

## Co-culture of Adult Mesenchymal Stem Cells and Nucleus Pulposus Cells in Bilaminar Pellets for Intervertebral Disc Regeneration

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### ABSTRACT

#### Background

Our goal is to optimize stem cell-based tissue engineering strategies in the context of the intervertebral disc environment. We explored the benefits of co-culturing nucleus pulposus cells (NPC) and adult mesenchymal stem cells (MSC) using a novel spherical bilaminar pellet culture system where one cell type is enclosed in a sphere of the other cell type. Our 3D system provides a structure that exploits embryonic processes such as tissue induction and condensation. We observed a unique phenomenon: the budding of co-culture pellets and the formation of satellite pellets that separate from the main pellet.

#### Methods

MSC and NPC co-culture pellets were formed with three different structural organizations. The first had random organization. The other two had bilaminar organization with either MSC inside and NPC outside or NPC inside and MSC outside.

#### Results

By 14 days, all co-culture pellets exhibited budding and spontaneously generated satellite pellets. The satellite pellets were composed of both cell types and, surprisingly, all had the same bilaminar organization with MSC on the inside and NPC on the outside. This organization was independent of the structure of the main pellet that the satellites stemmed from.

#### Conclusion

The main pellets generated satellite pellets that spontaneously organized into a bilaminar structure. This implies that structural organization occurs naturally in this cell culture system and may be inherently favorable for cell-based tissue engineering strategies. The occurrence of budding and the organization of satellite pellets may have important implications for the use of co-culture pellets in cell-based therapies for disc regeneration.

#### Clinical Relevance

From a therapeutic point of view, the generation of satellite pellets may be a beneficial feature that would serve to spread donor cells throughout the host matrix and restore normal matrix composition in a sustainable way, ultimately renewing tissue function.

**Key Words:** co-culture, bilaminar structure, intervertebral disc, nucleus replacement, tissue engineering. *SAS Journal*. June 2009;3:41–49. DOI: SASJ-2009-0005-NT

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### 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.<sup>1</sup> 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 common and an underlying cause of various spinal disorders.<sup>2</sup>

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 NP 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.<sup>3</sup> The feasibility of this approach has been demonstrated in several in vivo animal studies.<sup>4-6</sup> 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.<sup>7</sup> 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 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.<sup>8,9</sup> 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.<sup>8-16</sup> Several groups have looked at the concept of co-culture systems of nucleus pulposus cells (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 co-culture study and reported significant increases in proteoglycan synthesis and cell proliferation when non-degenerative NPC and MSC were cultured with direct cell-cell contact.<sup>17</sup> They reasoned that MSC were acting as feeder cells, which enhanced the ability of NPC to proliferate and secrete matrix. Richardson and colleagues also employed a similar 2D co-culture system and demonstrated that NPC cause MSC to differentiate into an NP-like phenotype as assessed by gene expression after FACS (fluorescence-activated cell sorting, Becton Dickinson, Franklin Lakes, New Jersey).<sup>18</sup> They observed that a 75% NPC / 25% MSC ratio was optimum for MSC differentiation, as indicated by SOX9, collagen 2, and aggrecan gene expression.<sup>18</sup> However, using a 3D co-culture 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.<sup>19</sup> More recently, Vadala and colleagues have shown that 3D unstructured co-culture of MSC and NPC upregulated key differentiation markers in MSC.<sup>20</sup> These papers have looked at the environmental triggers responsible for re-creating the condensation shape and the unique signaling due to co-culturing.

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 NPC and analyzed their behavior in culture. We observed a unique phenomenon: the budding of co-culture pellets and the formation of satellite pellets

that separate from the main pellet. Interestingly, the satellite pellets were composed of both cell types and had a specific organization in which the MSC were 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 co-culture pellets in cell-based therapies for disc regeneration.

## MATERIALS AND METHODS

### 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 to 6 hours 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 m osmolarity, and 5% fetal bovine serum (FBS)) at 37 °C with 5% CO<sub>2</sub>. Culture media was changed twice a week.

Commercially available human MSC were purchased (Lonza, Basel, Switzerland) 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<sub>2</sub>. Culture media was changed twice a week.

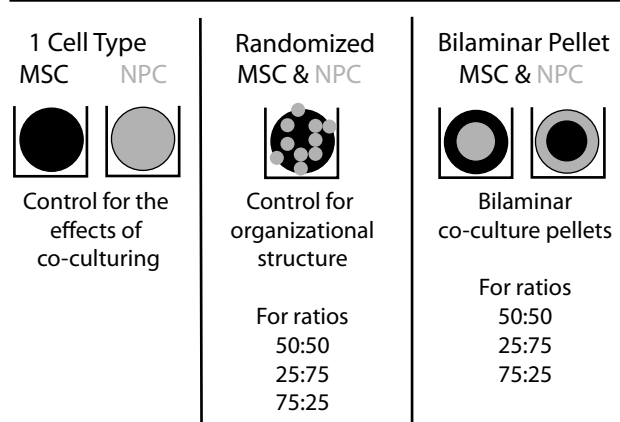
Human nucleus pulposus samples were obtained from a consenting 55-year-old female patient undergoing surgery for scoliosis. The tissue was digested and the cells expanded. In addition, bovine MSC were isolated from femur tissue and the cells were expanded.

### 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 structure. The pellets containing both MSC and NPC were formed with 3 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 15 mL polypropylene tube and centrifuged at low speeds (300 g) for 5 minutes. 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 minutes. In order to form the bilaminar organized pellets, the cell type that would form the inner sphere of the

pellet was added to a 15 mL polypropylene tube and centrifuged at low speed for 5 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 minutes. Organized pellets were formed for all 3 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 3 days with caps loosened to allow for gas exchange. After 3 days, the pellets became spherical and were transferred to Costar 24-well ultra low attachment microplates (Corning Inc., Lowell, Massachusetts) for the remainder of their culture time. Media was changed 3 times a week.

**Figure 1.**



The formation of co-culture 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.

**Histology**

**Cell Lineage Tracing for Frozen Sections**

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

**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 7 nm thickness. At this stage, the sections were immunostained using the human specific antibodies Lamp1 and Lamp 2 (Lysosomal-associated membrane proteins 1 and 2, Abcam plc, Cambridge,

England). The sections were then counterstained with hematoxylin.

**RESULTS**

**NPC Culture**

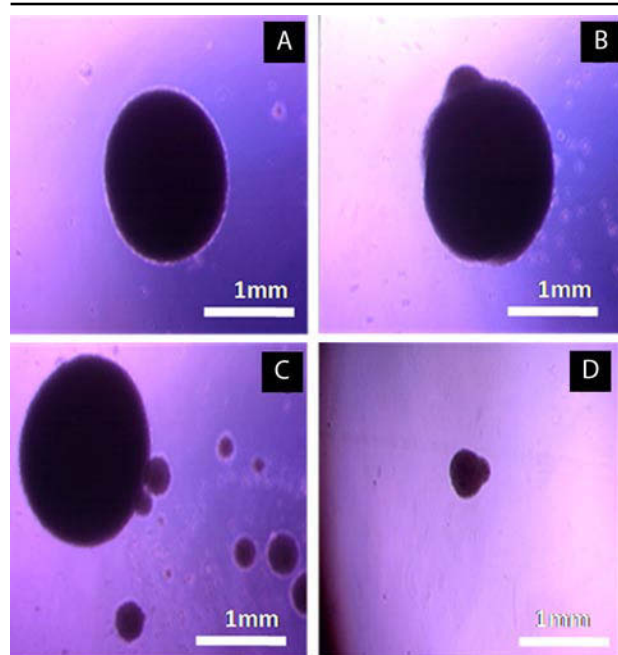
The NPC started as small rounded cells. They often required several days to attach to the tissue culture flask. Many of the cells never attached at all and were discarded. Though the cells were initially seeded in very small flasks (12.5 cm<sup>2</sup>, Falcon, Becton Dickinson, Franklin Lakes, New Jersey), 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**

Immediately after centrifugation the pellets appeared flattened. Spheres formed within 48 hours and reached a maximum size of approximately 2 mm in diameter. After one week of culture, co-culture pellets began to exhibit budding (Figure 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 3-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%

**Figure 2.**



Co-culture pellets budding and forming satellites.

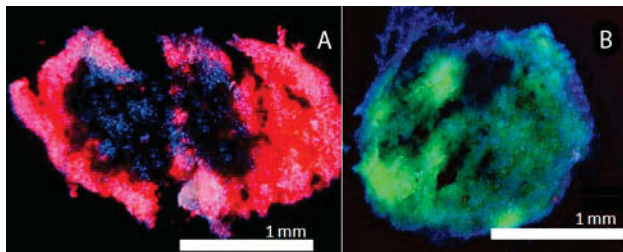


NPC pellets exhibited budding and satellite pellet formation within the same time frame as the co-culture pellets (obviously in this case the satellite pellets were composed entirely of NPC). The same-species pellets (eg, human-MSc and human-NPC) exhibited the same behavior as the cross-species pellets (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.

### Histology

Both the frozen (Figure 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 4). The histology of the satellite pellets confirmed that they were composed of both cell types. Most surprisingly, the satellite pellets all appeared to have the same structure with MSC on the inside and NPC on the outside (Figure 5). This structural organization was independent of the structure and ratio of the main pellet that they stemmed from.

**Figure 3.**



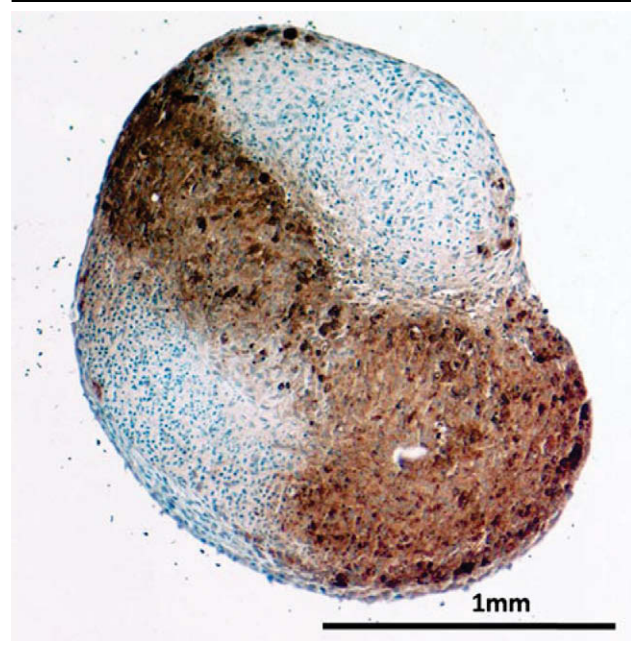
Frozen and paraffin histological sections.

### 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.<sup>18,19</sup>

Several groups have shown that unstructured co-culture offers some advantage over using MSC or NPC alone in the search of a cell-based strategy to regenerate the nucleus.<sup>17-20</sup> 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.<sup>17-20</sup> In our system, we have observed a novel phenomenon whereby co-culture 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

**Figure 4.**

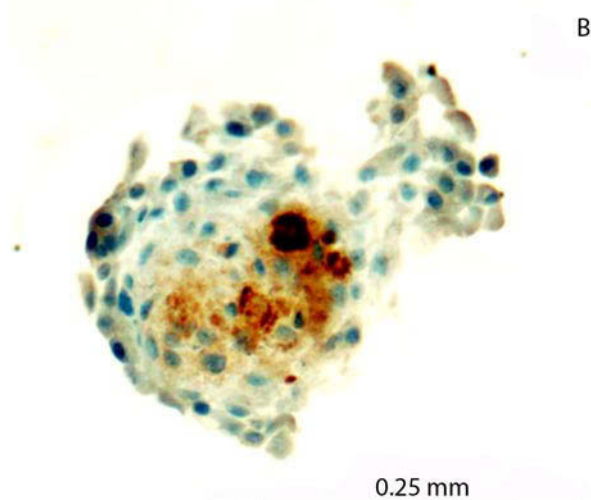
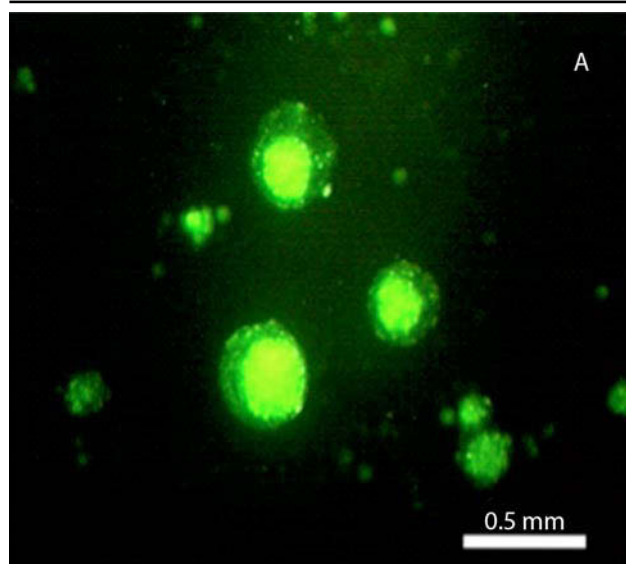


The main pellet maintains structure as satellites break off.

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.<sup>10-16</sup>

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 co-culture 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 co-cultured in any arrangement and in any ratio, will form bilaminar satellite pellets with MSC on the inside and NPC on the outside. This discovery 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 microenvironment is therefore more conducive to MSC differentiation. In addition, the NPC readily form an epithelial-like layer

**Figure 5.**

Satellite pellets exhibit the same structure: MSC inside and NPC outside.

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.<sup>2,21</sup> 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 renew tissue function.

This manuscript was the recipient of the Best Basic Science Paper at the meeting of the International Society for the Advancement of Spine Surgery in 2009.

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**EXTENDED REFERENCES****Upregulation of the viability of nucleus pulposus cells by bone marrow-derived stromal cells: significance of direct cell-to-cell contact in coculture system.**

*Yamamoto Y, Mochida J, Sakai D, Nakai T, Nishimura K, Kawada H, Hotta T.*

**STUDY DESIGN:** Upregulation of the viability of nucleus pulposus cells by coculture with bone marrow-derived stromal cells using a novel culture system. **OBJECTIVES:** The objective was to apply a novel coculture system having direct cell-to-cell contact between nucleus pulposus cells and bone marrow-derived stromal cells for stimulation of nucleus pulposus cells. **SUMMARY OF BACKGROUND DATA:** Reinsertion of nucleus pulposus cells was effective for treatment of intervertebral disc degeneration. However, obtaining highly viable nucleus pulposus cells was necessary to achieve successful results. Thus, an alternative method to upregulate the biologic and metabolic viabilities of nucleus pulposus cells was desired. **METHODS:** Nucleus pulposus cells and bone marrow-derived stromal cells were isolated from New Zealand white rabbits. A 6-well culture plate and insert with track-etched membrane having 0.4 microm pores at the bottom were used for coculture. Nucleus pulposus cells were monocultured, cocultured conventionally (having no direct cell-to-cell contact) with bone marrow-derived stromal cells, or cocultured having direct cell-to-cell contact with bone marrow-derived stromal cells. On day 4 of coculture, nucleus pulposus cells were evaluated for proliferation using WST-8 assay, deoxyribonucleic acid synthesis by measuring [H]-thymidine uptake, and proteoglycan synthesis by measuring [S]-sulfate uptake. We also quantified cytokines in supernatants from the culture system. **RESULTS:** Cell proliferation, deoxyribonucleic acid synthesis, and proteoglycan synthesis of nucleus pulposus cells were significantly upregulated in samples cocultured having direct cell-to-cell contact. Moreover, evaluations of supernatants revealed that growth factors associated with proliferation and cellular metabolism of nucleus pulposus cells were increased. **CONCLUSIONS:** Direct cell-to-cell contact in coculture system between nucleus pulposus cells and bone marrow-derived stromal cells accomplished significant upregulation in viability of nucleus pulposus cells.

**Intervertebral disc cell-mediated mesenchymal stem cell differentiation.**

*Richardson SM, Walker RV, Parker S, Rhodes NP, Hunt JA, Freemont AJ, Hoyland JA.*

Low back pain is one of the largest health problems in the Western world today, and intervertebral disc degeneration

has been identified as a main cause. Currently, treatments are symptomatic, but cell-based tissue engineering methods are realistic alternatives for tissue regeneration. However, the major problem for these strategies is the generation of a suitable population of cells. Adult bone marrow-derived mesenchymal stem cells (MSCs) are undifferentiated, multipotent cells that have the ability to differentiate into a number of cell types, including the chondrocyte-like cells found within the nucleus pulposus (NP) of the intervertebral disc; however, no method exists to differentiate these cells in an accessible monolayer environment. We have conducted coculture experiments to determine whether cells from the human NP can initiate the differentiation of human MSCs with or without cell-cell contact. Fluorescent labeling of the stem cell population and high-speed cell sorting after coculture with cell-cell contact allowed examination of individual cell populations. Real-time quantitative polymerase chain reaction showed significant increases in NP marker genes in stem cells when cells were cocultured with contact for 7 days, and this change was regulated by cell ratio. No significant change in NP marker gene expression in either NP cells or stem cells was observed when cells were cultured without contact, regardless of cell ratio. Thus, we have shown that human NP and MSC coculture with contact is a viable method for generating a large population of differentiated cells that could be used in cell-based tissue engineering therapies for regeneration of the degenerate intervertebral disc.

**Interaction of human mesenchymal stem cells with disc cells: changes in extracellular matrix biosynthesis.**

*Le Visage C, Kim SW, Tateno K, Sieber AN, Kostuik JP, Leong KW.*

**STUDY DESIGN:** To evaluate the in vitro interactions between human mesenchymal stem cells (MSCs) and degenerative disc cells. **OBJECTIVES:** To demonstrate the potential of MSCs in regulating the extracellular matrix synthesis of degenerative disc cells. **SUMMARY OF BACKGROUND DATA:** Culture of degenerative disc cells followed by their reinsertion into a disc can retard the degeneration process in an animal model. However, harvesting cells without accelerating degeneration is problematic. Autologous MSCs can be safely harvested from the bone marrow and transplanted into degenerative discs. **METHODS:** Human degenerative nucleus pulposus (NP), annulus fibrosus (AF) cells, and MSCs were cultured as pellets, and coculture pellets were formed by addition of MSCs to disc cells (50:50 ratio). Glycosaminoglycan (GAG) and DNA content were measured. Proteoglycan synthesis was analyzed by RT-PCR and western blot. Type II collagen expression was assessed by immunohistochemistry. **RESULTS:** Coculture pellets formed by the addition of MSCs to AF



cells were superior in size to all other pellets. AF/MSC pellets showed higher experimental GAG content than the predicted values represented by the sum of individual control pellets, with 10.2 versus 5.6 microg/pellet at week 3, respectively. The effect was not observed in the NP/MSC coculture, or when chondrogenic medium was used. Close contact between cells was necessary to obtain this enhancement of GAG content. Proteoglycan and collagen expression in both individual and coculture pellets was confirmed by PCR analysis and western blot. CONCLUSION: Addition of MSCs to AF cells resulted in an up-regulation of the proteoglycans synthesis. This study provides the rationale for further investigation of the potential of MSC therapy in treating intervertebral disc degeneration.

#### **Coculture of bone marrow mesenchymal stem cells and nucleus pulposus cells modulate gene expression profile without cell fusion.**

*Vadalà G, Studer RK, Sowa G, Spiezia F, Iucu C, Denaro V, Gilbertson LG, Kang JD.*

STUDY DESIGN: Changes in gene expression profile and cell fusion of mesenchymal stem cells (MSC) and nucleus pulposus cells (NPC) after coculture were analyzed. OBJECTIVE: To investigate the mechanisms of the interaction between NPC and MSC such as differentiation, stimulatory effect, and cell fusion. SUMMARY OF BACKGROUND DATA: Introduction of exogenous cells to supplement and replenish intervertebral disc cell population offers a potential approach to treat intervertebral disc degeneration (IDD). Recent evidences showed that intradiscal injection of MSC effectively alter the course of IDD in vivo, and the regenerative potential may result from up-regulated extracellular matrix protein synthesis mediated by MSC and NPC interaction. METHODS: Using a double labeling cell system and flow activated cell sorting, we quantitatively analyzed changes in the gene expression profile of human male MSC and female NPC after coculture in a 3-dimensional system that allows short distance paracrine interactions typical of the nucleus pulposus. Furthermore, we analyzed for cell fusion in the cell interaction by fluorescence in situ hybridization (FISH) for X and Y chromosomes, using a 3-dimensional culture system to allow cell-to-cell interactions conducive to cell fusion. RESULTS: Two weeks of coculture cell interaction in a 3-dimensional environment induces a change in MSCs towards a more chondrogenic gene expression profile indicating MSC differentiation, and NPC gene expression changes in matrix and chondrogenic genes demonstrating only a modest trophic effect of MSC on NPC. Moreover, FISH analysis demonstrated that cell fusion is not responsible for MSC plasticity in the interaction with NPCs. CONCLUSION: This study

clarifies the mechanism of MSCs and NPCs interaction in a 3-dimensional environment, excluding cell fusion. These data support the use of undifferentiated MSC for stem cell therapy for IDD treatment.

#### **All for one and one for all: condensations and the initiation of skeletal development.**

*Hall BK, Miyake T.*

Condensation is the pivotal stage in the development of skeletal and other mesenchymal tissues. It occurs when a previously dispersed population of cells gathers together to differentiate into a single cell/tissue type such as cartilage, bone, muscle, tendon, kidney, and lung and is the earliest stage during organ formation when tissue-specific genes are upregulated. We present a synopsis of our current understanding of how condensations are initiated and grown, how their boundaries and sizes are set, how condensation ceases, and how overt differentiation begins. Extracellular matrix molecules, cell surface receptors and cell adhesion molecules, such as fibronectin, tenascin, syndecan, and N-CAM, initiate condensation formation and set condensation boundaries. Hox genes (Hoxd-11-13) and other transcription factors (CFKH-1, MFH-1, osf-2), modulate the proliferation of cells within condensations. Cell adhesion is ensured indirectly through Hox genes (Hoxa-2, Hoxd-13), and directly via cell adhesion molecules (N-CAM and N-cadherin). Subsequent growth of condensations is regulated by BMPs, which activate Pax-2, Hoxa-2 and Hoxd-11 among other genes. Growth of a condensation ceases when Noggin inhibits BMP signalling, setting the stage for transition to the next stage of skeletal development, namely overt cell differentiation. BioEssays 22:138-147, 2000. Copyright 2000 John Wiley & Sons, Inc.



**SUPPLEMENTARY DATA**

**Table 1.** Top primary inpatient spine surgeries where DDD is the primary diagnosis (under 65)

| ICD-9 procedure code | Definition                        | %              |
|----------------------|-----------------------------------|----------------|
| 81.08                | Posterior lumbar fusion           | 52.50%         |
| 81.06                | Anterior lumbar fusion            | 27.27%         |
| 3.09                 | Decompression of the spinal canal | 4.80%          |
| 81.07                | Lateral lumbar fusion             | 4.67%          |
| 80.51                | Discectomy                        | 4.22%          |
|                      | Others                            | 6.54%          |
|                      | <b>Total</b>                      | <b>100.00%</b> |

**Table 2.** Degenerative Disc Disease Diagnosis by Age & Gender, United States, 2004-2007

| Age group | Males          |  | Females        |  |
|-----------|----------------|--|----------------|--|
|           | Total          | % of diagnoses by age under 65 (Males) | Total          | % of diagnoses by age under 65 (Females) |
| <1        | 4              | 0.00%                                  | 4              | 0.0%                                     |
| 1         | 3              | 0.00%                                  | 1              | 0.0%                                     |
| 2-4       | 11             | 0.01%                                  | 5              | 0.0%                                     |
| 5-9       | 24             | 0.02%                                  | 21             | 0.0%                                     |
| 10-14     | 173            | 0.12%                                  | 184            | 0.1%                                     |
| 15-19     | 1,151          | 0.79%                                  | 1,183          | 0.7%                                     |
| 20-24     | 2,241          | 1.53%                                  | 2,527          | 1.5%                                     |
| 25-29     | 5,573          | 3.81%                                  | 5,686          | 3.3%                                     |
| 30-34     | 10,220         | 6.99%                                  | 9,675          | 5.6%                                     |
| 35-39     | 15,317         | 10.48%                                 | 14,862         | 8.5%                                     |
| 40-44     | 19,478         | 13.33%                                 | 21,211         | 12.2%                                    |
| 45-49     | 23,823         | 16.30%                                 | 28,127         | 16.2%                                    |
| 50-54     | 24,781         | 16.95%                                 | 31,985         | 18.4%                                    |
| 55-59     | 23,362         | 15.98%                                 | 31,564         | 18.1%                                    |
| 60-64     | 20,010         | 13.69%                                 | 26,915         | 15.5%                                    |
|           | <b>146,171</b> |  | <b>173,950</b> |  |

Source: PearlDiver data derived from HIPAA-compliant insurance and private payer sources. These tables and charts are for information purposes only. The PearlDiver data has been obtained or derived from sources believed by PearlDiver to be reliable, but PearlDiver Technologies Inc. does not represent that the information is 100% accurate or complete. Any opinions or estimates which form any portion of the tables, charts or other aspects of this report represent the judgment of PearlDiver's analysts and management and are subject to change without notice. Copyright © 2009 PearlDiver Technologies Inc.