5 days to about 7 days, from about 1 week to about 2 weeks, or from about 2 weeks to about 3 weeks; then introduced into a treatment site in an individual.

[00170] Alternatively, a subject multi-layer cell composition can be maintained *in vitro* for a first period of time of from about 2 days to about 3 weeks, e.g., from about 2 days to about 5 days, from about 5 days to about 7 days, from about 1 week to about 2 weeks, or from about 2 weeks to about 3 weeks; and, after the first period of time *in vitro*, the individual multi-layer cell compositions (e.g., multi-layer cell units) can be used to form a subject intervertebral disc production composition *in vitro*; then the intervertebral disc production composition can be introduced into a treatment site in an individual.

[00171] In some embodiments, a subject multi-layer cell composition is maintained at a pressure that is above atmospheric pressure (e.g., above the average atmospheric pressure at sea level). For example, a subject multi-layer cell composition is maintained *in vitro* at a pressure of from about 0.5 MPa to about 1 MPa, from about 1 MPa to about 2 MPa, from about 2 MPa to about 3 MPa, from about 3 MPa to about 4 MPa, or from about 4 MPa to about 5 MPa.

[00172] As noted above, maintaining a subject intervertebral disc production composition *in vitro* and/or *in vivo* for a period of time results in the production of an intervertebral disc tissue (e.g., nucleus pulposus).

[00173] A subject intervertebral disc production composition can be introduced into a treatment site, where treatment sites include, e.g., an intervertebral disc that is damaged or diseased.

METHODS OF PRODUCING BONE

[00174] The present disclosure provides methods of producing bone *in vitro* and/or *in vivo*, the methods generally involving maintaining a subject bone production composition *in vitro* and/or *in vivo* under suitable conditions and for a suitable period of time to induce osteocyte differentiation of an MSC in the cell composition, such that bone is produced by the osteocyte(s). Maintaining a subject bone production composition *in vitro* or *in vivo* under suitable conditions and for a suitable period of time results in production of bone.

[00175] A subject bone production composition can be maintained at a temperature of from about 32°C to about 39°C, e.g., from about 32°C to about 35°C, from about 35°C to about 37°C, or from about 37°C to about 39°C.

[00176] A suitable period of time can be a period of time required for at least about 2%, at least about 5%, at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 60%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or more than 95% of the MSC to differentiate into osteocytes.

[00177] A suitable period of time can range from about 2 days to about 3 weeks, e.g., from about 2 days to about 5 days, from about 5 days to about 7 days, from about 1 week to about 2 weeks, or from about 2 weeks to about 3 weeks.

[00178] A subject bone production composition can be maintained *in vitro* for a first period of time, then maintained *in vivo* (e.g., maintained *in vivo* indefinitely). For example, subject bone production composition can be maintained *in vitro* for a first period of time, then introduced into a treatment site in an individual. For example, a subject bone production composition can be maintained *in vitro* for a first period of time of from about 2 days to about 3 weeks, e.g., from about 2 days to about 5 days, from about 5 days to about 7 days, from about 1 week to about 2 weeks, or from about 2 weeks to about 3 weeks; then introduced into a treatment site in an individual.

[00179] Alternatively, a subject multi-layer cell composition can be maintained *in vitro* for a first period of time of from about 2 days to about 3 weeks, e.g., from about 2 days to about 5 days, from about 5 days to about 7 days, from about 1 week to about 2 weeks, or from about 2 weeks to about 3 weeks; and, after the first period of time *in vitro*, the individual multi-layer cell compositions (e.g., multi-layer cell units) can be used to form a subject bone production composition *in vitro*; then the bone production composition can be introduced into a treatment site in an individual.

[00180] In some embodiments, a subject multi-layer cell composition is maintained at a pressure that is above atmospheric pressure (e.g., above the average atmospheric pressure at sea level). For example, a subject multi-layer cell composition is maintained *in vitro* at a pressure of from about 0.5 MPa to about 1 MPa, from about 1 MPa to about 2 MPa, from about 2 MPa to about 3 MPa, from about 3 MPa to about 4 MPa, or from about 4 MPa to about 5 MPa.

[00181] A subject bone production composition can be introduced into a treatment site, where treatment sites include, e.g., a site of damaged bone; a site of degenerated bone, and a site of diseased bone.

METHODS OF PRODUCING MUSCLE

[00182] The present disclosure provides methods of producing muscle *in vitro* and/or *in vivo*, the methods generally involving maintaining a subject muscle production composition *in vitro* and/or *in vivo* under suitable conditions and for a suitable period of time to induce myogenic differentiation of an MSC in the cell composition, such that muscle is produced by the myocytes and/or myoblasts. Maintaining a subject muscle production composition *in vitro* or *in vivo* under suitable conditions and for a suitable period of time results in production of muscle.

[00183] A subject muscle production composition can be maintained at a temperature of from about 32°C to about 39°C, e.g., from about 32°C to about 35°C, from about 35°C to about 37°C, or from about 37°C to about 39°C.

[00184] A suitable period of time can be a period of time required for at least about 2%, at least about 5%, at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 60%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or more than 95% of the MSC to differentiate into myocytes.

[00185] A suitable period of time can range from about 2 days to about 3 weeks, e.g., from about 2 days to about 5 days, from about 5 days to about 7 days, from about 1 week to about 2 weeks, or from about 2 weeks to about 3 weeks.

[00186] A subject muscle production composition can be maintained *in vitro* for a first period of time, then maintained *in vivo* (e.g., maintained *in vivo* indefinitely). For example, subject muscle production composition can be maintained *in vitro* for a first period of time, then introduced into a treatment site in an individual. For example, a subject muscle production composition can be maintained *in vitro* for a first period of time of from about 2 days to about 3 weeks, e.g., from about 2 days to about 5 days, from about 5 days to about 7 days, from about 1 week to about 2 weeks, or from about 2 weeks to about 3 weeks; then introduced into a treatment site in an individual.

[00187] Alternatively, a subject multi-layer cell composition can be maintained *in vitro* for a first period of time of from about 2 days to about 3 weeks, e.g., from about 2 days to about 5 days, from about 5 days to about 7 days, from about 1 week to about 2 weeks, or

from about 2 weeks to about 3 weeks; and, after the first period of time *in vitro*, the individual multi-layer cell compositions (e.g., multi-layer cell units) can be used to form a subject muscle production composition *in vitro*; then the muscle production composition can be introduced into a treatment site in an individual.

[00188] In some embodiments, a subject multi-layer cell composition is maintained at a pressure that is above atmospheric pressure (e.g., above the average atmospheric pressure at sea level). For example, a subject multi-layer cell composition is maintained *in vitro* at a pressure of from about 0.5 MPa to about 1 MPa, from about 1 MPa to about 2 MPa, from about 2 MPa to about 3 MPa, from about 3 MPa to about 4 MPa, or from about 4 MPa to about 5 MPa.

[00189] A subject muscle production composition can be introduced into a treatment site, where treatment sites include, e.g., a cardiac muscle damage or disease, a site of skeletal muscle damage or disease, a site of smooth muscle damage or disease.

SYSTEMS

[00190] The present disclosure provides a system for delivering a subject cartilage production composition to a treatment site in an individual. The present disclosure also provides a system for delivering a subject bone production composition to a treatment site in an individual.

[00191] A subject system comprises a delivery system that includes an injectable material. The injectable material can comprise: a) a subject multi-layer cell composition; and (b) a scaffold component. Thus, e.g., the injectable material can be a subject bone production composition, a subject cartilage production composition, a subject intervertebral disc tissue production composition, a subject muscle production composition, or a subject tendon production composition. The injectable material can also comprise: a) a first composition comprising a population of less-differentiated cells (e.g., MSC or other stem cell); b) a second composition comprising a population of more-differentiated cells (e.g., NPC, chondroblasts, chondrocytes, osteoblasts, osteocytes, myoblasts, myocytes, or tenocytes), where the first and the second compositions are physically separate from another.

[00192] Where a subject system is for delivering a subject cartilage production composition or a subject intervertebral disc tissue production composition to a treatment site in an individual, the system can include an injectable material that comprises: a) a subject multi-layer cell composition, wherein the cells in the first layer are chondrocytes (or chondroblasts) or nucleus pulposus cells; and b) a scaffold component. Where a

subject system is for delivering a subject bone production composition to a treatment site in an individual, the system can include an injectable material that comprises: a) a subject multi-layer cell composition, wherein the cells in the first layer are osteocytes (or osteoblasts); and b) a scaffold component. Where a subject system is for delivering a subject muscle production composition to a treatment site in an individual, the system can include an injectable material that comprises: a) a subject multi-layer cell composition, wherein the cells in the first layer are myoblasts or myocytes; and b) a scaffold component. Where a subject system is for delivering a subject tendon production composition to a treatment site in an individual, the system can include an injectable material that comprises: a) a subject multi-layer cell composition, wherein the cells in the first layer are tenocytes; and b) a scaffold component.

[00193] A suitable delivery system can include a syringe; a syringe and a needle; a syringe and a catheter; a syringe, a needle, and a catheter; a syringe and a flexible tubing; and the like. A syringe can include a single chamber, or two or more chambers. A suitable delivery system can include two or more syringes, e.g., a suitable delivery system can include two syringes; two syringes and two needles; two syringes, two needles, and a bifurcated tube; and the like.

[00194] A scaffold component can include two or more components that, when combined, result in formation of a macromolecular structure. An example is fibrin glue. For example, a scaffold component can comprise a first precursor material and a second precursor material; and the delivery system can include i) a first chamber comprising a subject multi-layer cell composition and the first precursor material; and ii) a second chamber comprising the second precursor material. In this example, the delivery system is adapted to mix the contents of the first chamber and the second chamber prior to delivery to the treatment site. As an example, the first precursor material comprises fibrinogen, and the second precursor material comprises thrombin. Alternatively, the first precursor material could be the thrombin component; and the second precursor material could be the fibrinogen component. Dual-chamber delivery systems are known in the art; for example, dual-chamber delivery systems that are suitable for use are described in, e.g., U.S. Pat. No. 6,454,786; U.S. Pat. No. 6,461,325; and U.S. Pat. No. 5,585,007.

[00195] Another example of a dual-chamber delivery system is one that comprises: a) a first composition comprising a population of less-differentiated cells (e.g., MSC or other stem cell); b) a second composition comprising a population of more-differentiated cells (e.g., NPC, chondroblasts, chondrocytes, osteoblasts, osteocytes, myoblasts, myocytes, or

tenocytes), where the first and the second compositions are physically separate from another, e.g., each in separate chambers of the dual-chamber delivery system. The ratio of the less-differentiated cells to the more-differentiated cells is generally greater than 1:1, as described above. The first composition and the second composition are maintained in separate chambers, then mixed just prior to introduction into a treatment site. A scaffold component can then be applied to the cells after introduction into the treatment site.

[00196] A scaffold component can include a first scaffold component that is a synthetic polymer comprising a photopolymerizable moiety and a second scaffold component comprising a photopolymerizable moiety. Suitable photopolymerizable components are described *supra*.

[00197] Suitable delivery systems include, e.g., a syringe or other vessel; and a needle or other conduit for introducing a subject bone production or cartilage production composition into a treatment site. A syringe can include a single chamber. A syringe can include two or more chambers. Alternatively, a suitable delivery system can include two syringes, each holding a composition to be admixed.

UTILITY

[00198] A subject multi-layer cell composition is useful for producing a subject cartilage production composition, a subject bone production composition, a subject intervertebral disc tissue production composition, a subject muscle production composition, or a subject tendon production composition. A subject cartilage production composition is useful for replacing, repairing, or regenerating cartilage *in vivo*, e.g., in an individual in need of cartilage replacement, repair, or regeneration. A subject intervertebral disc tissue production composition is useful for replacing, repairing, or regenerating intervertebral disc tissue *in vivo*, e.g., in an individual in need of replacement, repairing, or regeneration of intervertebral disc tissue. A subject bone production composition is useful for replacing, repairing, or regenerating bone *in vivo*, e.g., in an individual in need of bone replacement, repair, or regeneration. A subject cartilage production composition or a subject bone production composition can be introduced into a treatment site in an individual using a subject system for delivering such a composition. A subject muscle production composition is useful for replacing, repairing, or regenerating bone *in vivo*, e.g., in an individual in need of muscle replacement, repair, or regeneration. A subject tendon production composition is useful for replacing, repairing, or regenerating bone *in vivo*, e.g., in an individual in need of tendon replacement, repair, or regeneration.

Cartilage regeneration, replacement, and repair

[00199] As discussed above, a subject cartilage production composition is useful for replacing or regenerating cartilage *in vivo*, e.g., in an individual in need of cartilage replacement and/or regeneration. Such methods are useful in, for example, the repair of defects or lesions in cartilage tissue which is the result of degenerative wear such as that which results in arthritis, as well as other mechanical derangements which may be caused by trauma to the tissue, such as a displacement of torn meniscus tissue, meniscectomy, a taxation of a joint by a torn ligament, malignment of joints, bone fracture, or by hereditary disease. A subject cartilage regenerative method is also useful for remodeling cartilage matrix, such as in plastic or reconstructive surgery, as well as in periodontal surgery. A subject cartilage regenerative method can be used in conjunction with a reparative procedure, e.g., surgical repair of a meniscus, ligament, or cartilage.

[00200] As discussed above, in some instances, a subject cartilage production composition will include one or more scaffold components that are photopolymerizable. In such instances, a subject treatment method can involve: a) introducing a subject cartilage production composition into a treatment site in an individual; and b) exposing the introduced composition to a wavelength of light for such a time as to effect polymerization of the scaffold components.

[00201] As discussed above, in some instances a subject cartilage production composition will include two components that, when mixed, will form a fibrin glue. In such instances, a subject treatment method can involve: a) admixing a first composition comprising thrombin with a second composition comprising fibrinogen, where one of the compositions also includes a subject multi-layer cell composition, where the mixing results in a cartilage production admixture composition; and b) introducing the admixture composition into a treatment site in an individual. The time that elapses between the admixing and the introducing steps can be less than about 5 minutes.

[00202] A subject cartilage production composition can be introduced into an individual in need thereof to regenerate cartilage of a diarthroidal joint, such as a knee, an ankle, an elbow, a hip, a wrist, a knuckle of a finger, a knuckle of a toe, or a temporomandibular joint. The treatment can be directed to the meniscus of the joint, to the articular cartilage of the joint, or both. As another example, a subject cell composition can be used to treat a degenerative disorder of a knee, e.g., where the degenerative disorder is the result of traumatic injury (e.g., a sports injury or excessive wear) or osteoarthritis.

[00203] As another example, a subject cartilage production composition is introduced into an intervertebral disc, to treat degeneration of an intervertebral disc, and disorders resulting from degeneration of an intervertebral disc.

[00204] A subject cartilage production composition is useful to enhance attachment of a prosthetic device implanted in an individual. A subject cartilage production composition can also form a part of a prosthetic device, to be implanted into an individual. Prosthetic devices include, but are not limited to, an artificial meniscus, an artificial tendon, an artificial ligament, etc.

[00205] A subject cartilage production composition can be used for remodeling cartilage matrix, such as in plastic or reconstructive surgery. For example, a subject cartilage production composition can be used for remodeling cartilage in the external ear, in the nose, and the like.

Bone regeneration, replacement, and repair

[00206] As discussed above, a subject composition (e.g., a subject bone production system) is useful for repairing damaged bone or disease bone, and for replacing missing bone. For example, a subject bone production composition is useful for treating osteoporosis, for repairing bone fractures, and for carrying out bone reconstruction.

Muscle regeneration, replacement, and repair

[00207] As discussed above, a subject composition (e.g., a subject muscle production system) is useful for repairing damaged or disease muscle. Diseased muscle tissue includes ischemic cardiac muscle tissue; torn or otherwise damaged skeletal muscle tissue; diseased muscle tissue; etc.

SUBJECTS

[00208] Individuals who are suitable recipients for a subject cartilage production composition include, but are not limited to, individuals suffering from intervertebral disc degeneration; individuals suffering from arthritis of a joint, e.g., a diarthroidal joint; individuals in need of hip replacement; individuals in need of a prosthetic device; and individuals in need of tissue reconstruction, e.g., cartilage reconstruction.

[00209] Individuals who are suitable recipients for a subject bone production composition include, but are not limited to, individuals who have suffered a bone fracture but who are otherwise healthy; individuals who have suffered a bone fracture and who have osteogenesis imperfecta; individuals who have suffered bone loss due to osteoporosis; and individuals who have suffered bone loss due to trauma, or due to a surgical treatment.

EXAMPLES

[00210] The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use the present invention, and are not intended to limit the scope of what the inventors regard as their invention nor are they intended to represent that the experiments below are all or the only experiments performed. Efforts have been made to ensure accuracy with respect to numbers used (e.g. amounts, temperature, etc.) but some experimental errors and deviations should be accounted for. Unless indicated otherwise, parts are parts by weight, molecular weight is weight average molecular weight, temperature is in degrees Celsius, and pressure is at or near atmospheric. Standard abbreviations may be used, e.g., bp, base pair(s); kb, kilobase(s); pl, picoliter(s); s or sec, second(s); min, minute(s); h or hr, hour(s); aa, amino acid(s); kb, kilobase(s); bp, base pair(s); nt, nucleotide(s); i.m., intramuscular(ly); i.p., intraperitoneal(ly); s.c., subcutaneous(ly); and the like.

Example 1: Culturing MSCs in a bioreactor

[00211] Chondrogenic stimulation: bone marrow-derived MSCs (Cambrex Corp.) were suspended in 1.2% alginate gel at a concentration of 4 million cells/mL; beads were formed by dispensing dropwise into a calcium chloride bath. Beads were cultured in media with chondrogenic supplements and TGF-beta3 for 7 or 14 days.

[00212] Bioreactor assembly: a dialysis cassette (Slide-A-Lyzer, Pierce) was filled with 300uL of hyaluronan gel and submerged in cell culture media to allow for volume expansion and pressure buildup. An autoclavable porous metal clip surrounds the device to permit fluid exchange but prevent membrane rupture).

[00213] Undifferentiated MSCs or MSCs pre-cultured in alginate beads were suspended in fibrin glue (Tisseel VH, Baxter Corp.) at a concentration of $4x10^6$ cells/mL and a 100μL pellet was injected into the center of the cassette. Cassettes were cultured in media without chondrogenic supplements for 1 or 2 weeks. In addition to cell viability and histology, total proteoglycan content was assessed by dimethylmethylene blue (DMMB).

[00214] Cell viability of bioreactor cell pellets was improved when cells were pre-treated; groups E-H (Table 1) had 90-97% viability while groups A-D had 80-85% viability.

Table 1

Group	Pretreatment	Time in bioreactor	Average total GAG content (μg)
A	none	1 week	38 ± 16 (n = 2)
B	none	2 weeks	108 ± 66 (n = 6)

C	none	3 weeks	150 ± (n = 5)
D	none	4 weeks	255 (n = 1)
E	1 week	1 week	109 ± 62 (n = 5)
F	1 week	2 weeks	190 ± (n = 6)
G	2 weeks	2 weeks	251 ± (n = 5)
H	3 weeks	2 weeks	220 ± (n = 2)

[00215] Total glycosaminoglycan (GAG) content increased with increasing bioreactor culture time (Table 1); the change is significant when bioreactor culture time is doubled for cells that have been differentiated for 1 week prior to culture (group E vs. F, $p<0.03$). Although there was a trend of increasing GAG content with increased differentiation time (groups F vs. G vs. H) the change was not significant.

[00216] Safranin-O staining of fibrin-cell pellets showed little or no staining for undifferentiated groups cultured up to three weeks. After four weeks of culture, pellets showed isolated areas of faint staining. Groups pre-treated for one week before bioreactor culture also showed no staining, but robust staining was seen in group G.

Example 2: Co-culturing NPC and MSC

[00217] NPC and adult MSC were co-cultured in a 3-dimensional bi-layer culture system. The 3-dimensional system is a spherical, bilayer pellet with an inner sphere and an outer shell. Human MSC were obtained from a commercial source; NPC were obtained from mature bovine tail discs. One cell type was labeled with DiO. The bilayer pellet was formed in two centrifugation steps. Cells forming the inner sphere were centrifuged in 2 ml culture medium at 400 x g for 5 minutes in a 15-ml polypropylene tube. Cells forming the outer layer of the pellet were pipetted into the tube; and the cells were centrifuged at 400 x g for 5 minutes. Within 24 hours, the cells formed a rounded pellet that freely floated in the tube. Each pellet included about 5×10^5 cells.

[00218] Each pellet was cultured for 7 days, 14 days, or 21 days. After 7 days, 14 days, or 21 days, the cells in the pellets were sorted by fluorescence activated cell sorting into MSC and NPC. The sorted cells were assayed for levels of collagen type 2, aggrecan, and SOX9 using quantitative polymerase chain reaction. The amount of aggrecan produced (as assessed by GAG detection) was measured using a DMMB assay. The experimental groups are depicted schematically in **Figure 1**.

[00219] After 14 days, a spherical bi-layer pellet with MSC on the inside and NPC on the outside at a ratio of 25/75 produced significantly more GAG than all other conformations and ratios as measured by DMMB. MSC experienced signaling originating from the

center of the pellet spreading outwards as well as signaling from the interface of the two cell types propagating inwards.

Example 3: Structured coculture of adult stem cells and nucleus cells for disc regeneration

Materials and Methods

Cell Culture

[00220] Bovine NPC were isolated from caudal discs of healthy adult cows within 48 hours of sacrifice. The NP tissue was carefully separated by gross dissection and digested in 0.5% collagenase/dispase and 2% antibiotic/antimycotic in low glucose Dulbecco's Modified Eagle Medium (DMEM) at 37°C for 4-6hrs with constant stirring. The cells were then plated in tissue culture flasks and expanded to the fourth passage in NPC Media (DMEM with 1% antibiotic/antimycotic, 1.5% 400m Osmolarity, and 5% Fetal Bovine Serum (FBS)) at 37°C with 5% CO_2. Culture media was changed twice a week.

[00221] Commercially available human MSC were purchased (Lonza) and expanded to the sixth passage in monolayer culture using growth media (DMEM low glucose with 1% antibiotic/antimycotic and 10% FBS) at 37°C with 5% CO_2. Culture media was changed twice a week.

[00222] Human nucleus pulposus samples were obtained from a consenting 55 year-old female patient undergoing surgery for scoliosis. The tissue was digested and the cells expanded. In addition, bovine MSC were isolated from femur tissue and the cells were expanded.

Bilaminar Pellet formation

[00223] Human MSC and bovine NPC were used to make coculture pellets. The cross-species human-MSC /bovine-NPC pellets enabled one to trace the location of the cells via their lineage. Three different types of pellets were formed, each consisting of 500,000 cells total: pellets of 100% one cell type, pellets of MSC and NPC with randomized organization, and pellets of MSC and NPC organized into a bilaminar. The pellets containing both MSC and NPC were formed with three different cell number ratios of 25/75, 50/50, and 75/25 respectively (**Figure 1**). To produce the 100% one cell type pellets, 500,000 cells were pipetted into a 15mL polypropylene tube and centrifuged at low speeds (300g) for 5min. To create the randomized pellets, both cell types were added to the same tube, pipetted to ensure thorough mixing, and centrifuged at low speed for 5 min. In order to form the bilaminar organized pellets, the cell type that would form the

inner sphere of the pellet was added to a 15mL polypropylene tube and centrifuged at low speed for five minutes. Subsequently, the second cell type that would form the outer shell was gently added to the same tube. The cells were then centrifuged again at low speed for 5 min. Organized pellets were formed for all three ratios with MSC on the inside and NPC on the outside and vice versa. All pellets were cultured in 2 mL of growth media for three days with caps loosened to allow for gas exchange. After three days, the pellets became spherical and were transferred to ultra-low attachment 24 well plates (Corning) for the remainder of their culture time. Media were changed three times a week.

[00224] Same-species pellets were also made, as controls for species interactions. Pellets with human MSC and human NPC were made; and pellets with bovine MSC and bovine NPC were made.

Histology

Cell Lineage Tracing for Frozen Sections

[00225] Before being pelleted, cells were labeled with fluorescent cell membrane markers (either DiO or DiI, Invitrogen). After the pellets had reached their desired culture time, they were embedded in OCT Tissue-Tek (Sakura Finetek) and frozen sectioned at 7nm using a cryostat. Sections were then counterstained with the Hoescht dye, a fluorescent nuclear marker (Sigma). Images were taken using epi-fluorescent microscopy.

Immunohistochemistry with Paraffin Sections

[00226] At the end of the culture time, the pellets were fixed in 10% Buffered Formalin overnight and processed for paraffin sectioning at 7nm thickness. At this stage, the sections were immuno-stained using the human specific antibodies Lamp1 and Lamp2 (Abcam). The sections were then counterstained with hematoxylin.

Results

NPC culture

[00227] The NPC started as small rounded cells. They often required several days to attach to the tissue culture flask. Many of the cells never attached at all and were discarded. Though the cells were initially seeded in very small flasks (12.5 cm^2, Falcon), it took up to 2 weeks for the cells to reach confluence. Once the cells were confluent and passaged, their growth rate significantly increased, and they were easily expanded to the fourth passage.

Macroscopic observations

[00228] Immediately after centrifugation the pellets appeared flattened. Spheres formed within 48 hours and reached a maximum size of approximately 2mm in diameter. After

one week of culture, coculture pellets began to exhibit budding (**Figures 2A-D**). During the course of the second week of culture, these buds separated from the main pellet entirely to form numerous independent satellite pellets of various sizes. As the satellite pellets budded off of the main pellet, the main pellet did not noticeably decrease in size. At the three week time point, several of the larger satellite pellets also began to exhibit budding.

[00229] The 100% MSC pellets did not exhibit budding nor did any satellite pellets form at any point. The 100% NPC pellets exhibited budding and satellite pellet formation on the same time frame as the coculture pellets (in this case the satellite pellets were composed entirely of NPC). The same-species pellets (e.g. human-MSC and human-NPC) exhibited the same behavior as the cross-species pellets (human-MSC and bovine-NPC). There was no difference in the budding or satellite formation rate between the bilaminar coculture pellet and the random coculture pellets.

Histology

[00230] Both the frozen (**Figures 3A and 3B**) and paraffin histological sections confirmed that the main pellets maintained their structure throughout the culture time even as satellite pellets budded off them (**Figure 4**). The histology of the satellite-pellets confirmed that they were composed of both cell types. Most surprisingly, the satellite pellets appear to all have the same structure with MSC on the inside and NPC on the outside (**Figure 5A, 5B**). This structural organization was independent of the structure and ratio of the main pellet that from which the pellets stemmed.

[00231] **Figures 2A-D**. (A) Coculture pellet after three days of culture. (B) A budding cocluture pellet at one week of culture. (C) Coculture pellet after three weeks, several satellite pellets have formed and separated. (D) A budding satellite pellet after three weeks of culture.

[00232] **Figures 3A and 3B**. Frozen sections of bilaminar pellets after 3 weeks of culture. All cell nuclei are labeled with Hoescht dye (blue in original). A) Bilaminar pellet with MSC unstained and NPC dyed with DiI (red). The pellet is organized with 50% MCS on the inside and 50% NPC on the outside. B) Bilaminar pellet with MSC dyed with DiO (green in original) and NPC unstained. The pellet is organized with 75% MSC on the inside and 25% NPC on the outside.

[00233] **Figure 4**. Three week bilaminar pellet with MSC on the inside (brown in original) and NPC on the outside after undergoing budding and satellite pellet formation. This is a paraffin section stained using immunohistochemistry with the human specific

antibodies (darker; brown in original), Lamp1 and Lamp2, and counterstained with hematoxylin (lighter; blue in original). It is apparent that at three weeks, after undergoing budding numerous times, the pellet conserved it's original structure of having MSC on the inside and NPC on the outside.

[00234] **Figures 5A and 5B**. (A) Fluorescent microscopy image of a satellite pellet frozen section. MSC were pre-stained with DiO (green in original) before forming the main pellet. These satellite pellets formed from a random 50/50 coculture pellet. Here the center of the satellite pellet is clearly stained with DiO while the exterior is free of dye indicating that it is composed of both cell types and has the bilaminar organization of MSC inside and NPC outside. (B) Immunohistochemistry performed on a paraffin section of a satellite pellet also formed from a random 50/50 coculture pellet. The MSC at the center of the satellite pellet are stained with a human specific antibody (brown in original) and counterstained with hematoxylin. Once again, this demonstrates the bilaminar structure of the satellite pellets which have MSC on the inside and NPC on the outside.

Example 4: Further characterization of cell pellets

[00235] Bi-layer cell pellets were made as described above, with MSC ("naïve" cells) and NPC ("instructive" cells). Various parameters – including number of cells per pellet, GAG content per pellet – were measured as a function of 1) the ratio of MSC to NPC; 2) the configuration of MSC and NPC; 3) time in culture; 4) and presence of FBS in the culture medium. The results are shown in Figures 6-9.

[00236] **Figure 6** provides a graph of the DNA content at 1 week (wk), 2wk, 3wk of culture measure with Pico-green assay. The DNA content can be converted into the number of cells that were present in each pellet at the end of the culture time. All of the pellets started off with 0.5 million cells. Thus, this is a measure of the amount of cell proliferation at the different time points for the different groups. As shown in Figure 6, the groups with the most cell proliferation (highest DNA content at 3 weeks) are those with MSC on the inside and NPC on the outside.

[00237] **Figure 7** provides a graph of the GAG content as measured by dimethylmethylene blue (DMMB) assay. GAG refers to proteoglycan, one of the most important proteins in cartilage and intervertebral disc; the data are reported as GAG per pellet. Figure 7 provides the results for the ratio of MSC 25% and NPC at 75% after 2wk of culture. Figure 7 shows that the bilayer pellet with MSC on the inside made at least 30% more GAG than all other pellets. Asterisks denote statistically significant data.

[00238] **Figures 8A and 8B**. Figures 8A provides a graph similar to that shown in Figure 7 except that the data in Figure 8A are for 3wk time point. Figure 8A shows that the bilayer pellet with MSC inside produced the most GAG per pellet. Asterisks denote statistically significant data. Figure 8B provides a graph showing data for the 3wk time point with the ratio of 75% MSC and 25% NPC.

[00239] **Figure 9** depicts GAG production after two weeks of culture in media that contains 10% FBS and media that does not contain FBS. The data presented in Figure 9 demonstrates that the culture medium does not have any significant effect on GAG production by the bilaminar cell pellets.

Example 5: *In vivo* studies

[00240] An intervertebral disc of a rat was denucleated. A bilaminar cell pellet containing human MSC and bovine NPC at a 50:50 ratio was inserted into the site of denucleation, fibrin was added, and the annulus sutured. Two weeks later, the rat was sacrificed, and histological analysis of the disc was carried out. **Figure 10A** presents a view of the whole disc, showing the endplate, the annulus, and nucleus pulposus space. **Figure 10B** is a view of the nucleus pulposus space from Figure 10A. The pellet is seen on the right, and native tissue on the left. At the two-week time point, the pellet remained intact, the cells appeared to be viable, and the pellet and native tissues were merging.

[00241] While the present invention has been described with reference to the specific embodiments thereof, it should be understood by those skilled in the art that various changes may be made and equivalents may be substituted without departing from the true spirit and scope of the invention. In addition, many modifications may be made to adapt a particular situation, material, composition of matter, process, process step or steps, to the objective, spirit and scope of the present invention. All such modifications are intended to be within the scope of the claims appended her

[00242] Claims

What is claimed is:

1. A multi-layer three-dimensional cell composition comprising:

a) a first layer comprising a plurality of cells, wherein at least about 85% of the cells in the first layer are nucleus pulposus cells (NPC), chondroblasts, chondrocytes, osteoblasts, osteocytes, myoblasts, myocytes, or tenocytes; and

b) a second layer comprising a plurality of cells, wherein at least about 85% of the cells in the second layer are mesenchymal stem cells (MSC).

2. The cell composition of claim 1, wherein the cells of the first layer are obtained from donor tissue or are differentiated from a stem cell *in vitro*.

3. The cell composition of claim 1, wherein composition has a unit volume of from about 0.05 mm^3 to about 0.5 cm^3.

4. The cell composition of claim 1, wherein the composition has a substantially spherical form, a cuboidal form, or an irregular form.

5. The cell composition of claim 1, wherein the composition has a substantially spherical form, and wherein the first layer is completely surrounded by the second layer.

6. The cell composition of claim 1, wherein the composition has a substantially spherical form, and wherein the second layer is completely surrounded by the first layer.

7. The cell composition of claim 1, wherein the ratio of differentiated cells to MSC in the composition is 1:1.

8. The cell composition of claim 1, wherein the ratio of differentiated cells to MSC in the composition is greater than 1:1.

9. The cell composition of claim 1, wherein the ratio of MSC to differentiated cells in the composition is greater than 1:1.

10. The cell composition of claim 1, wherein the cells are present in the composition at a density of about 0.05×10^6 cells per mm^3 to about 10^7 cells per mm^3.

11. The cell composition of claim 1, wherein the MSC are isolated from a naturally-occurring source, or are derived by inducing a stem cell to differentiate into an MSC *in vitro*.

12. A muscloskeletal tissue production composition comprising:

a) a multi-layer cell composition of claim 1; and

b) a biologically compatible carrier.

13. The composition of claim 12, wherein the biologically compatible carrier comprises a scaffold component.

14. A cartilage production composition comprising:

a) a multi-layer cell composition of claim 1, wherein the cells in the first layer are chondrocytes and/or chondroblasts; and

b) a biologically compatible carrier.

15. The composition of claim 14, wherein the biologically compatible carrier comprises a scaffold component.

16. The composition of claim 14, wherein the composition comprises from about 1 multi-layer cell compositions to about 10^3 multi-layer cell compositions.

17. The composition of claim 14, wherein the composition is liquid at 22°C, and wherein said liquid forms a solid or semi-solid at 37°C.

18. The composition of claim 15, wherein said scaffold component is one or more of poly(ethylene glycol), a glycosaminoglycan, a fibrin glue component, an alginate, an agarose, and a collagen.

19. The composition of claim 15, wherein said scaffold component comprises a chondroitin sulfate component and/or a poly(ethylene glycol) component.

20. The composition of claim 19, wherein the chondroitin sulfate component comprises chondroitin-4-sulfate and chondroitin-6-sulfate.

21. The composition of claim 19, wherein the poly(ethylene glycol) component has an average molecular weight in the range of from about 2000 to about 10,000.

22. The composition of claim 14, further comprising at least one chondrogenic factor.

23. The composition of claim 22, wherein the at least one chondrogenic factor is one or more of a transforming growth factor-beta, inhibin A, chondrogenic stimulating activity factor, bone morphogenic protein-4, a vitamin A analog, growth and differentiation factor-5, and a fibroblast growth factor.

24. An intervertebral disc tissue production composition comprising:

a) a multi-layer cell composition of claim 1, wherein the cells in the first layer are NPC; and

b) a biologically compatible carrier.

25. The composition of claim 24, wherein the biologically compatible carrier comprises a scaffold component.

26. A muscle production composition comprising:

a) the multi-layer cell composition of claim 1, wherein the cells in the first layer are myoblasts or myoctes; and

b) a biologically compatible carrier.

27. The composition of claim 26, wherein the biologically compatible carrier comprises a scaffold component.

28. A bone production composition comprising:

a) a multi-layer cell composition of claim 1, wherein the cells in the first layer are osteoblasts; and

b) a biologically compatible carrier.

29. A system for delivering a musculoskeletal tissue-producing composition to a treatment site in an individual, the system comprising a delivery system comprising an injectable material, wherein the injectable material comprises:

a) a multi-layer cell composition of claim 1; and

b) a scaffold component.

30. The system of claim 29, wherein the scaffold component comprises a first precursor material and a second precursor material, and wherein the delivery system comprises:

i) a first chamber comprising the multi-layer cell composition and the first precursor material; and

ii) a second chamber comprising the second precursor material,

wherein the delivery system is adapted to mix the contents of the first chamber and the second chamber prior to delivery to the treatment site.

31. The system of claim 29, wherein the first precursor material is fibrinogen and wherein the second precursor material is thrombin.

32. The system of claim 29, wherein the scaffold component comprises a synthetic polymer comprising a photopolymerizable moiety and a glycosaminoglycan component comprising a photopolymerizable moiety.

33. The system of claim 32, wherein the photopolymerizable synthetic polymer is a poly(ethylene glycol), and wherein the photopolymerizable moiety is an acrylate, a diacrylate, an oligoacrylate, a dimethacrylate, or an oligomethoacrylate.

34. The system of claim 32, wherein the glycosaminoglycan component comprises chondroitin-4-sulfate and chondroitin-6-sulfate.

35. The system of claim 32, wherein the musculoskeletal tissue is an intervertebral disc tissue, cartilage, bone, muscle, or tendon.

36. A method of producing cartilage, the method comprising maintaining a multi-layer cell composition of claim 1 under conditions such that at least a portion of the MSC in the cell composition differentiate into chondrocytes, and wherein the chondrocytes synthesize cartilage components.

37. The method of claim 36, wherein the cartilage components comprise aggrecan and type II collagen.

38. The method of claim 36, wherein said maintaining is carried out *in vitro*.

39. The method of claim 38, wherein said maintaining is carried out for a period of time of from about 48 hours to about 3 weeks.

40. The method of claim 36, further comprising preparing a cartilage production composition comprising said multi-layer composition, and introducing said cartilage production composition into a treatment site in an individual.

41. The method of claim 40, wherein the treatment site is a diarthroidal joint.

42. The method of claim 40, wherein the treatment site is an intervertebral disc.

43. The method of claim 36, wherein said cartilage production composition comprises a photopolymerizable scaffold component, and wherein said method further comprises exposing the introduced cartilage production composition to ultraviolet light.

44. A method of producing a musculoskeletal tissue, the method comprising maintaining the multi-layer cell composition of claim 1 under conditions such that at least a portion of the MSC in the cell composition differentiates.

45. The method of claim 44, wherein the musculoskeletal tissue is an intervertebral disc tissue, cartilage, bone, muscle, or tendon.

46. The method of claim 44, wherein said maintaining is carried out *in vitro*.

47. The method of claim 46, wherein said maintaining is carried out for a period of time of from about 48 hours to about 3 weeks.

48. The method of claim 44, further comprising preparing a musculoskeletal tissue production composition comprising said multi-layer composition, and introducing said musculoskeletal tissue production composition into a treatment site in an individual.

49. The method of claim 48, wherein the treatment site is an intervertebral disc.

50. A method of making the multi-layer cell composition of claim 1, the method comprising:

a) forming a pellet of the first layer cells in a liquid medium in a tube having an inner surface that is substantially non-adherent for the cells; and

b) adding the second layer cells to the pellet,

wherein the pellet becomes suspended in the liquid medium, and wherein the second layer cells adhere to and surround the pellet.

ABSTRACT OF THE DISCLOSURE

The present disclosure provides compositions comprising musculoskeletal cells and mesenchymal stem cells in discrete regions. The present disclosure provides systems comprising a subject composition; and methods of using a subject composition to generate cartilage, bone, tendon, muscle, intervertebral disc, or other musculoskeletal tissues.

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