Conditioned Medium Derived from Notochordal Cell-Rich Nucleus Pulposus Tissue Stimulates Matrix Production by Canine Nucleus Pulposus Cells and Bone Marrow-Derived Stromal Cells

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Objectives: Conditioned medium derived from notochordal cell-rich nucleus pulposus tissue (NCCM) was previously shown to have a stimulatory effect on bone marrow stromal cells (BMSCs) and nucleus pulposus cells (NPCs) individually, in mixed species *in vitro* cell models. The objective of the current study was to assess the stimulatory effect of NCCM on NPCs in a homologous canine *in vitro* model and to investigate whether combined stimulation with NCCM and addition of BMSCs provides a synergistic stimulatory effect.

Methods: BMSCs and NPCs were harvested from chondrodystrophic dogs with confirmed early intervertebral disc (IVD) degeneration. NCCM was produced from NP tissue of nonchondrodystrophic dogs with healthy IVDs. BMSCs or NPCs alone (3×10^6 cells/mL) and NPCs+BMSCs (6×10^6 cells/mL; mixed 1:1) were cultured for 4 weeks in 1.2% alginate beads under base medium (BM), NCCM, or with addition of 10 ng/mL transforming growth factor- β 1 (TGF- β 1) as a positive control. Beads were assessed for glycosaminoglycan (GAG) and DNA contents by biochemical assays, GAG deposition by Alcian blue staining, and gene expression (aggrecan, versican, collagen 1 and 2, *SOX9*, A disintegrin and metalloproteinase with thrombospondin motifs 5 (ADAMTS5), and matrix metalloproteinase 13 [MMP13]) with real-time quantitative RT-PCR.

Results: NCCM increased NPC proliferation, proteoglycan production, and expression of genes associated with a healthy NP-like phenotype. BMSCs also showed increased proteoglycan production under NCCM, but these effects were not observed at the gene level. Combined stimulation of NPCs with NCCM and coculturing with BMSCs did not result in increased proteoglycan content compared to stimulation with NCCM alone.

Discussion: NCCM stimulates matrix production by both NPCs and BMSCs and directs NPCs toward a healthier phenotype. NCCM is therefore promising for IVD regeneration and identification of the bioactive components will be helpful to further develop this approach. In the current study, no synergistic effect of adding BMSCs was observed.

Introduction

THE INTERVERTEBRAL DISC (IVD) consists of a hydrated gel-like nucleus pulposus (NP), constrained by a collagenous fibrous outer layer, the annulus fibrosus. The NP consists mainly of proteoglycans embedded in a collagen network. The proteoglycans attract water, thereby creating a high osmotic environment, which is critical for transmitting loads and allowing flexibility to the spine. The degenerating IVD is characterized by a change of cell phenotype and

decreasing number of the resident nucleus pulposus cells (NPCs)¹; a shift in the NP matrix composition where collagen type 2 and proteoglycans are replaced by collagen type 1² and increased production of enzymes degrading the matrix.³ These changes result in a decrease of NP swelling pressure, and compressive loads are increasingly exerted on the annulus, which can eventually cause crack formation and rupture. IVD degeneration is associated with low back pain⁴ and current treatment methods mostly aim to alleviate the pain, but do not address the underlying causes of IVD degeneration.

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As degeneration is also characterized by a decreasing cell population, bone marrow stromal cells (BMSCs) have previously been proposed as a potential cell source to restore the IVD. In coculture with NPCs, BMSCs can acquire a phenotype consistent with that of NPCs.^{5,6} Furthermore, BMSCs transplantation into the IVD has shown to promote matrix synthesis and to delay or arrest degenerative changes, such as decrease in disc height or drop in water content.^{7–10} However, addition of BMSCs did not restore the IVD to a healthy state, indicating that additional or another type of stimulation is needed.

Alternatively, NP regeneration could also be achieved by stimulation of the remaining NPCs. Notochordal cells (NCs) are a promising alternative for NPC stimulation. They are presumed remnants of the embryonic notochord.¹¹ Although their exact role in the postdevelopmental disc is not identified, the observation that early disappearance of these cells in certain species (humans and chondrodystrophic dog breeds) coincides with the onset of IVD degeneration suggests NC involvement in IVD homeostasis.¹² Although species retaining their NC population, for example, nonchondrodystrophic dog breeds, also develop IVD degeneration, they do so in known isolated locations due to wear and tear of the IVD, whereas the majority of their discs remain healthy until the end of life.¹³ Some studies have already examined the stimulatory effect of NCs on NPCs. Culturing bovine NPCs in a canine NC-conditioned medium, produced from NCs in alginate beads, has shown to increase proliferation,¹⁴ proteoglycan synthesis,^{14,15} and enhanced expression of genes associated with the chondrogenic phenotype.¹⁶ Interestingly, a porcine conditioned medium from NCs in alginate beads or from notochordal cell-rich nucleus pulposus tissue (NCCM) also directed human BMSCs toward a chondrogenic phenotype and increased BMSC proteoglycan production.^{17,18} For human NPCs, the conditioned medium produced from porcine NCs in alginate beads provided a more efficient stimulation than NCCM from NCs maintained within NP tissue.¹⁹ For human BMSCs, however, NCCM produced from porcine NCs in NP tissue resulted in a higher increase in proteoglycan production and enhanced chondrogenic gene expression.¹⁸ Although encouraging for NCCM, it is still possible that some of these effects are partially due to the use of a heterologous experimental design.

In the field of NCs, the use of a canine model offers specific advantages. Nonchondrodystrophic dog breeds retain a healthy NC-rich NP throughout the largest part of their life. Chondrodystrophic breeds, however, lose their NC population and start developing IVD degeneration relatively early in life.¹³ These different dog breeds allow us to obtain healthy NC-rich NP tissue, as well as a homogeneous NPC population within the same species, contrary to other models where NCs and NPCs are obtained from different species. In cross-species models, effects of NCCM may be underestimated, since not all growth factors and cytokines are cross-species efficient. The strength of a homologous model is therefore to ensure that all factors derived from NC-rich NP tissue can have an effect on NPCs and BMSCs, which may not be the case for a cross-species model. Furthermore, dogs spontaneously develop IVD degeneration as humans, and it is known that the appearance of IVD degeneration and diagnostic and treatment methods are similar between humans and dogs.^{20–22}

Since a single-species *in vitro* model has not been used before, the first goal of this study is to confirm the stimu-

latory effects of NCCM on NPCs and BMSCs in a canine model. Furthermore, this study aims to investigate whether stimulation of NPCs with NCCM and BMSCs together has a synergistic effect compared to stimulation with BMSCs or NCCM alone.

Materials and Methods

NPCs and BMSCs were harvested from 2- to 2.5-year-old Beagles, sacrificed for unrelated experiments, which were approved by the ethics committee on animal experimentation of Utrecht University. To harvest NPCs, the IVDs were opened within hours after euthanasia under aseptic conditions and the NP tissue was quickly removed with a scalpel and forceps. The technique of harvesting NPs has been developed by experienced board-certified veterinary surgeons (Björn P. Meij and Marianna Tryfonidou), who are familiar with IVD-related spine surgeries in canine patients with IVD diseases. The technique was optimized to expose and retrieve 26 discs (6 cervical, 13 thoracolumbar, and 7 lumbar IVDs) within the timespan of 1 h in a canine cadaveric spine. Care was taken to only include NP tissue and not annulus fibrosus or endplate tissue. The tissue was then digested in 0.1% pronase (Roche Diagnostics, Mannheim, Germany) for 45 min at 37°C and subsequently in 0.15% collagenase type 2 (Worthington, Lakewood, NJ) for 16 h at 37°C. After digestion, the solution was filtered with a 70- μ m cell strainer (BD Biosciences, San Jose, CA) to collect the NPCs in the filtrate, which were centrifuged and resuspended in a freezing medium [DMEM/F-12 GlutaMAX (Gibco, Carlsbad, CA) with 10% fetal calf serum (FCS; Gibco) and 10% dimethyl sulfoxide (DMSO; Merck, Darmstadt, Germany)] and cryopreserved in liquid nitrogen until further use. BMSCs were harvested from the diaphysis of humeri, according to a previously described protocol,²³ and expanded in α MEM+10% FCS Gold (PAA Laboratories, Pasching, Austria)+1% penicillin/streptomycin (pen/strep) until subconfluency, at which time BMSCs were trypsinized and resuspended in the freezing medium for cryopreservation in liquid nitrogen at P0.

NCCM was produced from NC-rich NP tissue from 1- to 1.5-year-old nonchondrodystrophic mixed breed dogs (five donors, one donor per repetition). The IVDs were opened under aseptic conditions, and the NP tissue was taken out with a curette and forceps. The tissue was placed under serum-free conditions in high-glucose (hg) Dulbecco's modified Eagle's medium (DMEM; Gibco)+1% pen/strep without further additives at 1 g tissue per 30 mL medium and incubated for 4 days at 37°C, 5% CO₂, and 5% O₂. Subsequently, the medium was collected and filtered through a 70- μ m cell strainer to remove large pieces of tissue before being filtered with a 3 kDa cut-off filter tube (Amicon Ultra-15 Centrifugal filter; Merck). The strained material was resuspended in the same amount of new hgDMEM to obtain a final 1 $\times$ concentration and stored in aliquots at -80°C until further use.

BMSCs and NPCs were thawed and expanded for a single passage 1 week before the start of an experiment to have enough cells. NPCs were expanded in hgDMEM+10% FCS+1% pen/strep, and BMSCs were expanded in α MEM+10% FCS Gold+1% pen/strep, both at 37°C, 21% O₂, and 5% CO₂. NPCs and BMSCs alone were suspended

TABLE 1. EXPERIMENTAL GROUPS

	BM (cells/mL)	NCCM (cells/mL)	TGF (cells/mL)
NPCs	3×10^6	3×10^6	3×10^6
BMSCs	3×10^6	3×10^6	3×10^6
NPCS + BMSCs	3×10^6 + 3×10^6	3×10^6 + 3×10^6	3×10^6 + 3×10^6

BM, base medium; BMSC, bone marrow stromal cells; NCCM, notochordal cell-rich nucleus pulposus tissue; NPC, nucleus pulposus cell; TGF, transforming growth factor.

in 1.2% alginate (Sigma, Zwijndrecht, The Netherlands) at 3×10^6 cells/mL. For the mixture group (NPCs + BMSCs), NPCs and BMSCs were mixed at a 1:1 ratio and suspended at 6×10^6 cells/mL in 1.2% alginate. Alginate beads were produced according to a previously described protocol.²⁴ The beads were cultured for 4 weeks at 37°C, 5% CO₂, and 5% O₂, in three different medium groups for single-cell cultures (Table 1); the base medium [BM: hgDMEM + 5% stripped FCS (PAA Laboratories) + 1% pen/strep], conditioned medium (NCCM: supplemented with 5% stripped FCS + 1% pen/strep, one donor per repeat), or in the positive control medium [transforming growth factor (TGF): BM + 10 ng/mL TGF-β1 (PeproTech, Rocky Hill, NJ)], and in BM and NCCM for the coculture. Stripped FCS was used to diminish the confounding effect of the growth factors included in normal FCS. For each cell type (BMSC and NPC), two independent donors for BMSCs and NPCs were pooled per repetition, with a total of five repetitions. The medium was changed twice a week.

At day 1 and 28, two beads per group were placed in 10 μM calcein AM (excitation: 495 nm, emission: 515 nm; Sigma) and 10 μM propidium iodide (excitation: 535 nm, emission: 617 nm; Invitrogen, Carlsbad, CA) in phosphate-buffered saline. The beads were incubated in the dark for 30 min at 37°C, before cells were visualized for viability

with a confocal microscope (LSM 510Meta; Zeiss, Slie-drecht, The Netherlands).

At day 0 and 28, five beads per group were collected and stored at -80°C until assayed for biochemical contents. The samples were digested overnight at 60°C in papain solution (100 mM phosphate buffer, 5 mM L-cysteine, 5 mM ethylene diamine tetra-acetic acid, and 140 μg/mL papain; all from Sigma). From the digested samples, the glycosaminoglycan (GAG) content was analyzed with a dimethyl-methylene blue (DMMB) assay, modified from a previous protocol,²⁵ using shark cartilage chondroitin sulfate (Sigma) as reference, and the DNA content was measured with the Qubit Quantification Platform (Invitrogen). At day 28, three beads per independent sample were fixed overnight in 3.7% formalin at room temperature and embedded in paraffin. Sections with a thickness of 8 μm were cut and stained with Alcian blue (Sigma) for visualization of proteoglycan deposition and with hematoxylin for visualization of cell nuclei. Pictures were taken with a bright field microscope (Observer Z1; Carl Zeiss, Jena, Germany).

At day 0 and 28, five beads were collected, pooled, and incubated in 1 mL sodium citrate buffer [55 mM trisodiumcitrate-2-hydrate (Merck), 0.15 M sodium chloride (Merck), 25 mM HEPES (Sigma) in RNase-free water, pH adjusted to 7.4] for 5 min at room temperature to dissolve the alginate. After centrifugation, the cell pellet was lysed with 300 μL RLT buffer (Qiagen, Venlo, The Netherlands) and stored at -80°C. RNA was extracted and purified using the Qiagen mini-kit (Qiagen) with an on-column DNase digestion step. The quantity and purity of RNA were measured with a spectrophotometer (ND-1000; Isogen, de Meern, The Netherlands). The absence of genomic DNA contamination in isolated RNA was verified with a minus-RT control reaction (iCycler; Bio-Rad, Veenendaal, The Netherlands). cDNA was synthesized using the VILO-kit (Invitrogen). Genes of interest and their corresponding primer pairs are summarized in Table 2. 18S ribosomal RNA (18s) was selected as the reference gene from three tested

TABLE 2. PRIMER SEQUENCES FOR TARGET AND REFERENCE GENES USED IN RT-qPCR ASSAYS

Gene	Accession number	Oligonucleotide sequence	Product size (bp)	Annealing temperature (°C)
18s rDNA	NR_036642	Forward: CCTTCCTCAAAAAGTCTGGG Reverse: GTTCTCATCGTAGGGAGCAAG	95	61
ACAN	XM_005618252.1	Forward: GGACACTCCTTGCAATTTGAG Reverse: GTCATTCCACTCTCCCTTCTC	110	61
COL1A1	NM_001003090.1	Forward: GTGTGTACAGAACGGCCTCA Reverse: TCGCAAATCACGTCATCG	111	61
COL2A1	NM_001006951	Forward: GCAGCAAGAGCAAGGAC Reverse: TTCTGAGAGCCCTCGGT	150	61
VCAN	XM_003434417.2	Forward: TCTCAAGAAGCATCCTGTCTCAC Reverse: CCATCGGTCCAACGGAAGTC	116	61
SOX9	NM_001002978.1	Forward: CGCTCGCAGTACGACTACAC Reverse: GGGGTTCATGTAGGTGAAGG	105	63
MMP13	XM_536598.3	Forward: CCCAAGTGGAGGAAACTCA Reverse: CACCTCCTTCCAGACATTCAG	114	66
ADAMTS5	XM_846025.3	Forward: CTACTGCACAGGGAAGAG Reverse: GAACCCATTCCACAAATGTC	148	61

genes [*18S*, glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) and ribosomal protein *S19* (*RPS19*)] as it was the most stable gene in our experimental conditions. Gene expression was investigated using real-time PCR (CFX384; Bio-Rad) and expression is reported according to the $2^{-\Delta\Delta C_t}$ method.²⁶ The efficiency of each primer was tested for the used experimental conditions (ranging from 92.5% to 102.1%), and Pfaffl's correction was used to correct for differences in primer efficiency.

SPSS 21 (IBM, Armonk, NY) was used for statistical analysis. A Kruskal–Wallis test followed by Mann–Whitney *U post hoc* testing with a Bonferroni correction was used. Significant differences were assumed for $p < 0.05$.

Results

Stimulation of NPCs with NCCM

For one repetition, the DNA content at day 0 was far lower compared to other repetitions. For consistency, this repetition was excluded from analysis. Viability staining demonstrated only occasional cell death in all groups at day 0 and 28 (data not shown). At day 28, the DNA content decreased in BM, remained stable in NCCM, and doubled in the TGF group (Fig. 1a). The GAG content doubled in the NCCM group compared to the BM group and this was confirmed by Alcian blue staining with intense staining also in the immediate pericellular area (Fig. 1b). Addition of TGF caused a further increase in GAG production (Fig. 1a, b). Collagen type 1 gene expression was not affected by any of the medium conditions (Fig. 1c). After 28 days of culture, collagen type 2

and aggrecan gene expression was increased almost 10-fold with NCCM compared to BM, close to levels found with TGF stimulation at that time point. Versican and *SOX9* gene expression was significantly higher with BM and NCCM compared to TGF. *ADAMTS5* gene expression decreased significantly with NCCM compared to BM and decreased further with TGF. *MMP13* gene expression significantly decreased with TGF compared to BM and NCCM and tended to decrease in NCCM relative to BM ($p = 0.08$).

Stimulation of BMSCs with NCCM

In NCCM and BM groups, DNA content decreased compared to day 0, although only significantly in TGF. NCCM, however, did have an effect on GAG content (Fig. 2a), which increased ca. 40% compared to BM. This was also observed in Alcian blue staining (Fig. 2b). TGF had, in contrast with NPCs, no stimulatory effect on BMSCs, that is, GAG per bead content did not increase compared to BM. Collagen type 1 and versican gene expression slightly decreased, whereas collagen type 2, aggrecan, and *SOX9* increased, with no significant differences between medium conditions. *ADAMTS5* remained stable in BM and NCCM, but significantly increased with TGF. No differences between medium conditions on *MMP13* gene expression were observed.

Stimulation of NPCs with combined NCCM and BMSCs

For NPCs+BMSCs in BM and NCCM, the DNA content decreased significantly compared to day 0, to amounts

FIG. 1. (a) Nucleus pulposus cell (NPC) DNA (day 0 and 28) and glycosaminoglycan (GAG) (day 28) per bead content. (b) Alcian blue staining. (c) NPC gene expression relative to *18S* and normalized to day 0. In (a, c), bars indicate $p < 0.05$. Values are means + standard deviations, $n = 4$ per group. BM, base medium; NCCM, conditioned medium from notochordal cell-rich NP tissue; TGF, transforming growth factor $\beta 1$. Color images available online at www.liebertpub.com/tea

FIG. 2. (a) Bone marrow stromal cells (BMSC) DNA (day 0 and 28) and GAG (day 28) per bead content. (b) Alcian blue staining. (c) BMSC gene expression relative to 18S and normalized to day 0. In (a, c), bars indicate $p < 0.05$. Values are means + standard deviations, $n = 5$ per group. Color images available online at www.liebertpub.com/tea

similar to NPCs alone. Addition of BMSCs to NPCs did not alter GAG production, but combined with NCCM, the GAG content significantly increased compared to NPCs in BM (Fig. 3a). These results were confirmed by Alcian blue staining within intense staining in the immediate pericellular area and more diffuse blue staining in the alginate bead (Fig. 3b). Addition of BMSCs to NPCs, with or without NCCM, resulted in a significant decrease of collagen type 1 and versican gene expression compared to NPCs alone in BM (Fig. 3c). Collagen type 2 and aggrecan gene expression increased significantly with addition of NCCM, BMSCs, and their combination compared to NPCs alone in BM, although the highest upregulation was found in the NCCM-containing groups. ADAMTS5 gene expression significantly decreased for NPCs in NCCM compared to BM. However, addition of BMSCs significantly increased ADAMTS5 expression, while combined stimulation with NCCM and BMSCs did not significantly change ADAMTS5 gene expression compared to BM. No significant differences were observed between stimulatory conditions for *SOX9* and *MMP13*.

Discussion

This study confirmed that, in a canine model, NC-secreted factors had a stimulatory anabolic effect on NPCs and BMSCs. For NPCs, this was demonstrated by increased cell proliferation and GAG production, as well as enhanced expression of genes associated with a healthy NP phenotype, such as collagen type 2 and aggrecan. For BMSCs, the

stimulatory effect of NCCM was shown by the improved proteoglycan deposition. Furthermore, combined stimulation of NPCs with NCCM and addition of BMSCs did not have a synergistic effect compared to NCCM alone.

The stimulatory effect of NCCM on NPCs and BMSCs found in the current single species experiment is in line with other cross-species studies. For NPCs, previous studies have tested the effect of NCCM produced from canine NCs in alginate beads on bovine NPCs. Similar to the present findings, increases in proteoglycan production^{14–16} and cell proliferation¹⁴ were found. This effect was also found at the gene level. In the current study, the fact that aggrecan and collagen type 2 increased, while ADAMTS5 gene expression decreased, and MMP13 tended to decrease compared to BM, suggests that NCCM displays an anabolic as well as an anticatabolic effect on NPCs. After 28 days of culture, collagen type 2 and aggrecan gene expression levels of NPCs in NCCM approach the levels found with TGF- β 1 stimulation. Expression levels of *SOX9*, associated with a healthy NP phenotype,²⁷ and versican are even higher with NCCM than with TGF- β 1, indicating that NCCM may provide a more specific type of stimulation than TGF- β 1. However, since far more proteoglycans were produced with TGF- β 1 stimulation, it seems plausible that expression levels of anabolic genes peaked at some point before the end of the culture period when the gene expression was evaluated.

For BMSCs, a mixed model has been used in which human BMSC pellets were formed in the presence or absence of TGF- β 3 during the first day of culture and followed by assessment of the effects of bovine NCCM on the chondrogenically induced

FIG. 3. (a) NPC and NPC + BMSC DNA (day 0 and 28) and GAG (day 28) per bead content. (b) Alcian blue staining. (c) NPC + BMSC gene expression relative to *18S* and normalized to day 0. In (a, c), bars indicate $p < 0.05$. Values are means + standard deviations, $n = 4$ per group. Color images available online at www.liebertpub.com/tea

BMSCs. These studies reported improvement of proteoglycan production^{17,18} and expression of collagen type 2 and *SOX9* only in the presence of TGF- β ¹⁸ with NCCM. In the current study, however, the drop in DNA content in every medium group (although only significant with TGF- β 1) indicates that BMSCs did not thrive, possibly due to suboptimal culture conditions, such as the alginate bead culture method, hypoxia,²⁸ or the absence of chondrogenic medium, during the first day of culture. Still, stimulation of BMSCs with NCCM resulted in increased proteoglycan production, in line with the previous mixed model studies. Although collagen type 2, aggrecan, and *SOX9* gene expression increased compared to day 0, indicating BMSC differentiation, no differences were observed between medium conditions. As opposed to NPCs, BMSCs did not respond to the addition of TGF- β 1. This may be because in this study, only TGF- β 1 was added, without other commonly used additives to induce chondrogenesis of BMSCs, such as dexamethasone. NPCs do respond to the presence of NCCM, while BMSCs that were not pretreated with TGF- β to induce chondrogenic differentiation were less responsive. Based on these observations and given the distinct effects of NCCM on pretreated BMSCs as reported by Purmessur,¹⁸ we suggest that NCCM may have its regenerative effects mainly on cells within the chondrogenic lineage rather than on undifferentiated BMSCs.

Previous studies reported that in *in vivo* animal models with induced IVD degeneration, injection of BMSCs delayed or arrested degenerative changes such as the decreasing disc height index or the drop in water content.^{7–10} BMSCs can have a stimulatory effect in two ways. First, under proper conditions, BMSCs can differentiate toward an

NPC-like phenotype, after which they start producing proteoglycan themselves.^{6,29–31} Second, BMSCs can provide a stimulatory effect on NPC proliferation and proteoglycan production.^{6,32} Although in the current study, addition of BMSCs to NPCs in BM as well as in NCCM did not lead to an increased proteoglycan content, it did increase gene expression levels of collagen type 2 and aggrecan in BM. Furthermore, the highest GAG per DNA ratio was observed in the NPC + BMSC group in BM (18.3 ± 8.5 to 13.0 ± 5.8 for NPCs alone in BM and 15.9 ± 4.8 for NPCs in NCCM). These may be trophic effects of the BMSCs on the NPCs, which were also observed in cocultures of BMSCs with articular chondrocytes.^{33,34} These and other studies^{35,36} reported that in coculture, the minority of the BMSCs differentiated, whereas the remaining BMSCs underwent apoptosis. This is in line with the drop in DNA content in the BMSC monoculture and NPC-BMSC coculture in the current study. Although in the coculture twofold more cells were initially present compared with the monocultures, it is unlikely that the drop in DNA content in the coculture is caused by a nutrient deficiency, since DNA content for NPCs in a positive control medium reached levels similar to that of the initial DNA content in the coculture group, without any indication of cell death after 28 days of culture. This would not have been possible without enough nutrients to support this growth. Although the GAG content did not increase, BMSCs may still exert a stimulatory effect on NPCs. With optimized BMSC culture conditions, their addition to NPCs may also result in increased matrix production, and therefore, in addition, a synergistic effect of NCCM and BMSCs cannot yet be excluded.

TGF- β 1 provided a stronger stimulation to NPCs than NCCM, with substantially more proteoglycan production and expression of collagen type 2 and stronger inhibition of catabolic genes (ADAMTS5 and MMP13). Compared to TGF- β 1, the effect of NCCM may seem inconsequential. However, the concentration of TGF- β 1 used in the current study is far higher than physiological concentrations, and the unknown concentration of bioactive factors in NCCM may be orders of magnitude lower. Since production of NCCM for IVD regeneration purposes would require a large amount of NC-rich NP tissue, a more efficient method would be to identify the factor, or combination of factors, responsible for the stimulatory effect of NCCM. Analysis of the concentration of TGF- β 1, connective tissue growth factor,¹⁶ or other proposed candidates present in NCCM¹⁸ and evaluation of the stimulatory effect of this concentration could provide a basis for finding the bioactive ingredients in NCCM. Furthermore, the effect of NCCM has so far been shown in simplified models, where cells are taken from their native environment and seeded in alginate beads, which will likely affect the way cells respond to external stimuli. Also, characteristics of the discogenic environment, such as low pH and high osmolarity, are not incorporated in this study. These factors are, however, likely to affect cell behavior. To gain further insight in the biological significance of NCs in IVD regeneration, it is recommended to test the stimulatory effect of NCCM in tissue explant cultures.

Given that DMMB measurements showed that a substantial amount of GAGs was present in NCCM, we questioned whether this finding may have confounded the study results. These GAGs are likely to have had a minimal effect on determining the GAG content of the alginate beads. First, alginate beads were washed with phosphate-buffered saline before the digestion and subsequent DMMB measurements to ensure that loose GAGs from the NCCM were removed. Second, if GAGs would have adhered to the beads, even with washing, we would have expected to see a dense blue ring around the beads with the Alcian blue staining, which was not the case. Finally, we observed from the Alcian blue staining that the most intense blue staining (i.e., the highest GAG density) was located mainly pericellularly and in the areas with the highest cell density, gradually decreasing toward areas with a lower cell density. This finding indicates that the GAGs have actually been formed by the cells themselves and deposited in their pericellular matrix, rather than being deposited from the NCCM. Altogether, we therefore believe that the GAGs in NCCM had a negligible effect on the DMMB measurements in this study.

In conclusion, the current results show that in a canine model, NCCM stimulates NPC proliferation, GAG production, and expression of genes associated with a healthy NP phenotype. Although BMSCs also responded with increased GAG production, they appear less sensitive to NCCM stimulation than NPCs, indicating that BMSCs may need special conditions to enable them to respond optimally to NCCM stimulation. No effect of addition of BMSCs to NPCs was observed, possibly due to culture conditions that did not allow the BMSCs to thrive. Therefore, we cannot conclude on the stimulatory effect of BMSCs on NPCs. Nonetheless, the use of NC-secreted factors as a therapeutic approach for IVD regeneration, even with the addition of BMSCs, seems

promising, and identification of the bioactive factors in NCCM would be helpful to further develop this approach.

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Disclosure Statement

No competing financial interests exist.

References

1. Roberts, S., Evans, H., Trivedi, J., and Menage, J. Histology and pathology of the human intervertebral disc. *J Bone Joint Surg Am* **88**, 10, 2006.
2. Antoniou, J., Steffen, T., Nelson, F., Winterbottom, N., Hollander, A.P., Poole, R.A., Aebi, M., and Alini, M. The human lumbar intervertebral disc. *J Clin Invest* **98**, 996, 1996.
3. Le Maitre, C.L., Freemont, A.J., and Hoyland, J.A. Localization of degradative enzymes and their inhibitors in the degenerate human intervertebral disc. *J Pathol* **205**, 47, 2004.
4. Luoma, K., Riihimäki, H., Luukkonen, R., Raininko, R., Viikari-Juntura, E., and Lamminen, A. Low back pain in relation to lumbar disc degeneration. *Spine* **225**, 487, 2000.
5. Tao, F., Li, F., Li, G., and Pan, F. Differentiation of mesenchymal stem cells into nucleus pulposus cells *in vitro*. *J Huazhong Univ Sci Technol* **28**, 156, 2008.
6. Vadalà, G., Studer, R., Sowa, G., Spiezia, F., Iucu, C., Denaro, V., Gilbertson, L.G., and Kang, J.D. Coculture of bone marrow mesenchymal stem cells and nucleus pulposus cells modulate gene expression profile without cell fusion. *Spine* **33**, 870, 2008.
7. Yang, F., Leung, V.Y.L., Luk, K.D.K., Chan, D., and Cheung, K.M.C. Mesenchymal stem cells arrest intervertebral disc degeneration through chondrocytic differentiation and stimulation of endogenous cells. *Mol Ther* **17**, 1959, 2009.
8. Hiyama, A., Mochida, J., Iwashina, T., Omi, H., Watanabe, T., Serigano, K., Tamura, F., and Sakai, D. Transplantation of mesenchymal stem cells in a canine disc degeneration model. *J Orthop Res* **26**, 589, 2007.
9. Sakai, D., Mochida, J., Yamamoto, Y., Nomura, T., Okuma, M., Nishimura, K., Nakai, T., Ando, K., and Hotta, T. Transplantation of mesenchymal stem cells embedded in Atelocollagen® gel to the intervertebral disc: a potential therapeutic model for disc degeneration. *Biomaterials* **24**, 3531, 2003.
10. Sakai, D., Mochida, J., Iwashina, T., Hiyama, A., Omi, H., Imai, M., Nakai, T., Ando, K., and Hotta, T. Regenerative effects of transplanting mesenchymal stem cells embedded in atelocollagen to the degenerated intervertebral disc. *Biomaterials* **27**, 335, 2006.
11. Hunter, C.J., Matyas, J.R., and Duncan, N.A. The notochordal cell in the nucleus pulposus: a review in the context of tissue engineering. *Tissue Eng* **9**, 667, 2003.
12. Cappello, R., Bird, J., and Pfeiffer, D. Notochordal cell produce and assemble extracellular matrix in a distinct manner, which may be responsible for the maintenance of healthy nucleus pulposus. *Spine* **31**, 873, 2006.
13. Smolders, L.A., Bergknut, N., Grinwis, G.C., Hagman, R., Lagerstedt, A.S., Hazewinkel, H.A., Tryfonidou, M.A., and Meij, B.P. Intervertebral disc degeneration in the dog. Part

- 2: chondrodystrophic and non-chondrodystrophic breeds. *Vet J* **195**, 292, 2013.
14. Erwin, W.M., and Inman, R.D. Notochord cells regulate intervertebral disc chondrocyte proteoglycan production and proliferation. *Spine* **31**, 1094, 2006.
15. Aguiar, D.J., Johnson, S.L., and Oegema, T.R. Notochordal cells interact with nucleus pulposus cells: regulation of proteoglycan synthesis. *Exp Cell Res* **246**, 129, 1999.
16. Erwin, W.M., Ashman, K., O'Donnel, P., and Inman, R. Nucleus pulposus notochord cells secrete connective tissue growth factor and up-regulate proteoglycan expression by intervertebral disc chondrocytes. *Arthritis Rheum* **54**, 3859, 2006.
17. Korecki, C.L., Taboas, J.M., Tuan, R.S., and Iatridis, J.C. Notochordal cell conditioned medium stimulates mesenchymal stem cell differentiation toward a young nucleus pulposus phenotype. *Stem Cell Res Ther* **1**, 18, 2010.
18. Purmessur, D., Schek, R.M., Abbot, R.D., Ballif, B.A., Godburn, K.E., and Iatridis, J.C. Notochordal conditioned media from tissue increases proteoglycan accumulation and promotes a healthy nucleus pulposus phenotype in human mesenchymal stem cells. *Arthritis Res Ther* **13**, R81, 2011.
19. Abbott, R.D., Purmessur, D., Monsey, R.D., and Iatridis, J.C. Regenerative potential of TGF β 3+Dex and notochordal cell conditioned media on degenerated human intervertebral disc cells. *J Orthop Res* **30**, 482, 2012.
20. Bergknut, N., Rutges, J.P., Kranenburg, H.J., Smolders, L.A., Hagman, R., Smidt, H.J., Lagerstedt, A.S., Penning, L.C., Voorhout, G., Hazewinkel, H.A., Grinwis, G.C., Creemers, L.B., Meij, B.P., and Dhert, W.J. The dog as an animal model for intervertebral disc degeneration? *Spine* **37**, 351, 2012.
21. Bergknut, N., Smolders, L.A., Grinwis, G.C., Hagman, R., Lagerstedt, A.S., Hazewinkel, H.A., Tryfonidou, M.A., and Meij, B.P. Intervertebral disc degeneration in the dog. Part 1: anatomy and physiology of the intervertebral disc and characteristics of intervertebral disc degeneration. *Vet J* **195**, 282, 2013.
22. Lotz, J. Animal models of intervertebral disc degeneration: lessons learned. *Spine* **29**, 2742, 2004.
23. Tryfonidou, M.A., Schumann, S., Armeanu, S., Harichandan, A., Sivasubramanian, K., Mollenhauer, J., and Bühring, H. Update on canine MSC markers. *Cytometry A* **85A**, 379, 2014.
24. Guo, J.F., Jourdain, G.W., and MacCallum, D.K. Culture and growth characteristics of chondrocytes encapsulated in alginate beads. *Connect Tissue Res* **19**, 277, 1989.
25. Farndale, R.W., Sayers, C.A., and Barrett, A.J. A direct spectrophotometric microassay for sulfated glycosaminoglycans in cartilage cultures. *Connect Tissue Res* **9**, 247, 1982.
26. Pfaffl, M.W. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* **29**, e45, 2001.
27. Zhang, Y., An, H.S., Thonar, E.J.-M.A., Chubinskaya, S., He, T.-C., and Philips, F.M. Comparative effects of bone morphogenetic proteins and Sox9 overexpression on extracellular matrix metabolism of bovine nucleus pulposus cells. *Spine* **31**, 2173, 2006.
28. Potier, E., Ferreira, E., Meunier, A., Sedel, L., Logeart-Avramoglou, D., and Petite, H. Prolonged hypoxia concomitant with serum deprivation induces massive human mesenchymal stem cell death. *Tissue Eng* **13**, 1325, 2007.
29. Risbud, M., Albert, T.J., Guttapalli, A., Vresilovic, E.J., Hillibrand, A.S., Vaccaro, A.R., and Shapiro, I.M. Differentiation of mesenchymal stem cells towards a nucleus pulposus-like phenotype *in vitro*: implications for cell-based transplantation therapy. *Spine* **29**, 2627, 2004.
30. Steck, E., Bertram, H., Abel, R., Chen, B., Winter, A., and Richter, W. Induction of intervertebral disc-like cells from adult mesenchymal stem cells. *Stem Cells* **23**, 403, 2005.
31. Richardson, S.M., Walker, R.V., Parker, S., Rhodes, N.P., Hunt, J.A., Freemont, A.J., and Hoyland, J.A. Intervertebral disc cell-mediated mesenchymal stem cell differentiation. *Stem Cells* **24**, 707, 2006.
32. Watanabe, T., Sakai, D., Yamamoto, Y., Iwashina, T., Serigano, K., Tamura, F., and Mochizuki, J. Human nucleus pulposus cells significantly enhanced biological properties in a coculture system with direct cell-to-cell contact with autologous mesenchymal stem cells. *J Orthop Res* **28**, 623, 2010.
33. Wu, L., Leijten, J.H.C., Georgi, N., Post, J.N., Blitterswijk, C.A., and Karperien, M. Trophic effects of mesenchymal stem cells increase chondrocyte proliferation and matrix formation. *Tissue Eng Part A* **17**, 1425, 2011.
34. Wu, L., Leijten, J.C.H., Georgi, N., Post, J.N., Blitterswijk, C.A., and Karperien, M. Trophic effects of mesenchymal stem cells in chondrocyte co-cultures are independent of culture conditions and cell sources. *Tissue Eng Part A* **18**, 1542, 2012.
35. Wang, M., Rahnama, R., Cheng, T., Grotkopp, E., Jacobs, L., Limburg, S., Kim, H.T., and Kuo, A.C. Trophic stimulation of articular chondrocytes by late-passage mesenchymal stem cells in coculture. *J Orthop Res* **31**, 1936, 2013.
36. Song, I.H., Caplan, A.I., and Dennis, J.E. Dexamethasone inhibition of confluence-induced apoptosis in human mesenchymal stem cells. *J Orthop Res* **27**, 216, 2009.

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