Structured Coculture of Mesenchymal Stem Cells and Disc Cells Enhances Differentiation and Proliferation

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Key Words

Mesenchymal stem cell · Bilaminar pellet · Coculture · Proliferation · Differentiation

ture system increases MSC proliferation and differentiation as compared to single cell type or randomly mixed coculture controls.

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Abstract

Purpose: During in vivo stem cell differentiation, mature cells often induce the differentiation of nearby stem cells. Accordingly, prior studies indicate that a randomly mixed coculture can help transform mesenchymal stem cells (MSC) into nucleus pulposus cells (NPC). However, because in vivo signaling typically occurs heterotopically between adjacent cell layers, we hypothesized that a structurally organized coculture between MSC and NPC will result in greater cell differentiation and proliferation over single cell-type controls and cocultures with random organization. **Methods:** We developed a novel bilaminar cell pellet (BCP) system where a sphere of MSC is enclosed in a shell of NPC by successive centrifugation. Controls were made using single cell-type pellets and coculture pellets with random organization. The pellets were evaluated for DNA content, gene expression, and histology. **Results:** A bilaminar 3D organization enhanced cell proliferation and differentiation. BCP showed significantly more cell proliferation than pellets with one cell type and those with random organization. Enhanced differentiation of MSC within the BCP pellet relative to single cell-type pellets was demonstrated by quantitative RT-PCR, histology, and in situ hybridization. **Conclusions:** The BCP cul-

Introduction

Harnessing the regenerative potential of stem cells is an important tissue-engineering strategy. Consequently, developing effective techniques to direct stem cell differentiation has been an active area of research for many musculoskeletal tissues. For example, stem cell pellet approaches show promise as they selectively recapitulate

Abbreviations used in this paper

BCP	bilaminar cell pellet
DMEM	Dulbecco's modified Eagle medium
FBS	fetal bovine serum
MSC	mesenchymal stem cells
MSCin	MSC on the inside
MSCout	MSC on the outside
NP	nucleus pulposus
NPC	nucleus pulposus cells
NPCin	NPC on the inside
NPCout	NPC on the outside
SCP	single cell-type pellets

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aspects of the embryonic microenvironments for regenerative purposes [Caplan et al., 1997; Hall and Miyake, 2000; Lee et al., 2001; Ichinose et al., 2005; Eames and Schneider, 2008; Merrill et al., 2008].

Our approach focuses on the intervertebral discs of the spine. The intervertebral disc is composed of a peripheral annulus fibrosus and a central nucleus pulposus (NP). The NP contains chondrocyte-like cells (NPC) embedded in a matrix of proteoglycan and type II collagen that is highly hydrophilic and causes the tissue to swell and resist compression hydrostatically [Urban et al., 2000]. This avascular tissue is a high-pressure, ischemic environment that is challenging for cell function and survival and often leads to disc degeneration [Turner et al., 1992]. The goal of intervertebral disc repair is to re-establish pain-free motion by restoring its physical and biochemical properties.

Many regenerative strategies include delivering rejuvenated, synthetically active cells. Autologous mesenchymal stem cells (MSC) are attractive for this purpose because they can differentiate into NPC [Yoo et al., 1998]. This approach has been used in several *in vivo* studies, yet the long-term functional regeneration of adult discs has not been achieved [Crevensten et al., 2004; Sakai et al., 2005; Zhang et al., 2005].

The benefits of MSC/NPC coculture have been demonstrated by several groups. In these systems, paracrine signaling induces differentiation and proliferation of MSC and NPC [Yang et al., 2008], and beneficial effects are greater with cell-cell contact [Yamamoto et al., 2004; Le Visage et al., 2006]. For example, Richardson et al. [2006] demonstrated MSC differentiation into NPC in monolayer with MSC-NPC contact. They observed that a 75% NPC:25% MSC ratio was optimum for MSC differentiation, which was the basis of our selection of this ratio. This ratio also takes advantage of the fact that MSC are much easier to procure than their adult NPC counterpart, which is a practical consideration for future development of technology. Several studies have since made similar observations in 3D culture using a random mixture of MSC and NPC [Vadala et al., 2008; Chen et al., 2009; Wei et al., 2009]. These experiments highlight the beneficial signaling effects of coculture. However, the approaches do not take advantage of heterotypic signaling (across an interface) and homotypic signaling (within the layer of the same cell type) that accompanies 3D organization and is part of natural organ development processes.

We report here on a spherical bilaminar cell pellet (BCP) approach that structures cell-cell signaling be-

tween MSC and NPC in a 3D culture system where one cell type forms an inner sphere enclosed within a shell formed by the other cell type. Our hypothesis is that organized coculture between MSC and NPC will result in greater cell differentiation and proliferation over single cell-type controls and cocultures with random organization. To test our hypothesis, we used our recently described structured coculture pellet method that creates a 3D tissue induction interface between stem cells and disc cells [Allon et al., 2009].

Materials and Methods

Cell Culture

Adult bovine NPC were isolated from caudal discs of 4 different animals and digested in 0.5% collagenase and 2% antibiotic in low-glucose Dulbecco's modified Eagle medium (DMEM) at 37°C. The cells were then pooled and expanded to the 4th passage in NPC media [DMEM with 1% antibiotic/antimycotic, 1.5% 400 mosm, and 5% fetal bovine serum (FBS)] at 37°C with 5% CO₂. Commercially available human MSC were purchased (Lonza, Switzerland). Cells were sourced from three different donors, pooled, and expanded to the 7th passage in growth media (DMEM, 1% antibiotic, and 10% FBS) at 37°C with 5% CO₂. Human NPC were obtained from a consenting 55-year-old female patient undergoing surgery for scoliosis and processed similarly to the bovine NPC. In addition, bovine MSC were isolated from femur tissue.

Pellet Formation

Three different types of pellets were formed, each consisting of 500,000 cells in total: pellets of 100% of one cell type, pellets of MSC and NPC with randomized organization, and pellets of MSC and NPC organized in a BCP. The coculture pellets were formed with 3 different ratios of 25/75, 50/50, and 75/25, respectively (fig. 1). Human MSC were mixed with bovine NPC for all the experiments. The other combinations (human MSC with human NPC, bovine MSC with bovine NPC) were tested as controls for cross-species interactions. The human MSC and bovine NPC were selected for coculture as a means for tracing the cells at later times based on their species for both immunohistochemistry and for gene expression. This enabled us to isolate the behavior of the MSC and NPC in culture.

For the single cell-type pellets (SCP), cells were pipetted into a 15-ml polypropylene tube and centrifuged at low speeds (300 g) for 5 min. For the randomized pellets, both cell types were added to the same tube and centrifuged at 300 g for 5 min. For the BCP, the cell type that would form the inner sphere of the pellet was centrifuged at 300 g for 5 min. 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. BCP were formed with MSC on the inside (MSCin) and NPC 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 3 days and then transferred to ultra-low attachment 24-well plates (Corning).

Fig. 1. Schematic of the eleven different experimental groups indicating their compositions, structures, and ratios.

To control for the effects of FBS, some pellets were also cultured in serum-free media (high-glucose DMEM, 1% antibiotic/antimitotic, 1% insulin-transferrin-selenious acid mix, 100 μ g/ml sodium pyruvate, 40 μ g/ml L-proline, 50 μ g/ml L-ascorbic acid 2-phosphate, with 100 nM dexamethasone). In addition, MSC SCP were cultured for 3 weeks in chondrogenic media [serum-free media supplemented with 5 ng/ml of TGF- β_1 ; Pepro-Tech, Rocky Hill, N.J., USA]. They were compared to 75% MSCin:25% NPCout BCP both in growth media and chondrogenic media.

Measuring DNA Content

Pellets were digested in papain (20 U/ml in PBS) at 60°C overnight and assayed with a Quant-iT PicoGreen kit (Invitrogen, Calif., USA) to measure DNA content at 525 nm absorption. Overall, 11 pellet groups were generated (fig. 1) for 3 time points (1, 2 and 3 weeks). The sample size was $n = 10$ per group. DNA content was converted to cell numbers by approximating that 6 pg of DNA are in each cell.

Histology and in situ Hybridization

The pellets were fixed and paraffin embedded. The sections were stained with safranin O. Within the BCP, we localized the human MSCs using human specific antibodies Lamp1 and Lamp2 (Abcam, Cambridge, Mass., USA), and in all pellets we localized aggrecan using protein-specific antibodies (Santa Cruz Biotechnology, Santa Cruz, Calif., USA). In situ hybridization was used to identify aggrecan and collagen-producing cells [Albrecht et al., 1997]. Sections were hybridized with 35 S-labeled human riboprobes to *aggrecan* and *col2a1* and counterstained with Hoechst dye (Sigma, St. Louis, Mo., USA). Hybridization signals were detected using illuminated darkfield and the nuclear stain visualized with epifluorescence.

Quantitative RT-PCR

At 3 weeks, MSC SCP, NPC SCP, and 75% MSCin:25% NPCout were harvested for RNA extraction. In order to have sufficient amounts of RNA, 5 pellets were pooled per group per time point. There was a total of 5 pooled samples for each group. RNA was extracted with a QIAshredder kit and an RNeasy Mini Kit (Qia-

gen, Germany). The RNA was reverse transcribed using iScript (Bio-Rad Laboratories, Hercules, Calif., USA).

Primers were carefully selected to be species specific and only detect the gene expression of the human MSC in one instance and the bovine NPC in the other. The samples were analyzed using Taqman species-specific primers for the following human and bovine genes: β_2 -microglobulin, Sox9, aggrecan, collagen I (col1), collagen II (col2), collagen X (colX), and matrix metalloproteinase 13 (MMP13; Applied Biosystems, Foster City, Calif., USA).

All measurements were conducted in triplicate and each sample was normalized to the housekeeping gene β_2 -microglobulin which was invariant with respect to the condition. The ddCt method [Pfaffl, 2001] was used to measure the fold change of the human MSC and the bovine NPC separately, which we were able to distinguish using the species-specific primers. Two controls were used, one for each of the cell types: the control for the human MSC in the pellet was the 3-week human MSC SCP and the control for the bovine NPC in the pellet was the bovine 3-week NPC SCP. The fold change for all the genes mentioned above was calculated against the appropriate control in each instance.

Data Analysis

All statistical analyses were performed using JMP statistical software (Version 5.0). Standard analysis of variance procedures were performed to compare group means and to estimate the effect of the specimen group variables. The Tukey-Kramer test was used to determine pair-wise statistical differences. An alpha of 0.05 was used to interpret statistical significance.

Results

Controls for Cross-Species Interaction and the Effects of FBS

Consistent with prior reports [Allon et al., 2009], we observed no significant differences between single species and cross-species pellets; therefore, all the other experiments were performed using human MSC and bovine

Fig. 2. Number of cells per pellet for each time point, conformation, and ratio. Pellets are made with human MSC and bovine NPC cultured in growth media.

NPC. We also observed no significant differences between using growth media or serum-free media; therefore, all other experiments were performed using growth media.

DNA Content

At 1 week, there were no significant differences between groups (fig. 2). At 2 weeks, MSC and NPC SCP did not experience any significant increase in cell numbers. The coculture pellets had a significantly higher DNA content than SCP ($p < 0.0001$). In addition, the ratio of 50/50 significantly increased cell numbers over the ratio of 25% MSC:75% NPC ($p < 0.0001$).

At 3 weeks, structural organization and ratio were both significant predictors of cell numbers. The coculture groups 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 significantly higher than SCP ($p < 0.0001$). SCP and the randomized pellets experienced a 4-fold increase in cell number as compared to 1

week. By contrast, BCP experienced a 10-fold increase in cell number, with the 50% MSCin:50% NPCout reaching a 14-fold increase. Overall, the 50% MSCin:50% NPCout BCP had significantly more DNA content than all other pellets ($p < 0.0001$) except for 25% MSCin:75% NPCout that was not significantly different.

Histology and in situ Hybridization

The sections were stained with safranin O to qualitatively detect the presence of proteoglycan (red staining). The MSCin:NPCout pellets stained more vibrantly (fig. 3). The human specific antibody enabled us to confirm that the desired configuration was made and maintained throughout the culture time (fig. 4a–d).

For the MSCin:NPCout group at 1 week, the NPC expressed aggrecan, while the MSC did not (fig. 4a, g, m). However, at 3 weeks, aggrecan protein and RNA is localized throughout the pellet (fig. 4b, h, n). Col2 expression also increased throughout the pellet over time (fig. 4t).

For the NPCin:MSCout group, at 1 week the aggrecan protein and RNA localized to the pellet periphery and in

Fig. 3. a–c 3-week pellets stained with safranin O (red indicates proteoglycan). Pellets are made with human MSC and bovine NPC culture in growth media. **a** MSC pellets. **b** NPC pellets. **c** MSCin 50:NPCout 50 pellets.

Fig. 4. Immunohistochemistry (counterstained with hematoxylin). **a–f** Stained with Lamp1&2 (human specific) antibody. **g–l** Stained with Aggreacan antibody (counterstained with Hoechst dye). **m–r** The location of Aggreacan gene expression is shown. **s–x** The location of col2 gene expression is shown.

Fig. 5. a–c Quantitative RT-PCR with species-specific Taqman primers was performed on 3-week pellets: MSC, NPC, and 75% MSCin:25% NPCout. Readings were normalized to the housekeeping gene β_2 -microglobulin. For human genes in coculture pellets, the fold change was calculated using the MSC SCP (**a, b**). For bovine genes in coculture pellets, the fold change was calculated using the NPC SCP (**c**). * $p < 0.05$.

a central pocket where NPC were located (fig. 4c, i, o). At 3 weeks, the aggrecan was expressed throughout the pellet; however, the central NPC appear to have lower levels of RNA expression (fig. 4d, j, p). There appears to be a slight increase in expression of col2 on the periphery of the pellet at 3 weeks (fig. 4v).

MSC exhibited faint staining of aggrecan protein and no staining of aggrecan or collagen RNA (fig. 4e, k, q, w). NPC exhibited aggrecan protein staining throughout (fig. 4f, l, r) but aggrecan RNA was not uniform, with a pronounced ring of expression on the periphery of the pellet with some central staining. Col2 RNA was very faintly expressed (fig. 4x).

Quantitative RT-PCR

We were able to distinguish the gene expression of the MSC and NPC within the 75% MSCin:25% NPCout BCP by using species-specific primers. The fold change was calculated with the species-appropriate SCP (MSC for human and NPC for bovine). The MSC in the BCP had a 2-fold upregulation of aggrecan, a 675.5-fold upregulation of col2, a 190.3-fold upregulation of Sox9, a 2.7-fold downregulation of MMP13, and an 8.2-fold downregulation of colX ($p < 0.05$) (fig. 5). Col1 gene expression showed no significant differences.

The NPC in the BCP had a 2.2-fold downregulation of aggrecan, a 2.9-fold downregulation of col2, a 4.9-fold downregulation of col1, and a 2-fold upregulation of MMP13. The levels of Sox9 gene expression showed no difference.

Discussion

We envision that cell-based therapies will eventually be used to treat disc degeneration [Rengachary and Balabhadra, 2002; Allon et al., 2010], which is the focus and motivation of the continued efforts in the field. Since notochordal cells are neither readily available nor practical for disc tissue engineering, it is fortunate that other cell types have also been shown to stimulate nucleus cells for the purposes of disc regeneration. For example, nucleus cells can be 'reactivated' by coculture with annulus fibrosus cells and, upon reinsertion in vivo, the reactivated nucleus cells retarded disc degeneration [Okuma et al., 2000]. Conversely, native nucleus cells can signal donor

cells. For instance, under certain coculture conditions with nucleus cells, adipose stem cells can be induced toward the nucleus-like phenotype [Lu et al., 2007]. Similar results have been reported for MSCs where monolayer contact with nucleus cells can cause them to differentiate into nucleus-like cells as evidenced by upregulation of Sox9, aggrecan, and collagen II [Yamamoto et al., 2004; Le Visage et al., 2006; Richardson et al., 2006; Vadala et al., 2008]. Coculture studies with chondrocytes and MSCs suggest morphogenetic factors secreted by chondrocytes (TGF- β and IGF-1) induce MSC differentiation and proliferation [Liu et al., 2010]. Importantly, in these monolayer coculture situations, the quantity of disc matrix synthesized was dependent on the ratio of the respective cell type, with the optimal ratio being 3 nucleus cells for each MSC. However, since the density of nucleus cells in degenerated discs is low, this optimal cell ratio would be difficult to achieve in practice by injecting MSCs alone. In contrast, we have devised a BCP-based therapeutic approach, with the pellet composed of undifferentiated MSCs and mature NPCs, where the cell-cell interactions are preorchestrated in vitro.

The goal of this study was to assess whether a 3D bilaminar coculture of MSC and NPC, which allowed for structured homotypic and heterotypic interactions, would enhance cell proliferation and differentiation. We have previously shown that BCP produce significantly more aggrecan protein than SCP [Apple et al., 2008]. Our data here indicate that BCP also result in significantly more cell proliferation, have favorable gene expression profiles, and appear to enhance MSC differentiation.

MSC:NPC interactions significantly affected the total number of cells in the pellet (fig. 2). At 3 weeks, pellet structure and cell ratio both became statistically significant predictors of cell number. While future studies involving FACS sorting would help elucidate which cell type is proliferating, our results show that the pellets benefited from more structural organization to fully exploit the trophic effects of coculture. In addition, histologic analyses indicated a qualitative boost in matrix synthesis in the BCP group (fig. 3). These data together indicate an increase in proliferation and matrix synthesis which are both desirable for a tissue engineering solution.

Additionally, BCP induce MSC differentiation toward an NPC-like phenotype, with significant increases in the expression of aggrecan, col2, and Sox9 (fig. 5). Importantly, the downregulation of colX and MMP13 are indicative of the advantage of using coculture techniques in differentiating MSC as opposed to growth factors [Mueller et al., 2010]. MMP13 is commonly expressed in diseased

discs and causes further degeneration of the tissue; its up-regulation would hinder any therapeutic effect the BCP would provide. In situ hybridization provided further insight by showing that MSC within the MSCin:NPCout pellet began both expressing and secreting aggrecan over 3 weeks of culture time (fig. 4). The NPC within the MSCin:NPCout pellets downregulated aggrecan and col2 but maintained Sox9 gene expression levels. This may be indicative of the NPC's signaling role within the BCP as opposed to their purely secretory role in the NPC.

Overall, our experiments demonstrate an advantage to structured coculturing with MSCin and NPCout. This may be due to the fact that MSC propagate signals more readily amongst themselves than with other cell types [D'Andrea and Vittur, 1996; D'Andrea et al., 1998; Zhang et al., 2002; Valiunas et al., 2004], and that the internal microenvironment within the BCP may favor chondrogenic differentiation via growth-related pressurization and diminished oxygen availability. It is possible the BCP's organized signaling interface between MSC and NPC exploits this feature. Future studies will include testing BCP performance in culture conditions that more closely mimic the painful disc environment (ischemia and inflammation), as well as measuring paracrine effects on host disc cells. In previous work, we have shown in a rat model that BCP can rescue a degenerating disc with significantly better disc height, disc quality, and implanted cell retention at a 5-week time point than SCP and injected cells in a fibrin suspension [Allon et al., 2010]. Ultimately, we anticipate that beneficial BCP features reported here will provide measurable advantages for nucleus regeneration in preclinical studies with small and large animals.

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