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ORIGINAL ARTICLE

Notochordal Cell Matrix As a Therapeutic Agent for Intervertebral Disc Regeneration

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Notochordal cells (NCs) reside in the core of the healthy disc and produce soluble factors that can stimulate nucleus pulposus cells (NPCs). These NC-derived factors may be applied in intervertebral disc regeneration for treatment of low-back pain. However, identification of the active soluble factors is challenging. Therefore a novel approach to directly use porcine NC-rich NP matrix (NCM) is introduced. We explored porcine NCM's anabolic effects on bovine NPCs harvested from caudal discs of adolescent and adult (2–2.5 vs. 4–6 year old) cows. NC-conditioned medium (NCCM) and NCM were produced from porcine NC-rich NP tissue. Bovine NPCs were cultured in alginate beads for 4 weeks in base medium (BM), NCCM, and NCM to investigate NCM's regenerative potential. Porcine NCM increased glycosaminoglycan (GAG) content of both adolescent and adult bovine NPCs. This was through increased proliferation of adolescent bovine NPCs, whereas in adult bovine NPCs, it was mostly through increased GAG production per NPC. Furthermore, adolescent bovine NPCs were cultured in BM and porcine NCM treated with interleukin (IL)-1β to investigate NCM's potential in an inflammatory environment. Addition of IL-1β enhanced *IL1β* and *CXCL8 (IL8)* gene expression, while NCM diminished *IL1β* gene expression. IL-1β reduced GAG and DNA content, but the addition of NCM relative to BM improved GAG and DNA content. Altogether, porcine NCM exerts bovine NPC-age dependent effects, and NCM's anabolic effect on adult NPCs is stronger compared with NCCM. Furthermore, porcine NCM induced an anabolic response of bovine NPCs in an inflammatory environment and may have anti-inflammatory properties. Therefore, NCM has potential in a regenerative therapy for disc degeneration, and warrants additional *in vivo* studies.

Keywords: notochordal cells, nucleus pulposus, regenerative medicine, extracellular matrix

Introduction

Back pain is a leading cause for disability, and is closely related to intervertebral disc (IVD) degeneration.[1] Negatively charged proteoglycans attract positively charged ions, which in turn attract water into the core of the IVD, the nucleus pulposus (NP), providing the swelling pressure required for sustaining high compressive loads and proper IVD function.[2] With IVD degeneration, the nucleus pulposus cells (NPCs) are unable to maintain a healthy NP tissue.[3] As a result, the proteoglycan content decreases and collagen type II is slowly replaced by collagen type I. Thus, the NP becomes more fibrous and the swelling pressure decreases, resulting in annular loading/injury, decreased IVD height, and further degeneration of the spinal unit.

The degenerative changes in IVD tissue integrity have, in part, a molecular onset with an inflammatory component. Degenerating NPCs produce inflammatory cytokines such as interleukin (IL)-1β and tumor necrosis factor α (TNFα).[4,5] IL-6 and IL-8 produced by degenerating NPCs have been linked to low-back pain.[6] Furthermore, inflammatory cytokines stimulate the production of catabolic proteases, such as matrix metalloproteinases (MMP) and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS).[5,7,8] In addition, production of matrix proteins such as aggrecan and collagen type II is decreased in an inflammatory environment, reflecting the inability of NPCs in a degenerating IVD to restore the tissue to a healthy state.[7,8] Hence, the inflammatory environment in IVD degeneration may lead to back pain both directly as well as indirectly.

Current treatment methods for IVD degeneration aim to alleviate pain and do not address the underlying cause of IVD degeneration. Therefore, minimally invasive, regenerative therapies, such as cell or growth factor alone or in combination,

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are being investigated. Lately, within the field of regenerative medicine, notochordal cells (NCs) have received considerable attention due to their regenerative potential. Previous studies showed in various species and culture techniques that NC-conditioned medium (NCCM) stimulates NPC matrix production and proliferation, as well as differentiation of bone marrow-derived mesenchymal stem cells (BMSCs) toward a chondrogenic phenotype.[9–15] Porcine NCCM does not only stimulate isolated cells but also NPCs within their native environment as demonstrated in bovine NP explants.[16]

Although the *in vitro* effects of NCCM appear promising and even *in vivo* regenerative effects were observed after injection of NCCM in degeneration-induced rat IVDs,[17] direct injection of NCCM may not be feasible to restore the spontaneously degenerating human IVD. Alternatively, the active factors in NCCM could be identified and employed. Potentially interesting factors such as transforming growth factor β-1 and connective tissue growth factor (CTGF) have been identified,[11,17,18] but are being disputed as they exert, dependent on the tissue context, beneficial but also detrimental effects.[19] Noteworthy, increased expression of CTGF is observed in degenerated discs, but seems to fail in eliciting a reparative response in this environment.[20] Identification of NCCM's bioactive component appears difficult and time-consuming, hampering the development of an NC-based treatment for IVD degeneration. Furthermore, a combination of many factors may be involved, which would further complicate identification.

A promising approach for tissue regeneration and repair is the use of extracellular matrix. A well-known example is demineralized bone matrix (DBM), which is clinically applied in various forms, as it exerts bone regenerative effects due to the presence of osteoinductive proteins.[21,22] Similarly, NP extracellular matrix has been tested for its potential in IVD repair in various *in vitro* animal models. Bovine decellularized NP matrix, which *in situ* contains chondrocyte-like NPCs, has been shown to support human NPC and BMSC viability and matrix production.[23] Similarly, when cultured on a decellularized matrix produced by rabbit NPCs after *in vitro* expansion, human BMSCs increased expression of NPC markers and synthesized NP matrix.[24] Furthermore, porcine NP matrix, which is rich in NCs, induced differentiation of human induced pluripotent stem cells (hiPSCs) toward an NC-like phenotype, which can be employed in a cell-based regenerative therapy.[25,26] Although NP matrix from species that lose as well as species that maintain their NC population supported cell survival and stem cell differentiation, it is unknown whether the NC matrix exerts differential regenerative effects from NP matrix without NCs. In this respect, NCCM produced from porcine NP tissue exerted even greater matrix anabolic effect than human NCCM on NPCs isolated from human degenerated IVDs.[27] As such, porcine NP tissue, which is rich in NCs, may have a strong translation potential.

We propose to directly apply the NC-rich NP matrix (NCM) to facilitate biologic repair of the degenerating IVD, similar to DBM. NCM can be harvested from non-human sources, lyophilized and pulverized to a fine powder, which, if biologically active, would circumvent the need to specifically identify the active factors in NCCM. Therefore, in this study, we aim to test whether porcine NCM stimulates production of healthy NP matrix constituents by adolescent bovine NPCs. For this purpose, bovine NP matrix, used as an NC-free control for porcine NCM, was employed to explore whether the anabolic effects are tissue specific. Typically, older individuals are more likely to require an IVD regenerative therapy. Since aging has been associated with lower number of tissue progenitor cells and inherently a lower regenerative capacity,[28] we tested the anabolic effects of NCM not only on adolescent but also on adult bovine donors to investigate how aging affects NCM's regenerative potential. Furthermore, by mimicking the degenerative disc environment, we explore whether NCM has regenerative and anti-inflammatory potential in the presence of inflammatory stimuli.

Materials and Methods

Generation of porcine NCCM and NCM, and bovine NP matrix

NC-rich NP tissue was harvested from all lumbar, thoracic, and cervical IVDs of 12 pig spines (~3 month old), obtained from the local abattoir. The IVDs were opened aseptically, and the NP tissue was meticulously scooped out, careful not to include annulus fibrosus tissue, which was visually distinguished from the NP tissue. To produce NCCM, half of the NP tissue from each spine was cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM, 31966; Invitrogen, Carlsbad, CA) supplemented with 1% penicillin/streptomycin (P/S; 17-602E; Lonza, Basel, Switzerland) at 30 mL medium per gram of tissue as employed before.[13–15] The NP tissue was incubated for 4 days at 37°C, 5% CO_2, and 5% O_2. At the end of the incubation time, the NP tissue was removed from the conditioned medium by filtration with a 70-μm-pore-size cell strainer. Subsequently, the medium was filtered through a 3 kDa cutoff filter tube (Amicon Ultra-15 centrifugal filter, UFC900308; Merck, Darmstadt, Germany) and the residue was resuspended in the same amount of fresh low glucose (lg)DMEM (Invitrogen; 10567-014). To produce NCM, the other half of the NP tissue from each spine was lyophilized overnight and pulverized to a fine powder using a microdismembrator (Sartorius, Goetingen, Germany). Per spine, every other disc was used for NCCM/NCM so that NP tissue from all levels was homogeneously divided for NCCM and NCM production. NCCM and NCM were pooled and stored in aliquots at −80°C until further use. Bovine NP-derived tissue (bNP) matrix was harvested from caudal IVDs (Cd1–Cd5 from five 2–2.5 year-old cows, pooled), lyophilized, and pulverized similar to NCM.

Isolation of bovine NPCs

NPCs were harvested from the caudal IVDs (Cd1–Cd5) of adolescent (2–2.5 year old, 12 tails, 2 tails pooled per repeat) as well as adult cows (4–6 year old, 15 tails, 3 tails pooled per repeat), obtained from the local abattoir. The IVDs were opened under aseptic conditions, and the NP tissue was punched out, taking care not to include annulus fibrosus tissue. The NP tissue was enzymatically digested in 0.1% pronase (11459643001; Roche Diagnostics, Almere, the Netherlands) for 90 min, and subsequently in 0.025% collagenase type II (LS004177; Worthington, Lakewood, NJ) for 16 h, both at 37°C. After digestion, the cell suspension was filtered through a 70-μm–pore-size cell strainer

to remove remaining pieces of tissue. The NPCs were suspended in 1.2% alginate (180947; Sigma, Zwijndrecht, The Netherlands) at 3 million cells/mL. Alginate beads were produced according to a previously described protocol.[29] Briefly, 3 million cells were mixed with 1 mL alginate using an 18G mixing needle and the cell suspension was aspirated through a syringe. NPC-seeded alginate beads were produced by dropping the cell suspension in a 102 mM calcium chloride (Merck; 102378) solution. Subsequently, beads where washed thrice with 0.9% sodium chloride (Merck; 106404) solution before being transferred to the culture medium. Alginate bead culture was employed as it is a scaffold that has shown to maintain the typical NPC-like characteristics.[30]

Alginate bead culture

To test the regenerative potential of NCM, the alginate beads seeded with adolescent (2–2.5 year old) donors' NPCs were cultured in a base medium (BM: lgDMEM supplemented with 1% P/S, 1% ITS-1$^+$ [354352; Corning, Lasne, Belgium], 50 mg/mL ascorbic acid-2-phosphate [Sigma; A8960], 1.25 mg/mL bovine serum albumin [Roche; 10735078001], and 40 mg/ml L-proline [Sigma; P5607]), NCCM (same supplements as BM), or NCM dissolved in BM. In addition, the effects of porcine-derived NCM were compared to NC-poor bNP to demonstrate that the observed effects were tissue specific. The NCM and bNP concentration in medium were adjusted to obtain a similar protein concentration as NCCM ($\sim$0.4 mg total protein per mL). The anabolic potential of NCM in an inflammatory environment was investigated in the presence of an inflammatory stimulus. For this purpose, adolescent NPC-seeded alginate beads were cultured in either BM or NCM with and without 5 ng/mL IL-1β, added with each medium change. For each medium group $n=6$ independent repeats were cultured. Alginate beads were cultured for 28 days at 37°C, 5% CO_2, and 5% O_2 with 2–3 medium changes per week. Alginate beads were stored at −80°C for subsequent biochemical assays at day 0 and 28, (immuno)histochemical staining at day 28, and real-time quantitative PCR at day 4 and 28.

In a separate experiment, alginate beads seeded with adult (4–6 year old) bovine NPCs were cultured in BM, NCCM, and NCM (as described before, $n=5$ per group) to determine whether the age of bovine NPC donors affect NCM's anabolic effects. Alginate beads were cultured as described before. After culture, beads were stored at −80°C for biochemical assays at day 0 and 28 and (immuno)histochemical staining at day 28.

Biochemical content and (immuno)histochemical staining

Day 0 and 28 alginate beads were digested overnight at 60°C in papain solution (100 mM phosphate buffer [Sigma; P5244], 5 mM L-cysteine [Sigma; 200-157-7], 5 mM ethylene diamine tetra-acetic acid [Sigma; 03620], and 140 mg/mL papain [Sigma; P4762]). The digested samples were used to determine sulfated glycosaminoglycan (GAG), hydroxyproline, and DNA content. GAG content was analyzed with a dimethyl-methylene blue assay, modified from a previous protocol,[31] using shark cartilage chondroitin sulfate (Sigma; C4384) as reference. Hydroxyproline content was measured using the Chloramin-T assay[32] with a trans-4-hydroxyproline (Sigma; H54409) reference. DNA content was measured using the Qubit Quantification Platform (Invitrogen).

Per group, three alginate beads at day 28 were fixed overnight in 3.7% formalin at room temperature and embedded in paraffin. Eight micrometer thick sections were stained with Alcian blue and hematoxylin to visualize proteoglycan deposition and cell nuclei. For collagen immunohistochemistry, sections were first dewaxed using xylene and a series of decreasing ethanol concentration. Thereafter, sections were washed in phosphate-buffered saline (PBS) for 5 min and antigen retrieval was performed with citrate buffer for 20 min at 96°C for collagen type I staining, and with 0.05% pepsin in 10 mM HCl for 5 min at 37°C for collagen type II. Samples were washed again twice with PBS with 0.1% tween, and subsequently blocked with 10% normal goat serum for 30 min. Samples were then incubated overnight at 4°C with the primary antibody in 1% normal goat serum (NGS) in PBS (Abcam; Ab34710, 1:200 dilution for collagen type I and Abcam; Ab180697, 1:200 dilution for collagen type II). The next day, samples were washed twice for 5 min in PBS with 0.1% tween, followed by incubation with the secondary antibody (Molecular Probes anti-rabbit immunoglobulin G [IgG], A21428, 1:200 dilution for collagen type I and Molecular Probes anti-mouse IgG2a, A21137, 1:300 dilution for collagen type II) and 4′,6-diamidino-2-phenylindole (1:500 in PBS). Thereafter, samples were washed again twice in 0.1% tween in PBS and embedded using mowiol. Pictures were taken using a fluorescent microscope (Zeiss Axiovert 200M; Zeiss, Sliedrecht, The Netherlands). Positive control samples (bovine tendon for collagen type I and articular cartilage for collagen type II) were included, as well as negative controls for each sample (i.e., omission of the primary antibody). The negative controls showed no unspecific positive staining (Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/tea).

Gene expression

Real-time qPCR was performed to determine whether an inflammatory environment was established and whether NCM had anti-inflammatory properties. Alginate beads were dissolved in sodium citrate buffer (55 mM trisodiumcitrate-2-hydrate [Merck; 1064480500], 0.15 M sodium chloride, and 25 mM HEPES [Sigma; H3375] in RNAse-free water, pH adjusted to 7.4) for 5 min at room temperature. After centrifugation, the cell pellet was lysed in 300 μL Buffer RTL (74104; Qiagen, Venlo, The Netherlands) with 1% β-mercaptoethanol. RNA was extracted and purified using the Qiagen mini kit (Qiagen; 74104) with an on-column DNAse digestion step. A spectrophotometer (ND-1000; Isogen, de Meern, The Netherlands) was used to test the quantity and purity of isolated RNA. The absence of genomic DNA was verified with a minus-RT reaction (iCycler; Bio-Rad, Veenendaal, The Netherlands). cDNA was synthesized using the VILO kit (Invitrogen; 11754050). The tested genes and their corresponding primer pairs are listed in Table 1. The reference gene selected was 60S ribosomal protein L13 (*RPL13*), as its expression was most stable throughout all culture conditions. Gene expression was investigated using real-time PCR (CFX384; Bio-Rad) and expression is reported according to the $2^{-\Delta Ct}$ method, relative to expression of *RPL13*.

Table 1. Primer Sequences for Target and Reference Genes Used in Real-Time Quantitative Polymerase Chain Reaction Assays

Gene	*Accession number*	*Oligonucleotide sequence (5′→3′)*	*Product size (bp)*
RPL13	NM_001076998	FW: CTGCCCCACAAGACCAAG RV: TTGCGAGTAGGCTTCAGAC	140
IL-1β	NM_174093	FW: AGCATCCTTTCATTCATCTTTGAAG RV: GGGTGCGTCACACAGAAACTC	88
IL-6	NM_173923	FW: GGGCTCCCATGATTGTGGTA RV: GTGTGCCCAGTGGACAGGTT	69
IL-8	NM_173925.2	FW: TGCTTTTTTGTTTTCGGTTTTTG RV: AACAGGCACTCGGGAATCCT	71
TNFα	NM_173966	FW: ACACCATGAGCACCAAAAGC RV: GCAACCAGGAGGAAGGAGAA	130
ADAMTS-5	NM_001166515	FW: TCACTGCCTACTTAGCCCTGAA RV: GCTCCAACCGCTGTAGTTCAT	125
MMP-13	NM_174389	FW: CTTGTTGCTGCCCATGAGTT RV: TTGTCTGGCGTTTTGGGATG	197

The annealing temperature of all primer pairs was 60°C.
RPL13, 60S ribosomal protein L13; *IL-1β/6/8*, interleukin-1β/6/8; *TNFα*, tumor necrosis factor α; *ADAMTS-5*, a disintegrin and metalloproteinase with thrombospondin motifs 5; *MMP-13*, matrix metalloproteinase 13.

Statistics

Statistics were performed with Statistical Package for Social Sciences (SPSS, version 22; IBM, Armonk, NY). Normality was tested using the Shapiro–Wilk test. For biochemical and gene expression data, one-way analysis of variance (ANOVA) was performed, followed by independent *t*-tests *post hoc* testing with Bonferroni corrections. For gene expression data, a one-way, rather than a two-way ANOVA was performed at each time point, since only differences between medium groups were of interest and not the factor time. For differences in age-dependent biochemical data, independent *t*-tests with Bonferroni corrections were performed.

Results

The anabolic, tissue-specific effect of porcine NCM on adolescent bovine NPCs

The anabolic effect of NCM (i.e., from porcine NC-rich NP tissue) on adolescent (2–2.5 year old) bovine NPC donors was compared to that of porcine NCCM and bNP (i.e., from NC-poor bovine NP tissue). GAG per bead content increased significantly in samples cultured with NCCM, NCM, or bNP compared to BM cultures (Fig. 1a). In addition, GAG per bead content was significantly higher for samples cultured with NCM compared to bNP (Fig. 1a). DNA per bead at day 28 was significantly higher in all culture groups compared to day 0 (Fig. 1b). Furthermore, DNA per bead content at day 28 was significantly higher with NCM compared to all other culture groups (Fig. 1b). Due to the strong increase in DNA content, the GAG per DNA ratio of samples cultured with NCM was significantly lower compared to BM, whereas it was significantly higher for samples cultured with NCCM and bNP compared to the other groups (Fig. 1c). Hydroxyproline content increased significantly with NCM and bNP, but not with NCCM compared to BM (Fig. 1d). Alcian blue staining confirmed increased GAG deposition with NCCM and bNP, and especially NCM (Fig. 1e). In general, cell clusters appeared larger with NCM, indicating that more proliferation has occurred, which is consistent with the increased DNA content (Fig. 1e). Collagen immunostaining revealed a similar increase in collagen type II deposition with NCCM, NCM, and bNP compared to BM (Fig. 1e). However, presence of bNP resulted in an increased collagen type I deposition, whereas only pericellular (BM and NCM) or diffuse (NCCM) collagen type I was observed in other groups (Fig. 1e).

The potential of porcine NCM in an inflammatory environment

To test porcine NCM's regenerative potential in an inflammatory environment, alginate beads seeded with NPCs from adolescent bovine donors were cultured in BM and NCM with and without the addition of IL-1β. With NCM + IL-1β, the GAG content was significantly lower compared to NCM alone, but the GAG content was significantly higher compared to BM + IL-1β (Fig. 2a). The addition of IL-1β to BM and NCM resulted in a significantly lower DNA content compared to their respective counterparts without IL-1β (Fig. 2b). Nonetheless, DNA content was significantly higher with NCM + IL-1β compared to BM + IL-1β (Fig. 2b). Because of the strong increase in DNA content with NCM alone, the lowest GAG per DNA ratio was observed for this group (Fig. 2c). Hydroxyproline content with NCM was significantly higher compared to BM and BM + IL-1β, but not NCM + IL-1β (Fig. 2d). GAG content results were verified by Alcian blue staining with more intense matrix staining in the NCM and NCM + IL-1β compared to BM and BM + IL-1β, indicative of a higher proteoglycan deposition (Fig. 2e). Interestingly, collagen type II deposition was diminished in BM and NCM supplemented with IL-1β, compared to BM and NCM alone, respectively (Fig. 2e). Moreover, NCM and IL-1β separately did not affect collagen type I production deposition compared to BM, but their combination resulted in an increased collagen type I deposition in the territorial matrix (Fig. 2e).

The potential of porcine NCM in an inflammatory environment was further explored with the aid of gene expression profiling at early and late time points, that is, 4 and 28 days of culture (Fig. 3). Expression of *IL-1β* was significantly

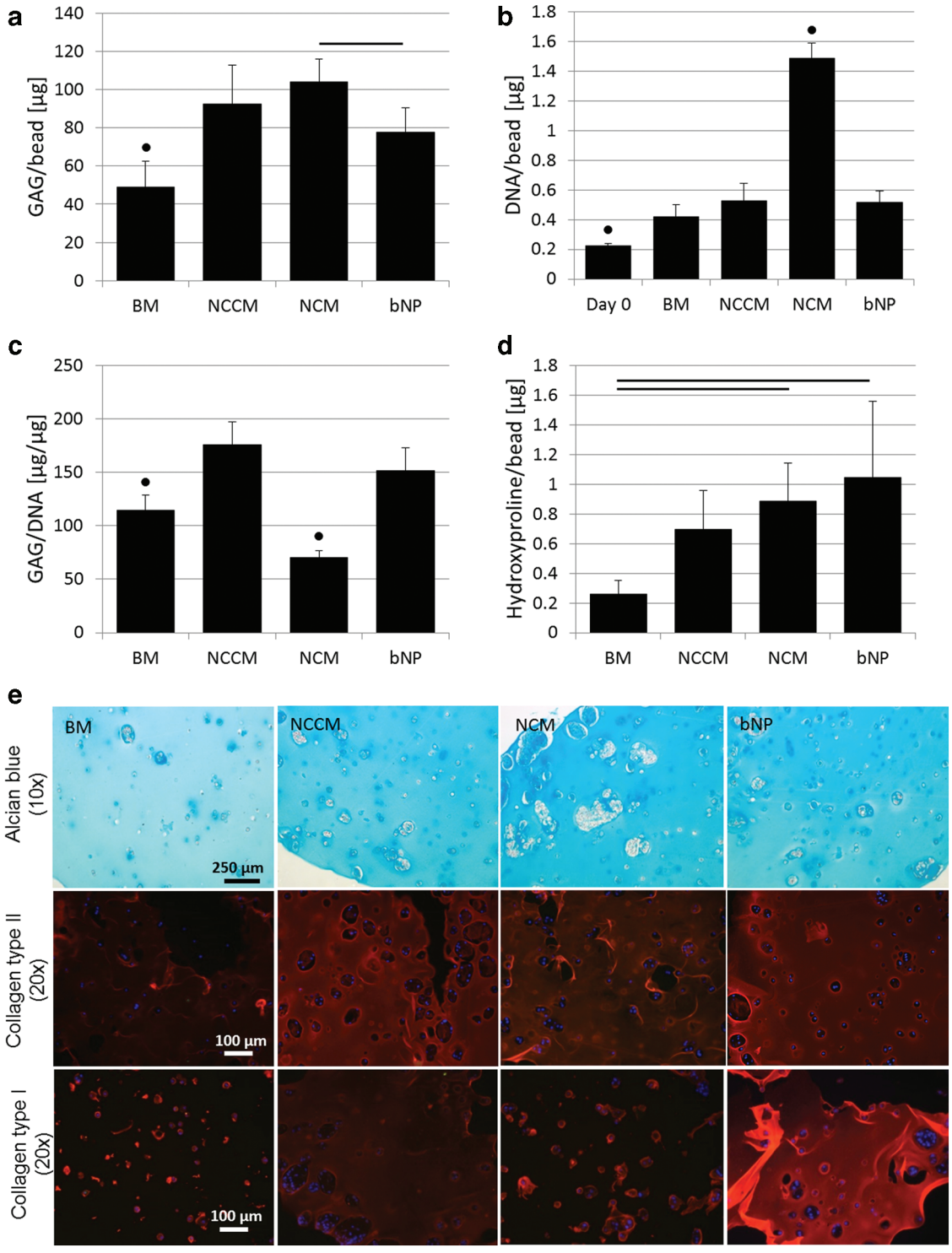

FIG. 1. NCM stimulates matrix production, mainly through strongly increased proliferation of adolescent (2–2.5 year-old) bovine NPCs. **(a)** GAG per alginate bead seeded with bovine NPCs, **(b)** DNA content per bead, **(c)** GAG per DNA, and **(d)** hydroxyproline content per bead. Values represent means + standard deviations, $n=6$ per group. *Bars* indicate $p<0.05$ between specified groups, "•" indicates $p<0.05$ from all other groups. **(e)** Histological Alcian blue staining confirms the increased GAG content with NCCM, bNP, and especially NCM compared to BM. NCCM, NCM, and bNP similarly enhance collagen type II production relative to BM, whereas bNP increases collagen type I production compared to BM, NCCM, and NCM. NC, notochordal cell; NP, nucleus pulposus; NCM, NC-rich NP matrix; NPC, nucleus pulposus cell; GAG, glycosaminoglycan; BM, base medium; NCCM, notochordal cell-conditioned medium; bNP, bovine nucleus pulposus powder. Color images are available online.

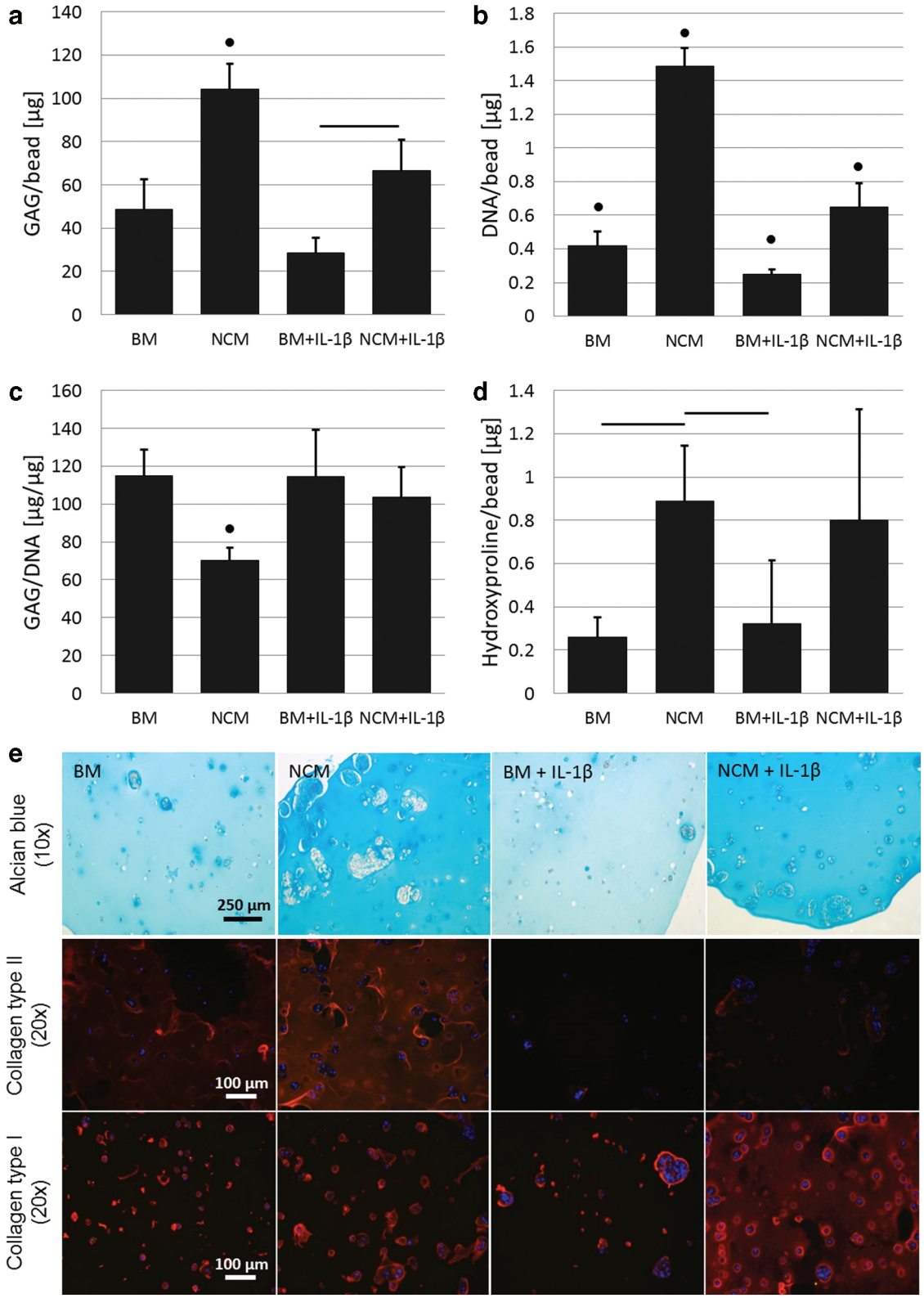

FIG. 2. NCM stimulates matrix production by adolescent (2–2.5 year old) bovine NPCs in the presence of an inflammatory stimulus. **(a)** GAG per alginate bead seeded with bovine NPCs, **(b)** DNA content per bead, **(c)** GAG per DNA, and **(d)** hydroxyproline content per bead. Values represent means + standard deviations, $n = 6$ per group. *Bars* indicate $p < 0.05$ between specified groups, "•" indicates $p < 0.05$ from all other groups. **(e)** Histological Alcian blue staining confirms increased GAG content with NCM with and without IL-1β compared to the BM control. Presence of IL-1β decreases collagen type II deposition in BM as well as NCM. Moreover, NCM combined with IL-1β increases collagen type I deposition, while IL-1β alone does not. IL, interleukin. Color images are available online.

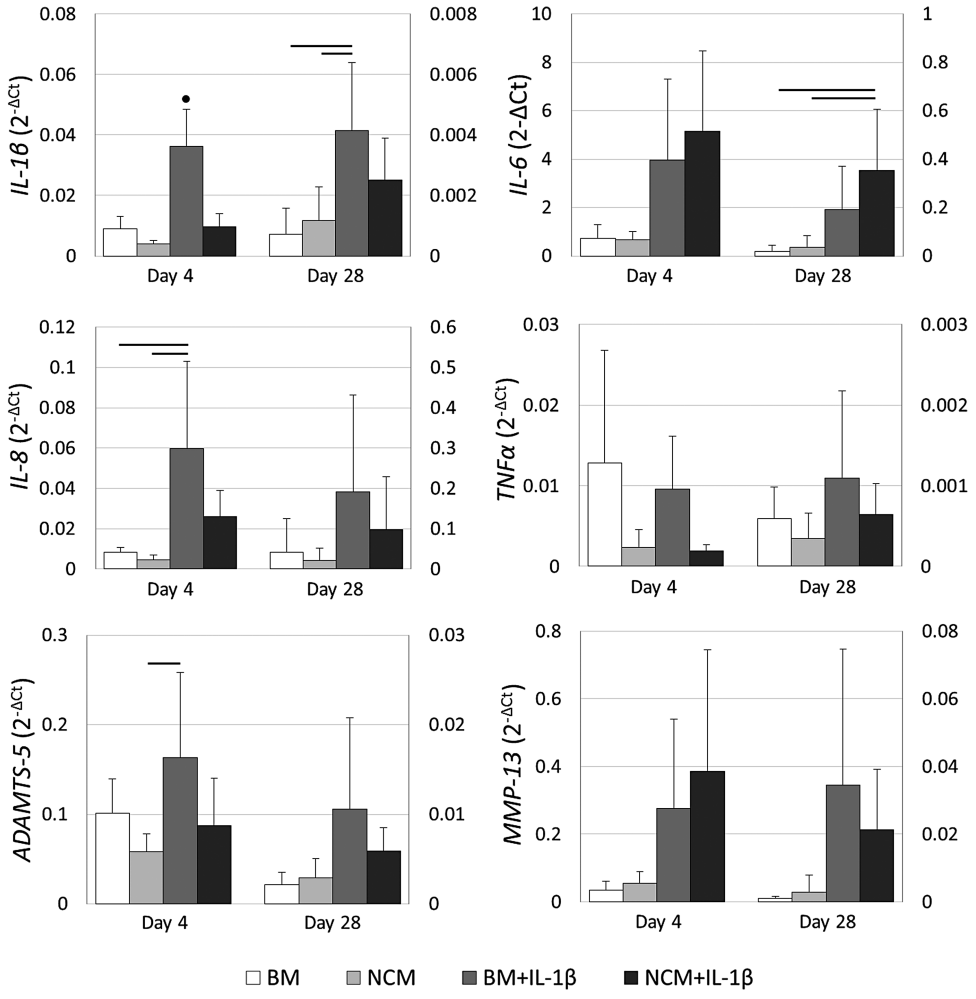

FIG. 3. NCM may have anti-inflammatory properties in inflammation-induced adolescent (2–2.5 year-old) NP cell seeded alginate beads. Expression levels are relative to RPL13. Values are means + standard deviations, $n = 5–6$ per group. *Bars* indicate $p < 0.05$ between specified groups, "•" indicates $p < 0.05$ from all other groups. TNFα, tumor necrosis factor α; ADAMTS-5, a disintegrin and metalloproteinase with thrombospondin motifs 5; MMP-13, matrix metalloproteinase 13; RPL13, 60S ribosomal protein L13.

upregulated with BM + IL-1β compared to all other culture groups at day 4, while at day 28, the same was true, except for NCM + IL-1β. At day 4, no significant differences in *IL-6* gene expression were observed, although its expression was significantly higher in NCM + IL-1β compared to BM and NCM alone at day 28. Expression of *IL-8* was significantly higher only at day 4 in BM + IL-1β compared to BM and NCM alone, but not in NCM + IL-1β. No significant differences were observed for expression of *TNFα* at either day 4 or 28. Expression of *ADAMTS-5* was significantly higher in BM + IL-1β compared to NCM alone only at day 4, while NCM + IL-1β did not significantly differ from other groups. Also, *MMP-13* gene expression did not significantly differ between culture groups at either day 4 or 28.

The anabolic potential of porcine NCM on adult bovine NPCs

Given the distinct proliferative and thereby matrix anabolic response of adolescent bovine NPCs to porcine NCM, the anabolic potential of NCCM and NCM was tested also on NPCs from adult (4–6 year old) bovine donors. The latter are expected to be less responsive due to aging and better represent the human situation. While in the presence of NCCM, the GAG content significantly increased compared to BM, in the presence of NCM, the GAG content increased even further compared to BM and NCCM (Fig. 4a). DNA content increased significantly with NCCM compared to day 0, but also increased further with NCM, where DNA per bead content was significantly higher compared to all other groups (Fig. 4b). Together, this resulted in a significantly increased GAG per DNA content with NCM compared to BM (Fig. 4c). Hydroxyproline content, indicative for collagen content, also increased significantly with NCCM and NCM compared to BM (Fig. 4d). Biochemical content results were confirmed histologically (Fig. 4e). Alcian blue staining intensity increased with NCCM, and especially NCM compared to BM, indicating a higher proteoglycan density. No appropriate collagen staining images for the BM group could be obtained, because the samples washed out during the staining procedure. This was likely due to limited matrix deposited to keep the sample intact. Nonetheless, collagen immunostaining indicated similar collagen type II deposition for NCM and NCCM, whereas collagen type I staining appeared less intense with NCM compared to NCCM.

The distinct response of adolescent and adult bovine NPCs to porcine NCCM and NCM

To characterize the differential response of adolescent and adult NPCs, GAG, DNA, and GAG per DNA content of

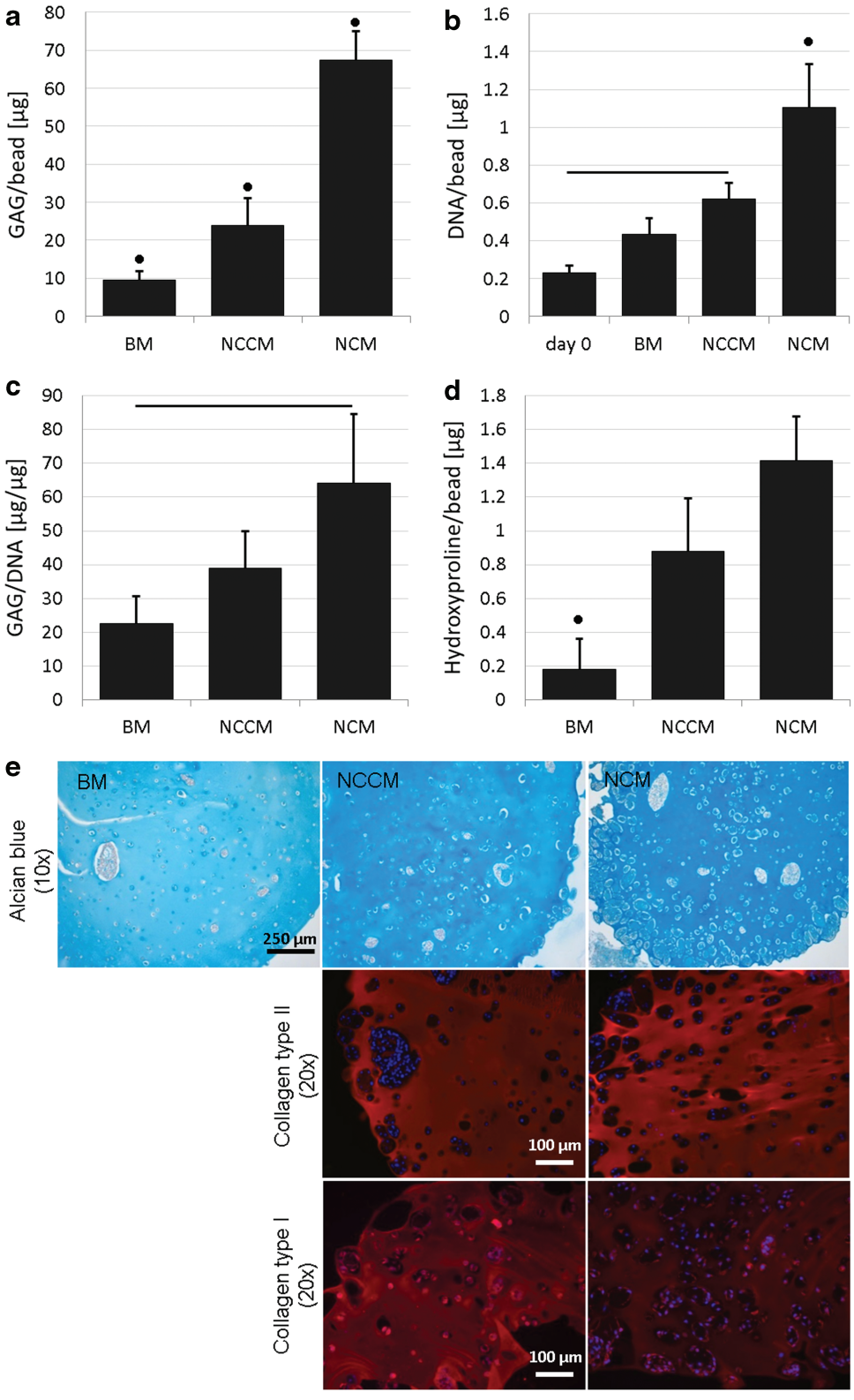

FIG. 4. Porcine NCM induced a distinct anabolic response of adult (4–6 year old) bovine NPCs. **(a)** GAG and **(b)** DNA content per alginate bead seeded with bovine NPCs, **(c)** GAG per DNA, and **(d)** hydroxyproline content per bead. Values represent means + standard deviations, $n = 5$ per group. *Bars* indicate $p < 0.05$ between specified groups, "•" indicates $p < 0.05$ from all other groups. **(e)** Alcian blue staining confirms increased GAG deposition with NCCM, and especially NCM compared to BM. Collagen immunohistochemistry shows a similar staining intensity between NCCM and NCM for collagen type II, but decreased collagen type I staining intensity with NCM compared to NCCM. Color images are available online.

alginate beads cultured in NCCM and NCM were normalized to their respective BM controls. No differences were observed in normalized GAG, DNA, or GAG per DNA content between adolescent and adult NPCs in response to NCCM (Fig. 5a). With the addition of NCM, however, a significantly higher normalized GAG content and GAG per DNA ratio was observed for NPCs from adult donors compared to adolescent donors (Fig. 5b). No significant difference in normalized DNA content was observed between adolescent and adult NPCs (Fig. 5b).

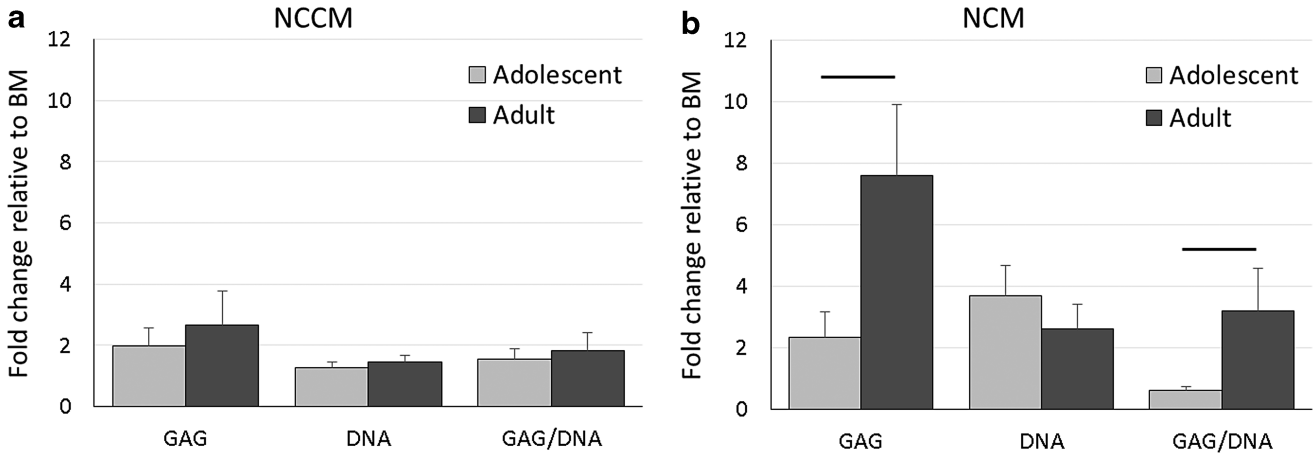

FIG. 5. Porcine NCCM and NCM had distinct effects on bovine NPCs from adolescent (2–2.5 year old) and adult (4–6 year old) donors. GAG, DNA, and GAG per DNA content of NPC-seeded alginate beads cultured in **(a)** NCCM and **(b)** NCM normalized to BM. Normalization was performed for each repeat separately. Values represent means + standard deviations, $n = 6$ per group for adolescent groups and $n = 5$ for adult groups. *Bars* indicate $p < 0.05$ between specified groups.

Discussion

In this study, a novel approach to directly use porcine NCM as an alternative to porcine NCCM for an IVD regenerative strategy is introduced. We aimed to determine whether porcine-derived NCM can stimulate bovine NPCs to produce healthy NP matrix constituents, as was previously demonstrated for NCCM.[10,12–15,27,33,34] The regenerative potential of NCM was similar to that of NCCM for adolescent NPCs and even stronger for adult NPCs. Furthermore, the regenerative effects seemed to be tissue dependent, as shown by higher GAG content and increased cell proliferation in seeded cells compared to bNP, while bNP stimulated deposition of collagen type I typically present in degenerative fibrotic processes. In the presence of an inflammatory stimulus, representing the degenerative disc environment, the matrix anabolic properties of NCM were largely maintained.

Porcine NCM has distinct anabolic effects on NPCs from adolescent versus adult bovine donors

In this study, the regenerative potential of porcine NCM was tested on NPCs from adolescent or adult bovine donors, as the latter were suspected to be less responsive and more representative for the human situation. Differences between adolescent and adult NPCs became evident from BM cultures, showing higher GAG content for adolescent compared to adult NPCs (49 ± 14 vs. $9 \pm 2\,\mu g$/bead, respectively) at a similar DNA content (0.42 ± 0.08 vs. $0.44 \pm 0.08\,\mu g$/bead), underscoring their different regenerative potential. Notably, NCM induced a significantly higher increase in GAG content and GAG per DNA ratio relative to BM for adult compared to adolescent NPCs, whereas this differential effect was not observed for NCCM. These findings suggest that NCM has the potency to stimulate not only adolescent NPCs but also NPCs from older, less responsive donors. Moreover, while increased GAG content was achieved, at least in part, through increased proliferation of adolescent NPCs, NCM strongly stimulated the per-cell matrix production by NPCs from adult donors. Arguably, stimulation of GAG production per cell, rather than through increased cell proliferation may be the preferred mechanism of a regenerative therapy, considering the NP *in vivo* may not be able to support such increase in cellularity due to limited nutrient availability.[35] Aside from GAGs, NCM appeared to stimulate collagen type II deposition relative to BM for adolescent NPCs, whereas collagen type I was not affected. For adult NPCs, collagen type II staining was similar with NCM and NCCM, whereas collagen type I staining was consistently less intense with NCM compared to NCCM. This remains to be corroborated by quantitative analysis of the tissue collagens. Altogether, these findings suggest that NCM induces deposition of matrix that is in line with that of healthy NP tissue.

NCM has stronger anabolic and proliferative effects compared to NCCM

NCM showed significantly stronger matrix anabolic (adult donors) and proliferative (adolescent and adult donors) effects on bovine NPCs compared to NCCM. There are several possible explanations for these findings. First, with NCCM production, specific matrix-bound proteins may remain attached to the NP tissue[36] and get discarded after NCCM incubation. Such proteins are included in NCM, where they could be responsible for its stronger anabolic effect. Second, directly after harvesting NP tissue, it is lyophilized and stored frozen for NCM production, hence its proteins may be better preserved compared to the proteins secreted in medium during NCCM incubation. Similarly, extracellular vesicles derived from NCCM had an anabolic effect on canine NPCs.[34] These factors and their active contents may as well be better preserved in lyophilized form, and partly account for the stronger effect of NCM. Last, NPCs in bovine NP tissue, which was allowed to swell freely, produced IL-6 and PGE2.[37] Possibly, such factors are also produced with free swelling of porcine NP tissue during NCCM production and introduced in the NCCM-NPC culture, limiting NCCM's stimulatory effect. Altogether, these findings imply that bioactive factors in lyophilized NC-rich NP tissue are better preserved relative to the factors in NCCM.

NCM has tissue-specific regenerative effects

Considering the well-known differences between species in matrix composition and phenotype of cells present within a healthy IVD, we questioned whether the anabolic effects observed are specifically related to NCs and their matrix present within the NP tissue. Therefore, the stimulatory effect of porcine NCM on NPCs was compared to that of bNP that was derived from healthy bovine IVDs that do not contain NCs, but the typical chondrocyte-like cells. Although the strongest increase in GAG and DNA content was observed with NCM, the GAG content also increased with the addition of bNP. This suggests that factors are present within the bovine matrix as well that stimulate matrix production by the NPCs. Nonetheless, the higher GAG content and the strong increase in DNA content with NCM, but not with bNP, suggest that the proliferative effect is specific to the healthy porcine NP tissue. Furthermore, in the absence of an inflammatory stimulus, NCM seemed to stimulate mainly collagen type II production, whereas collagen type I was observed only pericellularly. However, besides collagen type II, bNP also strongly stimulated deposition of collagen type I, implying that the type of matrix deposited in the presence of NCM is more in line with that of healthy NP tissue. Ideally, the differential effects of NC-rich and NC-free matrix are investigated using tissue from the same species. However, pigs retain their NCs throughout their lives, whereas cows lose their NC population already before birth, which makes a same-species comparison difficult. As such, other species-specific differences than the presence/absence of NCs (e.g., matrix components and degenerative state) could have affected these results. Nonetheless, as observed before with NCCM,[27] the stronger regenerative effects of NCM than bNP on bovine NPCs underscore the cross-species effects, and suggest a promising translational potential for clinical application in humans.

NCM's anabolic effects are largely maintained in an inflammatory environment

Inflammation contributes to IVD degeneration and inflammatory mediators may affect the efficacy of a regenerative treatment for IVD degeneration. Therefore, this study explored the effects of porcine NCM also in the presence of an inflammatory stimulus. Addition of IL-1β indeed resulted in an inflammatory environment as evident by a decrease in DNA content and increased inflammatory gene expression. Furthermore, the inflammatory stimulus administered at a supraphysiological concentration reduced NCM's matrix anabolic effects, as also observed for human NPCs.[7] Nonetheless the matrix anabolic effects of NCM were still evident compared to BM, with marked GAG and collagen type II deposition. Moreover, expression of *IL-1β* with NCM + IL-1β was significantly lower compared to BM + IL-1β at day 4.These findings suggest that NCM has anti-inflammatory properties and NCM may have regenerative potential even in an *in vivo* inflammatory environment, that is, the degenerating IVD.

Future directions

Aside from NCM's stronger anabolic effects on adult NPCs, its use may be advantageous over NCCM or growth factor treatment. By using NCM, the tedious process of identifying the (combination of) bioactive factors in NCCM is circumvented. Furthermore, NCM is easily obtained and readily available, and its use may be more cost-effective than growth factor-based treatment strategies. NCM may be administered in combination with a swelling hydrogel, such as that developed by Sivan *et al.*[38] Such combination may directly restore the degenerated IVD's mechanical functionality, while inducing regenerative effects over time. Moreover, as porcine NP matrix previously demonstrated its ability to differentiate hiPSCs toward an NC-like phenotype,[25,26] combined application of NCM with progenitor cells may augment its effect. However, before NCM can be applied *in vivo*, additional processing is required. Porcine DNA harbors endogenous viruses that are not infectious to the host, but can come to expression upon transplantation in other species.[39] As such, genetic material needs to be removed, while retaining as much as possible of the matrix and proteins.

Conclusions

In this study, the use of porcine NCM as a novel regenerative strategy for IVD degeneration was investigated. NCM stimulates matrix production of adolescent bovine NPCs through increased cell proliferation, whereas it stimulates per-cell matrix production by adult NPCs, which may be beneficial for its application as a regenerative therapy. Although the stimulatory effect of NCM was not as strong in an inflammatory environment, it still induced an anabolic response of NPCs. These findings highlight the potential of NCM as a regenerative treatment for IVD degeneration, and warrants additional studies in an *in vivo* model of IVD degeneration.

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