

Can Notochordal Cells Promote Bone Marrow Stromal Cell Potential for Nucleus Pulposus Enrichment? A Simplified *In Vitro* System

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Bone marrow stromal cells (BMSCs) have shown promising potential to stop intervertebral disc degeneration in several animal models. In order to restore a healthy state, though, this potential should be further stimulated. Notochordal cells (NCs), influential in disc development, have been shown to stimulate BMSC differentiation, but it is unclear how this effect will translate in an environment where resident disc cells (nucleus pulposus cells [NPCs]) could also influence BMSCs. The goal of this study was, therefore, to evaluate the effects of NCs on BMSCs when cocultured with NPCs, in a simplified 3D *in vitro* system. Bovine BMSCs and NPCs were mixed (Mix) and seeded into alginate beads. Using culture inserts, the Mix was then cocultured with porcine NCs (alginate beads) and compared to coculture with empty beads or porcine skin fibroblasts (SFs, alginate beads). NPCs alone were also cocultured with NCs, and BMSCs alone cultured under chondrogenic conditions. The effects of coculture conditions on cell viability, matrix production (proteoglycan and collagen), and gene expression of disc markers (aggrecan, type II collagen, and SOX9) were assessed after 4 weeks of culture. The NC phenotype and gene expression profile were also analyzed. Coculture with NCs did not significantly influence cell viability, proteoglycan production, or disc marker gene expression of the Mix. When compared to NPCs, the Mix produced the same amount of proteoglycan and displayed a higher expression of disc marker, indicating a stimulation of the BMSCs (and/or NPCs) in the Mix. Additionally, during the 4 weeks of culture, the NC phenotype changed drastically (morphology, gene expression profile). These results show that NCs might not be as stimulatory for BMSCs in an NPC-rich environment, as believed from individual cultures. This absence of effects could be explained by a mild stimulation provided by (de)differentiating NCs and the costimulation of BMSCs and NPCs by each other.

Introduction

INTERVERTEBRAL DISC DEGENERATION, is heavily associated with chronic back pain,^{1,2} a common disorder with a tremendous socioeconomic impact. As the first signs of degeneration are characterized by matrix degradation and a declining number of the cells of the central core of the disc (nucleus pulposus [NP]),³ it has been proposed to complement this declining cell population with exogenous cells.⁴ These exogenous cells should produce a healthy disc matrix and possess a phenotype close to the NP cells (NPCs), which are chondrocyte-like cells.

Bone marrow stromal cells (BMSCs), also known as mesenchymal stem cells, are one of the most promising candidates for a clinical application of cell-based disc regeneration. They have been extensively studied since the late 1970s, in particular, for their chondrogenic differenti-

ation^{5,6} and they have been used in numerous clinical trials for cartilage repair.⁷ As NP and articular cartilage share some similarities, such as high proteoglycan and type II collagen contents, BMSCs have been proposed for disc regeneration.^{8,9} In fact, several studies have already shown, *in vitro*, that BMSCs can acquire a phenotype close to the one of NPCs and produce the disc matrix.¹⁰⁻¹² Moreover, BMSCs injected into discs, in which degeneration is induced by aspiration¹³⁻¹⁶ or digestion¹⁷ of the NP, have been able to partially reverse the degenerative process in rabbit,^{13,14} sheep,¹⁷ pig,¹⁶ and dog¹⁵ models. In all these studies, however, injected discs did not reach the histological or MRI scores of healthy controls. These studies showed that BMSC injection can efficiently stimulate disc matrix production, but also suggest that BMSC potential needs to be further promoted if one wants to restore the disc to a healthy state.

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When talking about extensive disc matrix production, it is logical to look back at the disc development and growth when such large amounts of matrix are produced. At these stages, the NP contains another cell type that will disappear during growth in human: the notochordal cells (NCs). These cells attract more and more attention from the disc scientific community as they seem heavily involved in disc homeostasis and their (natural) disappearance may ultimately cause disc degeneration.¹⁸ Most importantly here, NCs have been shown to stimulate not only NPCs^{19–21} but also BMSCs. Purnessur *et al.* and Korecki *et al.*, indeed, showed that factors secreted by porcine NCs can promote the synthesis of proteoglycans (main component of disc matrix with collagen) and the gene expression of discogenic markers (aggrecan and type II collagen) by human BMSCs.^{22,23}

It is, however, unclear how NCs can further stimulate the potential of BMSCs when these cells are injected in an environment containing NPCs, as NPCs can themselves promote differentiation of BMSCs.^{24–26} Using a simplified *in vitro* model, the goal of this study was to investigate the potential of NCs to further stimulate BMSCs when in an NPC-rich environment. To do so, bovine BMSCs were co-cultured with fresh bovine NPCs in alginate beads (Mix). Using an indirect coculture system, the Mix was cultured in the presence of porcine NCs, empty alginate beads (negative control), or skin fibroblasts (SFs, control for the specific effects of NCs). To assess the differentiation and/or activity of the BMSC/NPC mixture under NC stimulation, the Mix was compared to fully differentiated cells (NPCs) co-cultured with NCs. Finally, BMSCs were cultured under standard chondrogenic conditions to ascertain the sensitivity to stimulation of the used BMSCs.

Materials and Methods

Cell sources

All the slaughterhouse materials were obtained in accordance with local regulations.

Bovine BMSCs were obtained from bone marrow aspirates of young cows (4–12 month old; $n=4$, using 1 per repeat). BMSCs were isolated by adhesion²⁷ and expanded

up to P1 in a plain medium (high-glucose Dulbecco's modified Eagle's medium [hgDMEM; Gibco Invitrogen, Carlsbad, CA]+1% penicillin/streptomycin [P/S; Lonza, Basel, Switzerland]) supplemented with 10% fetal bovine serum (FBS; Gibco Invitrogen; batch selected for BMSC growth and differentiation) before freezing. A fresh batch of BMSCs were thawed and cultured up to P3 for each experiment. Cells from each donor were cultured separately.

Bovine NPCs were obtained from caudal intervertebral discs harvested from young cows (22–26 month old; $n=12$, pooling 3 per repeat). Discs were dissected and the NPs harvested under sterile conditions. NPs were then digested following a sequential pronase/collagenase type 2 (Worthington, Lakewood, NJ) digestion.²¹ Using a cell strainer, cells smaller than 100 μm were kept after digestion and rinsed in a plain medium before use.

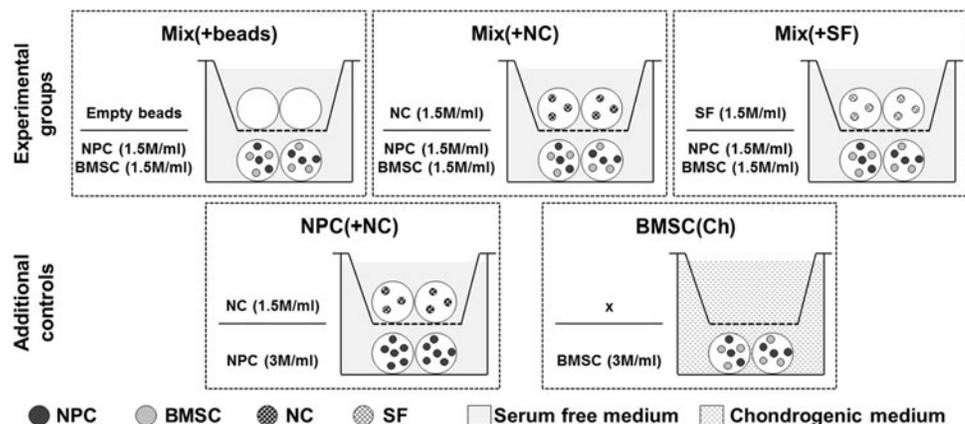
Porcine NCs were isolated from lumbar and thoracic intervertebral discs harvested from young pigs (≤ 10 weeks; $n=8$, pooling 2 per repeat). Discs were dissected and the NP harvested under sterile conditions. NPs were then digested following a sequential pronase/collagenase P digestion²¹: 60 min in 0.3% Pronase (Calbiochem, Darmstadt, Germany) at 37°C in a plain medium; 16 h in 0.05% collagenase P (Roche, Basel, Switzerland) at 37°C in a plain medium. Using a cell strainer, cells smaller than 100 μm were kept after digestion and rinsed in a plain medium before use.

Porcine SFs were extracted from ears of pigs (6–8 month old; $n=8$, pooling 2 per repeat). Sterile skin biopsies were digested following a sequential trypsin/collagenase digestion.²⁸ Obtained cells were rinsed, seeded into flasks, and expanded up to P1 in a plain medium+10% FBS before freezing. Fresh batches were thawed and cultured up to P3 for each experiment.

Cell seeding and culture

To obtain enough cells for the groups described in Figure 1, NPCs from three donors and NCs and SFs from two donors were pooled per repeat ($n=4$). Before seeding, cells were prestained to differentiate the different cell types during culture. BMSCs were stained with 10 μM Vybrant™

FIG. 1. Groups and culture conditions. Bovine bone marrow stromal cells (BMSCs), nucleus pulposus cells (NPCs), porcine notochordal cells (NCs), and skin fibroblasts (SFs) were seeded in alginate beads at 1.5 or 3 million (M) cells/mL. In Mix groups, BMSCs and NPCs were mixed 1:1. BMSCs were prestained with carboxy-fluorescein diacetate, succinimidyl-ester when NPCs, SFs, and NCs remained unstained. For the chondrogenic medium, a serum-free medium was complemented with 10 ng/mL transforming growth factor β_3 , 0.1 μM dexamethasone.



carboxy-fluorescein diacetate, succinimidyl-ester (CFDA-SE; Molecular Probes, Carlsbad, CA) according to the manufacturer's instructions. NPCs, NCs, and SFs remained unstained. BMSCs and NPCs were mixed at 50%/50% (Mix)^{24,29,30} and seeded into alginate beads^{31,32} at 3 million cells/mL. NPCs and BMSCs alone were also seeded at 3 million cells/mL. NCs and SFs were used at a concentration of 1.5 million cells/mL. Produced beads were then (co)cultured according to Figure 1 using cell culture inserts with a pore size of 1 μ m (BD Falcon™). Cells were cultured for 4 weeks under 2% O₂, 5% CO₂, and 93% N₂ in hgDMEM+1% P/S+1% ITS-1⁺ (BD Bioscience, Breda, The Netherlands)+1.25 mg/mL bovine serum albumin (Roche)+50 μ g/mL ascorbic acid 2-phosphate (Sigma, Zwijndrecht, The Netherlands)+40 μ g/mL L-proline (Sigma)+100 μ g/mL sodium pyruvate (Gibco Invitrogen). The BMSCs alone were cultured under chondrogenic conditions (Ch) (culture medium supplemented with 0.1 μ M dexamethasone [Sigma] and 10 ng/mL transforming growth factor [TGF β]₃; Peprotech, Rocky Hill, NJ).³³

Cell viability

At day 1 and 29, beads ($n=3$ beads/repeat/group) were washed and incubated in a 10 μ M calcein blue (Sigma)/10 μ M propidium iodide (Gibco Invitrogen) solution for 1 h at 37°C. Cells were then imaged in the center of the beads at a depth of 200 μ m using a confocal microscope (CLSM 510 Meta; Zeiss, Sliedrecht, The Netherlands).

Cartilaginous matrix formation and cell proliferation

At day 0 and 29, five beads per repeat and group were pooled and digested in a papain solution at 60°C for 16 h (150 mM NaCl [Merck, Darmstadt, Germany], 789 μ g/mL L-cystein [Sigma], 5 mM Na₂EDTA.2H₂O [Sigma], 55 mM

Na₃citrate.2H₂O [Aldrich, Zwijndrecht, The Netherlands], and 125 μ g/mL papain [Sigma]). Digested samples were then used to determine their content of sulfated glycosaminoglycans (GAGs), as a measure of proteoglycans, hydroxyproline (HYP), as a measure for collagen content, and DNA. The GAG content was determined with the dimethylmethylene blue assay, adapted for alginate presence.³⁴ Shark cartilage chondroitin sulfate (Sigma) was used as reference and digested with empty alginate beads (i.e., alginate concentration identical for references and experimental samples). The HYP content was measured using the Chloramin-T assay³⁵ and a trans-4-hydroxyproline (Sigma) reference. The DNA content was measured using the Hoechst dye method³⁶ with a calf thymus DNA reference (Sigma).

At day 29, beads ($n=3$ beads/repeat/group) were also embedded in cryo-compound and snap-frozen in liquid nitrogen. Ten-micrometer-thick cryosections were cut in the middle of the beads. Sections were then thawed, incubated 5 min in 0.1 M CaCl₂ at room temperature (RT), and fixed in buffered formalin 3.7% for 3 min at RT. Sections were then rinsed in 3% glacial acetic acid (Merck) and stained in the Alcian blue solution (Sigma; 1%, pH=1.0 for alginate presence) for 30 min at 37°C. Sections were then rinsed in 0.05 M CaCl₂ and counterstained in Mayer's hematoxylin (Sigma) for 10 min at RT. The stained sections were rinsed in 0.05 M CaCl₂ before mounting in Mowiol (Merck) and observed with a brightfield microscope (Observer Z1; Zeiss).

Gene expression

At day 0 and 29, 9–15 beads per repeat and group were pooled, snap-frozen in liquid nitrogen, and stored at –80°C until RNA isolation. The frozen beads were disrupted with a microdismembrator (Sartorius, Goettingen, Germany)³⁷ and

TABLE 1. PRIMER SEQUENCES FOR TARGET AND REFERENCE GENES USED IN REVERSE TRANSCRIPTION-QUANTITATIVE POLYMERASE CHAIN REACTION ASSAYS

Gene	GenBank accession gene number	Sequence 5'-3'	Product size (bp)	Source
<i>GAPDH</i>	NM_001034034	GCGTGAACCACGAGAAGTATAA CCCTCCACGATGCCAAAGT	119	49
<i>18S</i>	Not available	Not available	Not available	PD
<i>HPRT1</i>	AF176419	TGCTGAGGATTTGGAGAAGG CAACAGGTCGGCAAAGAAGT	154	50
<i>SOX9</i>	AF278703	ACGCCGAGCTCAGCAAGA CACGAACGGCCGCTTCT	70	51
<i>COL2A1</i>	NM_001001135	CCAGCGTCCCCAAGAAGA CCAGGTTGTCATCTCCATAGC	114	PD
<i>ACAN</i>	NM_173981	GGGAAACCTCTGGACTCTTTG AGATATTTCTCCATAAAAACCCTGATG	122	PD
<i>COL10A1</i>	NM_174634	ATATTCTCACCGAGCTAACACAA TTCAGCTACCTCCATATGCA	89	PD
<i>T</i>	NM_00119298	AAGAACGGCAGGAGGATGT CCAGCAGGAAGGAGTACATG	82	PD
<i>CK8</i>	NM_001159615	TGCTGGAGGGAGAGGAGAG CTGTAGTTGAAGCCTGGAGTC	118	PD
<i>CK18</i>	NM_001192095	AAGAACGGCAGGAGGATGT CCAGCAGGAAGGAGTACATG	82	PD

PD: primers designed by and ordered from Primer Design Ltd. (Southampton, United Kingdom).

GAPDH, glyceraldehyde 3-phosphate dehydrogenase; *HPRT1*, hypoxanthine phosphoribosyltransferase; *SOX9*, SRY (sex determining region Y)-box 9; *COL2A1*, type II collagen, alpha 1; *ACAN*, aggrecan; *COL10A1*, type X collagen, alpha 1; *T*, Brachyury; *CK8*, cytokeratin-8; *CK18*, cytokeratin-18.

FIG. 2. Assessment of cell viability and proliferation. (A) DNA content (μg) per bead at day 1 and 29. Values are mean \pm standard deviation; $n=4$ per group; * $p<0.0001$ versus day 1; + $p<0.02$ versus BMSC(Ch), Mix(+beads), and Mix(+NC). The Mix(+NC) was stained at day 1 (B) and day 28 (C, D) with calcein blue (blue fluorescence) for living cells and with propidium iodide (red fluorescence) for dead cells. BMSCs were prestained with CFDA-SE (green fluorescence). Representative of four repeats. Scale bar is 100 μm (B, C) and 20 μm (D). Color images available online at www.liebertpub.com/tea

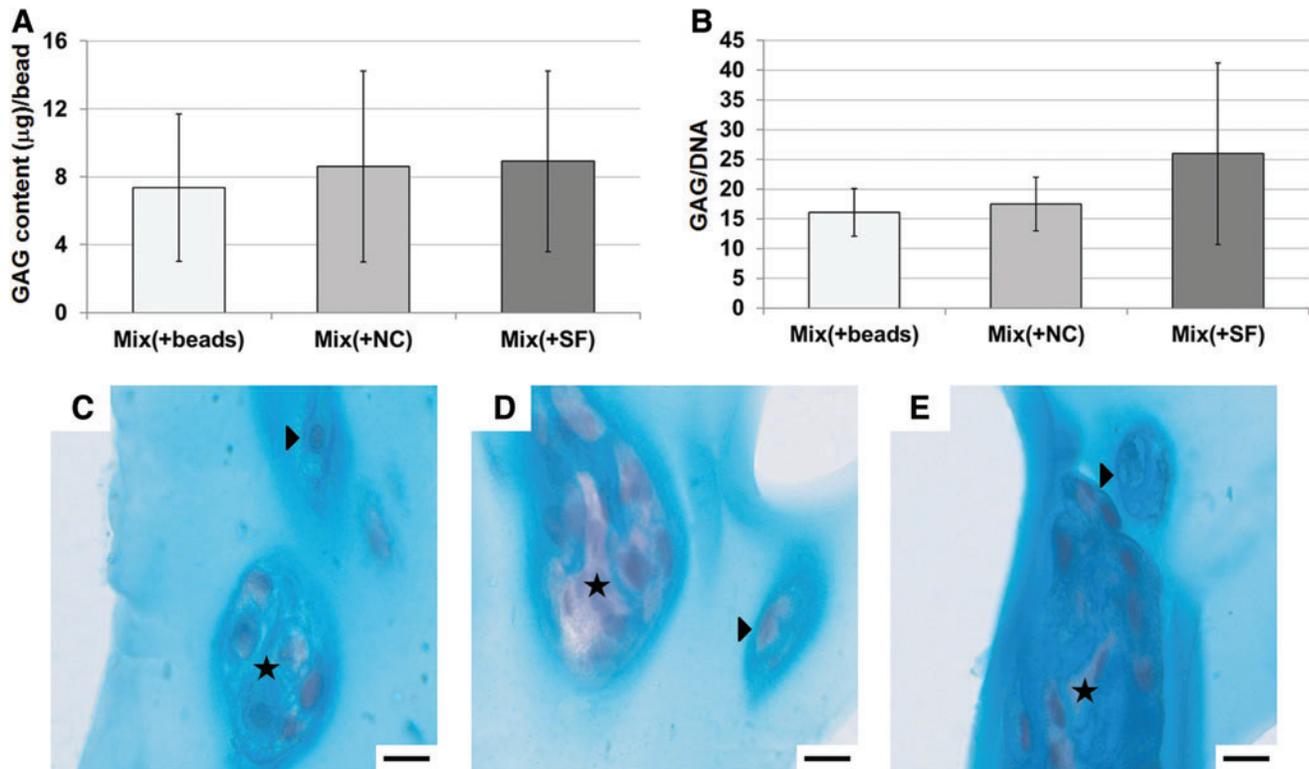
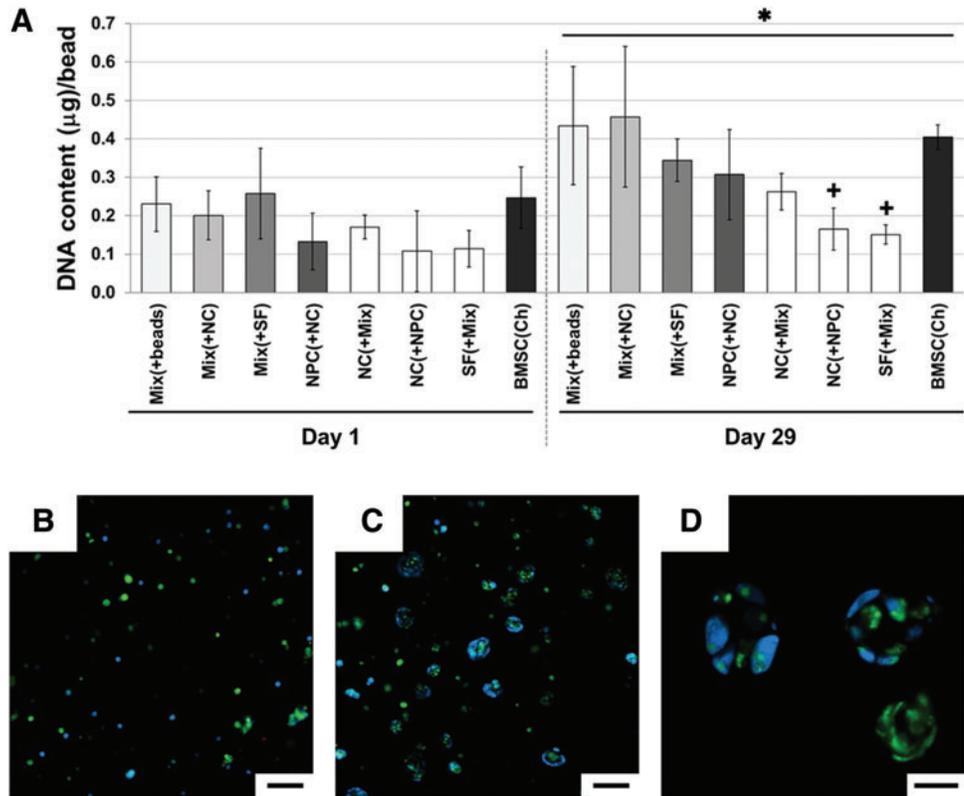


FIG. 3. Assessment of disc matrix production. Matrix production of Mix cocultured with empty beads, NCs, or SFs. The Mix (BMSC/NPC) was cultured for 29 days in a serum-free medium and with either empty alginate beads [Mix(+beads)], NCs [Mix(+NC)], or SFs [Mix(+SF)]. Glycosaminoglycan (GAG) content (μg) per bead (A) and relative to DNA (μg) (B) was used as a measure for proteoglycan production at day 29. Values are mean \pm standard deviation. $n=4$ per group. GAG deposition was assessed at day 29 for Mix(+beads) (C), Mix(+NC) (D), and Mix(+SF) (E) with Alcian blue staining. Representative of four repeats per group. Scale bar is 10 μm . Stars are aggregates as observed in Figure 1D, arrow heads are individual cells. Please note that light blue is alginate. Color images available online at www.liebertpub.com/tea

RNA was extracted using TRIzol[®] (Gibco Invitrogen) and purified using the RNeasy[®] mini-kit (Qiagen, Venlo, The Netherlands) with an on column DNase digestion step. Quantity and purity of isolated RNA were measured by spectrophotometry (ND-1000; Isogen, De Meern, The Netherlands) and integrity by gel electrophoresis. The absence of genomic DNA was validated using primers for glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Two hundred nanograms of total RNA was then reverse transcribed (Superscript[®] Vilo[™]; Gibco Invitrogen) for the Mix, NPC, and BMSC groups; 100 ng for the NC groups. Gene expression levels of type II collagen (*COL2A1*), aggrecan (*ACAN*), SRY (sex determining region Y)-box 9 (*SOX9*), and type X collagen (*COL10A1*) were assessed for the Mix, NPC, and BMSC groups with SYBR green qPCR (CFX384[™]; Biorad, Hercules, CA) (see Table 1 for primer list). Gene expression levels of Brachyury (*T*), cytokeratin-8 (*CK8*)/-18 (*CK18*) were assessed for the NC groups (see Table 1 for primer list). *18S* was selected as the reference gene as the most stable gene throughout our experimental conditions from three tested genes (*HPRT1*, *GAPDH*, and *18S*). Expression of gene of interest is reported as relative to *18S* expression and normalized to day 0 expression levels ($2^{-\Delta\Delta C_t}$ method) using efficiency-corrected comparative quantitation.³⁸ Primer efficiency was determined in every polymerase chain reaction run. When gene expression was not detected, the Ct value was set to 40 to conduct the statistical analysis.

Statistical analyses

All statistical analyses were performed with IBM[®] SPSS[®] (v21.0; IBM Corp., Armonk, NY). General linear regression models based on ANOVAs were used to examine the effects of groups and days of culture and their interactions on the variable DNA content. Full factorial models were fitted to the data, and then a backwards-stepwise procedure was used to remove the nonsignificant effects. For each significant effect, a Tukey-HSD *post hoc* test was conducted. A *p*-value <0.05 was considered significant. A nonparametric Kruskal–Wallis test followed by a *post hoc* Mann–Whitney U test were used to examine the effects of coculture conditions or days of culture on the GAG and HYP contents and gene expression data. A *p*-value <0.05 was considered significant.

Results

Cell viability and proliferation

At day 1, all groups displayed good cell survival, similar to Mix(+NC) (Fig. 2B). In Mix, NPCs (blue cells) and BMSCs (green cells) appeared well dispersed, with a 1:1 ratio (Fig. 2B). At day 29, all cell types survived and proliferated (Fig. 2A). The formation of aggregates could be observed (Fig. 2C, D) in all, but without differences between Mix groups. These aggregates appeared to contain a majority of BMSCs prestained in green (Fig. 2D).

Effects of NCs on BMSCs cocultured with NPCs

Addition of NCs (or SFs) to BMSCs cocultured with NPCs did not affect the overall production of proteoglycans

(Fig. 3A). Proteoglycan production at the cell level (GAG/DNA) was also not affected by the culture conditions (Fig. 3B). GAGs were deposited within the aggregates observed in Figure 1D as well as around individual cells (Fig. 3C–E), with no effects of the coculture conditions. Collagen production was only detected at very low levels for all Mix groups (all around 0.3 μg/bead; data not shown).

Similarly, NCs (or SFs) did not influence the gene expression of *ACAN* (main type of proteoglycan in the NP) and *COL2A1* (main type of collagen in the NP) either after 4 or 29 days of coculture (Fig. 4A and B, respectively). Coculture with NCs, slightly but not significantly, increased the expression of *SOX9*, an early marker of BMSC

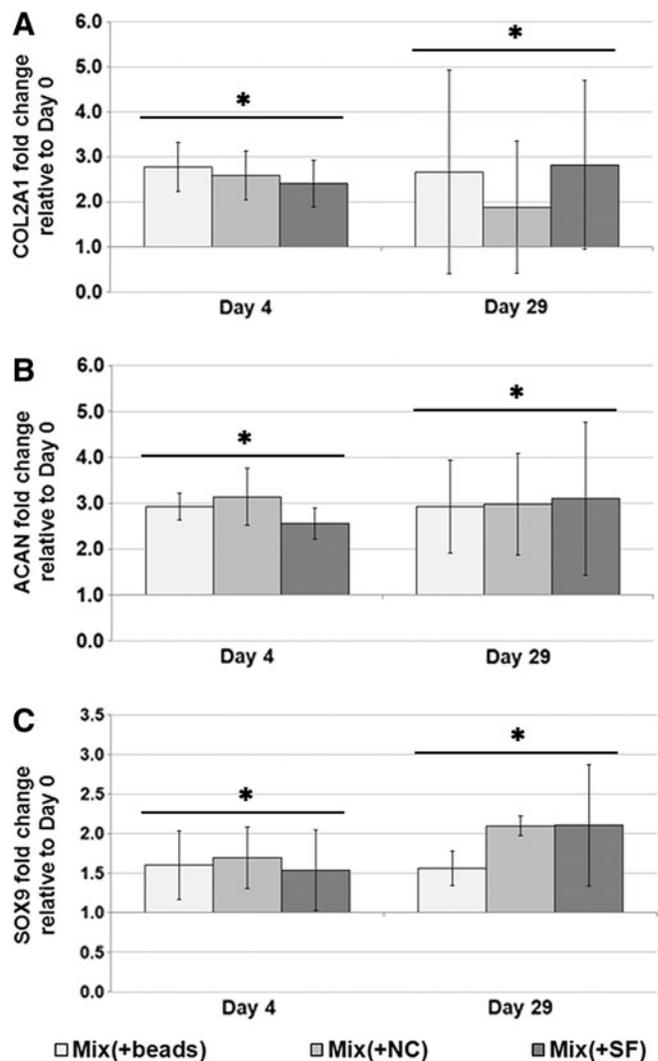


FIG. 4. Assessment of disc marker gene expression. Gene expression profiles of Mix cocultured with empty beads, NCs, or SFs. The Mix (BMSC/NPC) was cultured for 29 days in a serum-free medium and with either empty alginate beads [Mix(+beads)], NCs [Mix(+NC)], or SFs [Mix(+SF)]. Type II collagen, alpha 1 (*COL2A1*) (A), aggrecan (*ACAN*) (B), and sex determining region Y-box 9 (*SOX9*) (C) gene expression relative to *18S* and normalized to day 0 values ($2^{-\Delta\Delta C_t}$ method). Values are mean \pm standard deviation. *n* = 4 per group. **p* < 0.0001 versus day 0, except for *COL2A1* day 29 where *p* < 0.006.

chondrogenic differentiation, after 29 days of exposure (Fig. 4C). This effect, however, was also observed with SFs.

Differentiation/activity of the Mix compared to differentiated cells

BMSCs and NPCs cocultured with NCs [Mix(+NC)] produced the same amount of proteoglycan at the cell level (GAG/DNA) than NPCs cocultured with NCs [NPC(+NC)] or BMSCs maintained under chondrogenic culture conditions [BMSC(Ch)] (Fig. 5A). Collagen production (HYP/DNA), on the other hand, although similar to NPCs, was lower than BMSC(Ch) (Fig. 5B). *COL2A1* and *ACAN* gene expression levels were higher for Mix(+NC) than NPC(+NC), but both remained lower than levels observed for BMSC(Ch), at both day 4 and 29 (Fig. 5C). *SOX9*, on the contrary, was expressed at the same levels for both Mix(+NC) and BMSC(Ch). *COL10A1*, a marker for hypertrophic chondrocyte, was not detected in NPC(+NC) when it was slightly upregulated at day 29 in the Mix, but to a lower extent than for BMSC(Ch).

NC phenotype in long-term culture

At day 1, NCs appeared well dispersed in the alginate beads (Fig. 6A) and displayed their typical morphology: large cells containing big vacuoles (Fig. 6D). They also highly expressed brachyury, cytokeratin-8/-18 (data not shown). At day 4, although NCs still displayed their original morphology (Fig. 6B, E), they showed an important decrease in brachyury and cytokeratin-8/-18 expression levels (Fig. 6G). At day 29, NCs remained viable in the serum-free conditions used (Fig. 6C), but lost their initial phenotype to become round and small (Fig. 6F) with a lower expression of brachyury and cytokeratin-8/-18 (Fig. 6G).

Discussion

In this study, the ability of NCs to stimulate BMSC discogenic potential in an NPC-rich environment was explored. Exposure of BMSCs mixed with NPCs (Mix) to NCs did not affect disc matrix production in the long term (Fig. 3) nor did it influence disc marker gene expression in the short or

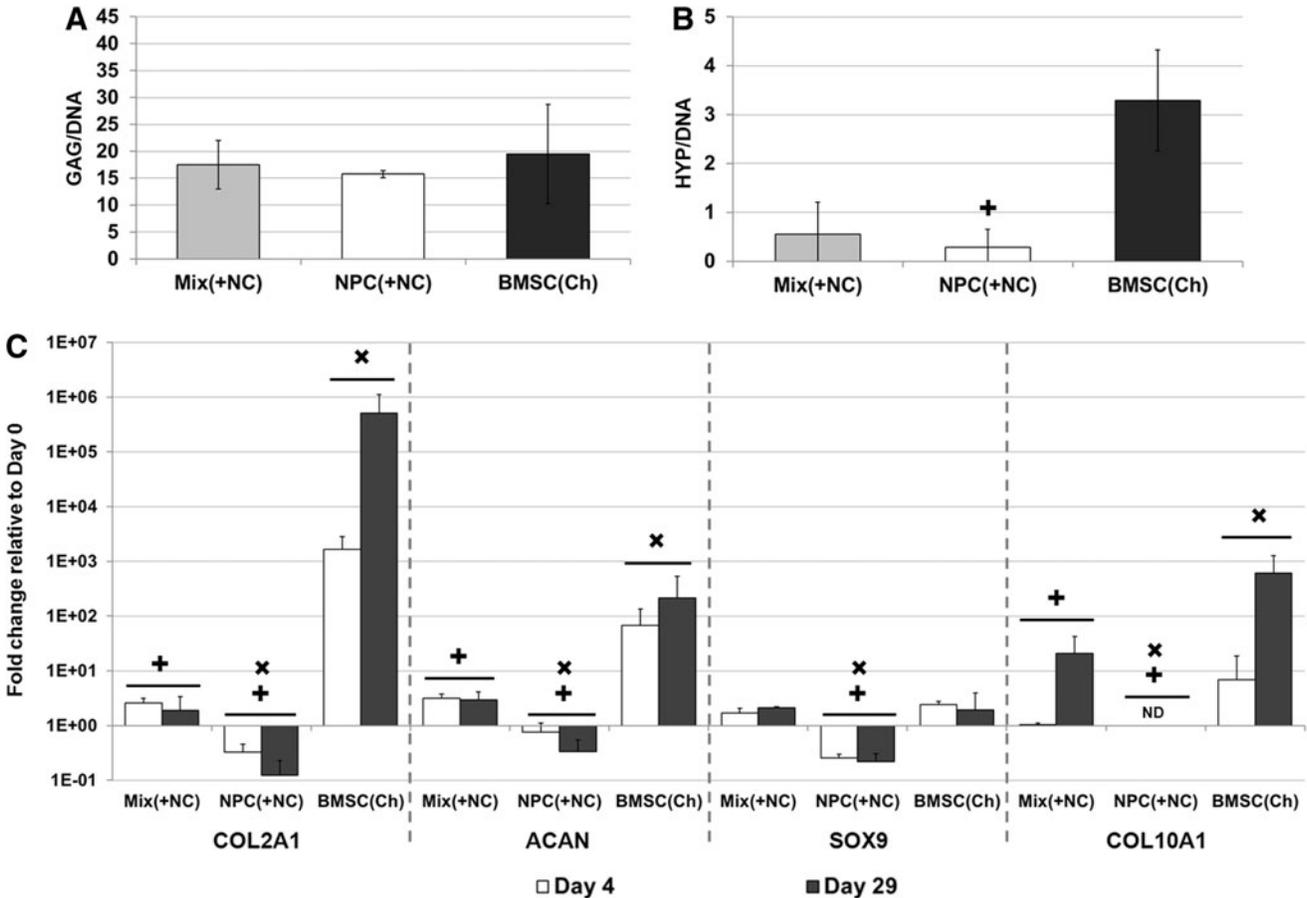


FIG. 5. Matrix production and gene expression profiles of Mix compared to NPCs and BMSC(Ch). The Mix (BMSC/NPC) and NPCs were cultured for 29 days with NCs (+NC) in a serum-free medium; BMSCs alone were cultured under chondrogenic conditions [BMSC(Ch)]. At day 29, GAG (μg) relative to DNA (μg) (A) and hydroxyproline (HYP) (μg) relative to DNA (μg) (B) were used as a measure for proteoglycan and collagen production, respectively. Values are mean \pm standard deviation. $n=4$ per group. $^+p<0.03$ compared to BMSC(Ch). (C) At day 4 and 29, gene expression of *COL2A1*, *ACAN*, *SOX9*, and *COL10A1* was evaluated. Expression levels are relative to *18S* and normalized to day 0 expression ($2^{-\Delta\Delta C_t}$ method). Values are mean \pm standard deviation. $n=4$ per group. Please note the logarithmic scale for both mean and standard deviation. $^+p<0.0002$ compared to BMSC(Ch); $^x p<0.0002$ compared to Mix(+NC).

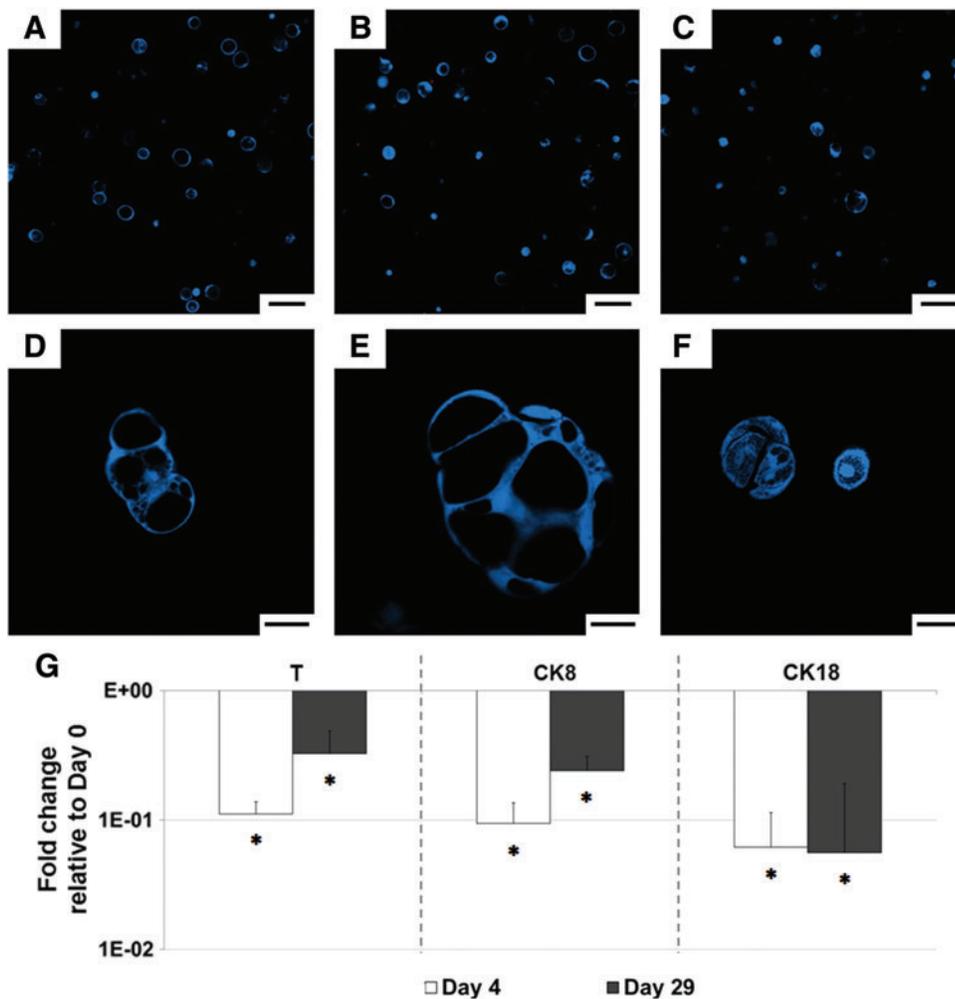


FIG. 6. NC phenotype in long-term culture. NCs were cocultured with Mix for 28 days in a serum-free medium. Cell morphology and viability was assessed at day 1 (A, D), 4 (B, E), and 29 (C, F) using calcein blue (blue fluorescence) for living cells and propidium iodide (red fluorescence) for dead cells and confocal imaging. Representative of four repeats. Scale bar is 100 μ m (A–C) and 20 μ m (D–F). (G) Gene expression of Brachyury (*T*), cytokeratin-8/-18 (*CK8*, *CK18*) relative to *18S* and normalized to day 0 expression ($2^{-\Delta\Delta C_t}$ method). Values are mean \pm standard deviation. $n=4$ per group. Please note the logarithmic scale for both means and standard deviations. * $p < 0.0002$ compared to day 0. Color images available online at www.liebertpub.com/tea

long term (Fig. 4). The absence of effects on the gene expression of *COL2A1*, *ACAN*, or *SOX9* has been reported before on NPCs^{19,20} and BMSCs²² and thus is not unanticipated. The GAG production, on the other hand, has been shown to be stimulated by NCs in NPCs^{19,20} or BMSCs.²²

This lack of substantial effects on the Mix could be explained by the use of dispersed NCs seeded in alginate to stimulate the other cells. When present in the nucleus tissue, NCs indeed appear as clustered cells. The loss of this natural organization during cell isolation may impact their stimulative potential. Porcine NCs dispersed into alginate have been reported to have a lesser effect on human BMSCs²² than native NC-rich tissue fragments. It is, however, not so clear how the NC's cellular organization influences the NC impact on other cells. Several studies reported that NCs digested out of the tissue and seeded into alginate, similarly to what was done in the present study, can successfully stimulate human²⁰ or bovine^{19,21,39} NPCs as well as human BMSCs²³ (Table 2). The disruption of the physiological organization of the NCs during cell isolation, though, might be one explanation of their loss of phenotype during long-term culture. Still pertaining to cell–cell interactions, the lack of effect of the NCs can be due to the absence of cell–cell contact between these cells and the BMSCs/NPCs. Coculture of BMSCs and NPCs with direct cell–cell contact, indeed, has

been shown to be more efficient than one without.^{25,40} However, the coculture studies using NCs demonstrated that a cell–cell contact was not necessary for the NCs to stimulate BMSCs or NPCs (Table 2). The use of a conditioned medium unequivocally shows that NCs are acting via secreted factors and not cell–cell connection.^{20,22,23,39,41}

The ratio of NCs to Mix used here could also be too low to initiate a substantial response from BMSCs and NPCs (NCs:Mix = 1:2). When several studies looked at the ratios of BMSCs and NPCs,^{24,25,42,43} very few focused on the NCs/NPCs or NC/BMSC ratios. To the author's best knowledge, only Gantenbein-Ritter *et al.* evaluated the effects of different ratios of NCs/NPCs in a midterm (2 weeks) culture.¹⁹ When they reported a more pronounced effect of a 1:1 ratio, the coculture of NCs:NPCs at 1:3 led to almost the same amount of GAG/DNA than this optimal ratio.

Another explanation could be a change of NC phenotype in the long-term culture: variation in gene expression of brachyury and cytokeratin-8/-18 already at day 4 and loss of vacuoles at day 29 (Fig. 6). Although there are no specific markers for NCs, brachyury and cytokeratin-8/-18 can be used to assess the NC phenotype as they are much more expressed in NCs than other disc cells.^{44,45} As the serum-free medium supplemented with ITS, as used here, has been shown to maintain a relatively stable phenotype of NCs

TABLE 2. CULTURE CONDITIONS FOR NPC/NC, BMSC/NC, AND NPC/NC COCULTURE IN PREVIOUS STUDIES

Cell culture system	Coculture conditions	BMSCs (days of culture)	NPCs (days of culture)	NCs (days of culture)	Ref.
NPC/NC					
3D: alginate beads	Indirect coculture ^a , without cell–cell contact		Bovine, no degeneration (14)	Porcine, dispersed (14)	19
3D: alginate beads	NC conditioned medium ^b		Human, grade 4–5 degenerated (7)	Porcine, dispersed in alginate or tissue fragments (4)	20
3D: alginate beads	Mixed populations ^c NC conditioned medium ^b		Bovine, no degeneration (4)	Canine, dispersed (3–4)	21
3D: alginate beads	NC conditioned medium ^b		Bovine, no degeneration (1)	Canine, dispersed (4)	39
3D: alginate beads	NC conditioned medium ^b		Bovine, no degeneration (3)	Canine, dispersed (4)	41
BMSC/NC					
3D: cell pellet	NC conditioned medium ^b	Human (21)		Porcine, dispersed in alginate or tissue fragments (4)	22
3D: cell pellet	NC conditioned medium ^b	Human (7)		Porcine, dispersed (4)	23
BMSC/NPC					
3D: cell pellet	Direct coculture ^d	Human (14)	Human, grade 3–5 degenerated (14)		24
2D: monolayer	Indirect coculture ^a , with or without cell–cell contact	Human (7)	Human, no degeneration (7)		25
2D: monolayer	Direct coculture ^d	Human (7)	Human, degenerated and no degeneration (7)		26
3D: alginate beads and pellet	Mixed populations ^c and direct coculture ^d	Human (14)	Human, grade 2–3 degenerated (14)		30
3D: alginate beads	Indirect coculture ^a , without cell–cell contact	Rabbit (10)	Rabbit, no degeneration (10)		42
3D: cell pellet	Direct coculture ^d	Human (28)	Human, grade 2–4 degenerated (28)		43
2D: monolayer	Indirect coculture ^a , with or without cell–cell contact	Human (7)	Human, grade 2–4 degenerated (7)		40

^aThe two cell populations are separated by a membrane, allowing or no cell–cell contact.

^bThe culture medium is conditioned for 2–4 days by NCs and then used to culture BMSCs or NPCs, no cell–cell contact is possible.

^cThe two cell populations are mixed together before seeding in a hydrogel; cell–cell contact is possible, but very limited as the cells are encapsulated in the hydrogel apart of each other.

^dThe two cell populations are mixed together and seeded on 2D surfaces or used in 3D pellets, cell–cell contact is possible. BMSC, bone marrow stromal cell; NC, notochordal cell; NPC, nucleus pulposus cell.

when cultured alone,⁴⁶ culture conditions used should be favorable for NC phenotype maintenance. The change observed here might therefore have been caused by the coculture with the Mix. The influence of coculture on NCs has previously been observed on *COL2A1* and *ACAN* gene expression when porcine NCs were cocultured with bovine NPCs.¹⁹ The hypothesis that (de)differentiating NCs in long-term culture only provide a small stimulatory signal is supported by the observation that GAG production by NPCs is only weakly stimulated by a 2-week coculture with NCs (1.2×basal medium)¹⁹ when compared to a culture in a medium conditioned by NCs, for which NCs were only cultured for 4 days (2×basal medium).²⁰ Although the difference in strength may come from the different systems used, to the author's best knowledge, strong stimulation of BMSCs or NPCs by NCs has only been reported in systems (coculture or conditioned medium) where NCs were cultured for less than 4 days^{20–23,41} (Table 2). Due to the absence of a NPC group to compare with NPC(+NC), it is not possible to draw a definitive conclusion here on the absence of stimulation by the NCs: is it due to the dedif-

ferentiation of NCs during long-term culture or to the desensitization of cells in the Mix to a moderate signal provided by NCs? The latter is supported by the previously cited study where NCs in long-term culture can provide, even if weak, a stimulatory signal as shown before with NPCs.¹⁹ Another explanation yet, for the dearth of NC effects, could be the use of healthy NPCs instead of ones with an altered phenotype as found in a degenerating disc. Indeed, these cells may be more sensitive to an external stimulation as they are in need of external cellular support to maintain the nucleus matrix. Several short-term studies, however, demonstrated that healthy NPCs could respond, even if sometimes moderately, to the NC stimulation^{19,21,39,41} (Table 2). Moreover, studies on NPC/BMSC coculture using human NPCs isolated from discs of different degeneration stages showed that NPCs are as, or even more, sensitive to BMSC addition when they are isolated from less degenerated discs.^{40,43} These observations, combined with the ability of healthy NPCs to respond to NCs, indicate that NPCs isolated from healthy bovine discs used here should be able to respond to external stimulation.

Thus, it appears that the absence of effects by NCs is most likely due to the strong stimulation of BMSCs and NPCs by each other in the Mix, causing a desensitization of these cells to any additional signal than can be provided by NCs. The Mix, indeed, produced significant amount of GAGs (Fig. 3) and showed upregulated gene expression of *COL2A1* and *ACAN* (Fig. 4), under all the culture conditions used. The amount of proteoglycans produced by individual cells (GAG/DNA) in the Mix was similar to the one of fully differentiated cells, either NPCs or BMSCs under chondrogenic conditions (Fig. 5A). This high matrix production can then be the result of the differentiation of BMSCs or the stimulation of NPCs. Aggregates formed at day 29 are predominantly composed of BMSCs (Fig. 2D), and GAGs were deposited around and within these aggregates (Fig. 3C–E), suggesting BMSC differentiation. This idea is supported by the gene expression profile of the Mix when compared to NPCs and differentiated BMSCs (Fig. 5C). Expression levels of the Mix appeared, indeed, as the resultant of a mixture of NPCs (showing lower expression than the Mix) and differentiated BMSCs (displaying levels higher than the Mix). Thus, these observations support the idea that, in the Mix, BMSC differentiation is induced by coculture with NPCs, as shown before with human BMSCs and NPCs into 2D^{25,26,40} and 3D³⁰ models (Table 2). If so, the induced differentiation appears toward a NPC, rather than a chondrocyte, phenotype. The collagen production by the Mix was, indeed, similar to NPCs but lower than BMSCs under chondrogenic conditions. An interesting observation when it is known that articular cartilage displayed a higher collagen/proteoglycan ratio than the intervertebral disc.⁴⁷ Although it appears that the high amount of matrix produced by the Mix is (in part) due to the differentiation of BMSCs, we cannot rule out the stimulation of NPCs by BMSCs in the present study.

Another limitation of the present study is the (over)simplification of the disc environment. *In vivo*, this environment is much more complex: low pH and oxygen, high osmolarity, and mechanical load. All these factors could influence the behavior of BMSCs, such as shown with pH,⁴⁸ and their sensitivity to NC stimulation. Moreover, some of the results present large standard deviations that may impede the statistical detection of a beneficial effect of the NCs. The spread in age of the donors for the BMSCs (4–12 months), and to a lesser extent for the NPCs (22–26 months), may account for such data dispersion. None of the repeat, however, showed an increase in proteoglycan production or gene expression of *COL2A1* and *ACAN* when NCs were added compared to the addition of SFs. The use of juvenile donors to isolate BMSCs may also constitute a bias in the present study. Indeed, such young cells may have a higher proliferation rate and sensitivity to external stimulation than adult or old counterparts. Nonetheless, if that is so, the use of juvenile BMSCs can appear as the best case scenario to obtain a response to NC coculture, and still no positive effects were observed.

In conclusion, this study confirmed that the coculture of BMSCs and NPCs leads to a strong stimulation of disc matrix production and cell phenotype. Adding NCs, however, did not stimulate further these outcomes, as suggested from individual cocultures of NCs/BMSCs or NCs/NPCs. This absence of effects could be explained by the fact that

either NCs lost their effects in culture or are only providing a moderate stimulation when the BMSCs/NPCs are already strongly stimulated by each other. To assess the full potential of NCs for disc regeneration, their direct use may not be so effective and any stimulatory mechanisms they may possess, for example molecular factors, should be identified and optimally applied.

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Disclosure Statement

Both authors declare that they have no competing interests.

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