Soluble factors from the notochordal-rich intervertebral disc inhibit endothelial cell invasion and vessel formation in the presence and absence of pro-inflammatory cytokines

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Summary

Background: Chronic low back pain can be associated with the pathological ingrowth of blood vessels and nerves into intervertebral discs (IVDs). The notochord patterns the IVD during development and is a source of anti-angiogenic soluble factors such as Noggin and Chondroitin sulfate (CS). These factors may form the basis for a new minimally invasive strategy to target angiogenesis in the IVD.

Objective: To examine the anti-angiogenic potential of soluble factors from notochordal cells (NCs) and candidates Noggin and CS under healthy culture conditions and in the presence of pro-inflammatory mediators.

Design: NC conditioned media (NCCM) was generated from porcine NC-rich nucleus pulposus tissue. To assess the effects of NCCM, CS and Noggin on angiogenesis, cell invasion and tubular formation assays were performed using human umbilical vein endothelial cells (HUVECs) ± tumor necrosis factor alpha (TNFα [10 ng/ml]), vascular endothelial growth factor (VEGF)-A, MMP-7, interleukin-6 (IL-6) and IL-8 mRNA levels were assessed using qRT-PCR.

Results: NCCM (10 & 100%), CS (10 and 100 μg) and Noggin (10 and 100 ng) significantly decreased cell invasion of HUVECs with and without TNFα. NCCM 10% and Noggin 10 ng inhibited tubular formation with and without TNFα and CS 100 μg inhibited tubules in Basal conditions whereas CS 10 μg inhibited tubules with TNFα. NCCM significantly decreased VEGF-A, MMP-7 and IL-6 mRNA levels in HUVECs with and without TNFα. CS and Noggin had no effects on gene expression.

Conclusions: We provide the first evidence that soluble factors from NCs can inhibit angiogenesis by suppressing VEGF signaling. Notochordal-derived ligands are a promising minimally invasive strategy targeting neurovascular ingrowth and pain in the degenerated IVD.

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Introduction

Chronic low back pain affects 70–85% of the population at some point during their lives and is the leading cause of disability worldwide with costs exceeding $100 billion a year in the United States alone. Back pain is strongly associated with intervertebral disc (IVD) degeneration and the development of strategies to target discogenic back pain is a research priority. Current therapies are restricted to invasive surgical procedures or therapies that focus on temporary analgesia rather than targeting the mechanisms underlying the source of the pain. The ingrowth of nociceptive nerve fibers and blood vessels has been identified in symptomatic IVDs. Thus neurovascular ingrowth in the IVD is a novel and underexplored therapeutic target.

The IVD is the largest avascular organ in the body, resulting in a hypoxic cellular microenvironment. Hypoxia promotes neoangiogenesis in health (wound healing) and disease (cancer) posing the question: how can the healthy hypoxic IVD maintain an avascular structure if it is surrounded by vascularized spinal tissues? Proteoglycans such as aggrecan inhibit endothelial migration in vitro and the healthy intact IVD is a rich-source of these large aggregating matrix proteins. Such proteoglycans also
provide the IVD with water imbibing properties and the high magnitudes of pressurization in the IVD may inhibit blood vessels from growing in. However during degeneration, there is an increase in structural disruption (annular fissures), depletion of proteoglycans, and loss of pressurization. These changes, combined with the hypoxic nature of the IVD, provide a permissive microenvironment that favors neo-angiogenesis. Studies have demonstrated that nerve fibers expressing the nerve growth factor (NGF) receptor, TrkA grow alongside blood vessels expressing the ligand NGF in tissue isolated from painful discs.

The diseased IVD is a source of key angiogenic factors such as vascular endothelial growth factor (VEGF), tumor necrosis factor alpha (TNFα), interleukin-8 (IL-8), IL-6 and MMP-7 and such factors may play a role in neo-angiogenic processes in painful disc degeneration. VEGF is a key angiogenic growth factor that stimulates endothelial cell proliferation, migration, and blood vessel formation. It is modulated by pro-inflammatory cytokines and hypoxia inducible proteins in vitro. TNFα influences angiogenesis through the up-regulation of pro-angiogenic factors VEGF, IL-6, and MMP-7 in IVD cells and also induces angiogenic sprouting in endothelial cells. Other cytokines such as IL-6 can stimulate the production of VEGF while IL-8 enhances endothelial cell survival, proliferation, and migration. Matrix enzymes like MMP-7 promote angiogenesis through the release and activation of pro-angiogenic cytokines sequestered in extracellular matrix and accelerate endothelial cell proliferation and vessel formation.

Recapitulation of the processes that occur during embryonic patterning of the IVD may help inform symptom-modifying treatments for painful disc degeneration. The notochord is an embryonic structure of chordates that patterns the spine and secretes anti-angiogenic factors such as Noggin and Chordin. The notochord also synthesizes proteoglycans, including chondroitin sulfated (CS) proteoglycans, which are involved in repulsion of dorsal root ganglion cells. Such proteoglycans can also inhibit angiogenesis in adults. Notochordal cells (NCs) isolated from the immature nucleus pulposus (NP) secrete soluble factors that can inhibit neurite outgrowth while maintaining neural cell viability and were also reported to have anti-inflammatory effects suppressing IL-6, IL-8, and nitric oxide synthesis in the IVD. In this work, we evaluate if NCs secrete soluble factors that can target neovascularization associated with discogenic back pain.

We propose a hypothetical model describing how NC conditioned medium (NCCM) can be utilized to target neurovascular

![Fig. 1. Schematic of the hypothetical model: treatment of painful neurovascular ingrowth. Soluble factors found in NCCM from the healthy immature IVD, specifically CS and Noggin, can inhibit angiogenesis via inhibition of endothelial cell invasion and blood vessel formation in both a (1) healthy and (2) degenerate environment through (3) inhibition of pro-angiogenic cytokines VEGF, MMP-7, IL-6, and IL-8. We propose that vascular ingrowth is the precursor to subsequent nerve ingrowth and pain and that inhibition of angiogenesis is an important clinical target that could result in therapies with sustained efficacy.](image-url)
ingrowth in discogenic back pain and to identify therapeutic candidates (Fig. 1). While the inhibitory effects of aggrecan derived from adult human NP tissue and non-degenerate human NP cells themselves have been assessed on neurovascular growth in vitro, it remains unknown if NCCM would have similar affects. Hypothesis 1 is that NCCM contains soluble factors including CS and Noggin that can inhibit angiogenesis through suppression of endothelial cell invasion and blood vessel formation (Fig. 1). Pro-inflammatory mediators are increased in painful degenerate IVDs and are also associated with angiogenesis. Hypothesis 2 is that NCCM, Noggin and CS can suppress endothelial invasion and blood vessel formation in the presence of TNFα. Hypothesis 3 is that NCCM and therapeutic candidates inhibit angiogenesis through suppression of pro-angiogenic factors VEGF, MMP-7, IL-8 and IL-6. Angiogenesis and nerve ingrowth are often integrated processes. We propose that neo-angiogenesis is an initiator of discogenic back pain as it promotes sensory nerve ingrowth into the IVD and suggest that direct repulsion of neovascularization may be an important part of therapies to relieve discogenic pain.

Methods

Generation of NCCM

NCCM was generated from NC-rich NP tissue aseptically dissected from eight porcine spines (6–8 weeks of age) obtained within 24 h of death (Animal Facility Research 87 Inc., Boylston, MA, USA). The isolated tissue was incubated in high glucose DMEM, 1× insulin transferrin & selenium, salt solution (5 M NaCl/0.4 M KCl) and 50 µg/ml ascorbic acid for 4 days in hypoxia (1% O2, 5% CO2 and 37°C). NCCM was filtered using 3000 MW Amicon Ultra-15 Centrifugal Filter (Millipore, # UFC900324) to remove soluble factors and to remove small metabolites and waste products. The filtrate was re-suspended in Medium 200PRF (Med200) (Life Technologies, #M200PRF500) and stored at −80°C.

Human umbilical vein endothelial cell (HUVEC) culture

Primary HUVECs (Life Technologies, #CO1510C) were cultured according to the manufacturer’s instructions in Medium 200 supplemented with Low Serum Growth Supplement (LSGS) (Life Technologies, #S00310) until 80% confluent. The final concentrations of the components in the supplemented medium (basal medium) were 2% fetal bovine serum, 1 µg/ml hydrocortisone, 10 ng/ml human epidermal growth factor, 3 ng/ml basic fibroblast growth factor, and 10 µg/ml heparin. HUVECs at passages 1–3 were used for all experiments.

Effects of NCCM on endothelial cell invasion

HUVECs were serum-starved (0% FBS, 0.2% BSA) overnight and seeded in Basal medium without FBS at a density of 30,000 cells per well into the top insert plate of a 96 well Millipore Multiscreen-MIC Plate (8.0 µm pores, Millipore, #MAMCS810) pre-coated with 50 µl Matrigel (BD Biosciences, #356234) at a concentration of 800 µg/ml. HUVECs were incubated for 16 h (5% CO2 and 37°C) with the following treatment conditions added to the receiver plate in quadruplicate: (1) Basal medium and (2) NCCM at 10% and 100% doses (n = 4). All conditions were supplemented with FBS at a final concentration of 5%, to serve as a chemo-attractant, and LSGS. After 16 h, the receiver plate was removed, and the cells in the insert plate were incubated for 15 min with 0.1% 2 mM Ethidium Homodimer-1 stock (Life Technologies, #E1169) and 0.2% 4 mM Calcein AM stock (Life Technologies, #C1430) in Basal medium without FBS. Twenty images of the bottom surface of the insert plate membrane were taken per group at 5× magnification and percent area fluorescence were measured as a measurement of cell invasion analyzed (see Image analysis).

Effects of NCCM on endothelial tubular formation

HUVECs were seeded at 80,000 cells per well in a 24 well plate coated with 100 µl Geltrex LDEV-Free, a reduced growth factor basement membrane matrix (Life Technologies, #A1413202). HUVECs were incubated for 24 h (5% CO2, 37°C) with the following treatment conditions in triplicate: (1) Basal medium and (2) NCCM (n = 4) at 10% and 100% doses. All groups were supplemented with LSGS, which together with Geltrex, induced tubular formation. After 24 h, cells were incubated for 15 min with Ethidium and Calcein as above. Fifteen images were taken per group at 5× magnification and tubule length analyzed (see Image analysis).

CS and Noggin additive and blocking studies on endothelial cell invasion and tubular formation

Additive studies examined the following treatment conditions: (1) Basal medium, (2) CS (Sigma, #C9819) at 10 and 100 µg/ml29, and (3) Noggin (R&D Systems #6057-NG-025/CF) at 10 and 100 ng/ml35 for both the cell invasion assay and tubular formation assays. For blocking studies, NCCM and basal medium (n = 4) were treated with 0.05 U/ml Chondroitinase ABC (Chon-ABC) (Sigma #C3667) at 37°C for 30 min to digest CS and dermatan sulfate (DS). Our previous work has shown that CS forms the major glycosaminoglycan (GAG) in NCCM36. HUVECs were then treated with: (1) Basal medium, (2) Basal medium with Chon-ABC, (3) NCCM (100%), and (4) NCCM (100%) with Chon-ABC, for both the cell invasion and tubular formation assays. Currently, there is no direct method available to inactivate or block Noggin in conditioned media and so a blocking study for Noggin could not be performed.

Effects of NCCM, CS and Noggin on cell viability

Images (15 images @ 5× magnification) of Calcein (green = live cells) and Ethidium (red = dead cells) stained endothelial cells from the tubular formation assay were used to assess cell viability. Percent live cells were quantified from the combined totals of live and dead cells using Image J.

Effects of NCCM, CS and Noggin on endothelial cell invasion, tubular formation and gene expression in the presence of TNFα

HUVECs were seeded, incubated and analyzed as above for the cell invasion and tubular assays. Groups included (1) Basal medium, (2) NCCM at 10% and 100% doses, (3) CS at 10 and 100 µg/ml and (4) rhNoggin at 10 and 100 ng/ml, with or without recombinant human TNFα (Life Technologies, #PH3015) (10 ng/ml) including a TNFα control group. For assessment of gene expression, HUVECs from the tubular assay were lysed with Trizol Reagent, RNA extracted (Macherey–Nagel, #740902.50) and cDNA synthesized (Life Technologies, #11754-250) according to manufacturers instructions. qRT-PCR was performed using Taqman Gene Expression Assays (Life Technologies, #4331182) for VEGF (hs00900055_m1), MMP-7 (hs01042796_m1), IL-6 (hs00985639_m1), IL-8 (hs00174103_m1) and 18S (hs03928990_g1) using a ΔΔCT method36. ΔΔCT are expressed as the mean fold change normalized to the housekeeping gene 18S and either to the Basal or TNFα control media samples.
To examine the expression of NGF released into the media a human NGF-beta sandwich ELISA (Peprotech # 900-K60) was also performed on Basal and TNFα control media samples.

**Image analysis**

To quantify cell invasion and tubular formation assays, images were captured using a fluorescent inverted Zeiss microscope (Emission/excitation: Calcein = 494/517 nm and ETH = 528/617 nm). Zeiss Auto Measurement Software was used to determine the percent area fluorescence for the invasion assay and length of tubules for the tubular assay (Fig. 2). The “Area percent” Zeiss Auto Measurement Field Feature was used to determine percent area of fluorescence and is defined as the total area of fluorescence in relation to the area of the total image. The “Fibrelength” Zeiss Auto Measurement Region Feature was used to determine the length of tubules and is defined as the length of a fiber-like region in the unit of the scaling assigned to the image.

**Statistics**

As a Gaussians distribution could not be assumed, a non-parametric multiple comparison Kruskal–Wallis test with a post hoc Dunns test was performed between Basal and the respective controls using Graphpad Prism.

**Fig. 2.** Image analysis of angiogenesis assays: cell invasion and tubular formation. Cell invasion is an in vitro measure of cell migration through a matrix barrier that occurs during angiogenesis in vivo. Tubular formation represents the organization and assembly of endothelial cells into blood vessels. Representative images used to analyze HUVEC invasion and tubular formation assays. Images were captured at 5× magnification using a Zeiss inverted microscope and Axiovision software after staining with green-fluorescent calcein-AM and red-fluorescent ethidium homodimer-1. Zeiss Auto Measurement Program was used to (1) define and measure the area of fluorescence to determine cell invasion and (2) measure the length of tubules excluding branching points of formed tubular networks, without human bias.

**Fig. 3.** NCCM, CS and Noggin inhibit endothelial cell invasion. HUVECs cultured under basal conditions invaded through the Matrigel basement membrane and migrated to the bottom of the well insert. Addition of NCCM at both 10% and 100% significantly inhibited endothelial cell invasion compared to basal control, \( P = 0.0024 \) (A) (error bars represent CIs: Basal [85.67, 92.78], NCCM 10 [44.78, 59.95], NCCM 100 [52.37, 67.27]). Addition of CS at 10 and 100 µg significantly inhibited cell invasion compared to basal control, \( P = 0.0032 \) (B) (CI: Basal [47.83, 57.50], CS10 [14.18, 27.98], CS100 [19.15, 27.07]). Addition of Noggin at 10 and 100 ng significantly inhibited cell invasion compared to basal control, \( P = 0.0021 \) (C) (CI: Basal [60.63, 76.48], Noggin10 [28.42, 38.83], Noggin100 [35.06, 60.31]). All scale bars correspond to 200 µm.
groups (NCCM 10%, NCCM 100%, CS 10 mg, CS 100 mg, Noggin 10 ng, Noggin 100 ng) for tubular, cell invasion and gene expression assays. A Mann Whitney U test was used to assess protein expression (IL-1β, TNFα, IL-6 and IL-8) comparing TNFα treatment with Basal control. For NCCM, biological replicates were n = 4, however, as treatments with CS and Noggin groups were not based on biological replicates, the mean value of 3 and 4 experimental replicates were used for tubular and invasion assays, respectively (Supplemental data 1). All values are expressed as the mean ± the 95% confidence interval (95% CI). P values <0.05 were considered significant.

Results

NCCM inhibits cell invasion and endothelial tubular formation

NCCM at both 10% and 100% doses significantly inhibited cell invasion compared to Basal controls (P = 0.0024) [Fig. 3(A)]. Both doses of NCCM significantly decreased the length of tubules and increased the fragmentation of tubular networks compared to Basal control (P = 0.0244) [Fig. 4(A)].

CS and Noggin inhibit endothelial cell invasion and tubular formation

To investigate the role of CS, additive studies were performed with doses of 10 and 100 µg/ml. Both doses significantly inhibited cell invasion (P = 0.0032) [Fig. 3(B)]. CS at 100 µg/ml significantly decreased the length of tubules formed compared to Basal controls (P = 0.0349), however, CS at 10 µg/ml did not significantly inhibit tubule length [Fig. 4(B)]. When examining the effects of Noggin, both doses (10 and 100 ng/ml) significantly inhibited cell invasion (P = 0.0021) [Fig. 3(C)]. Noggin at 10 ng/ml significantly decreased the length of tubules formed compared to Basal controls (P = 0.0343), however, Noggin at 100 ng/ml did not inhibit tubule length [Fig. 4(C)].

Blocking studies for CS were performed in which NCCM (100%) was digested with Chon-ABC and used to treat HUVECs for both the cell invasion and tubular formation assays. NCCM significantly inhibited cell invasion and decreased the length of tubules compared to Basal controls (P = 0.0070 and P = 0.0193, respectively). NCCM treated with Chon-ABC restored cell invasion and tubule length to basal levels with no differences between Basal control, Basal control with Chon-ABC, and NCCM with Chon-ABC in either assay (Fig. 5).

NCCM, CS and Noggin maintain cell viability

NCCM and Noggin at both doses and 100% and 100% concentrations significantly inhibited cell invasion compared to Basal controls (P = 0.0024) [Fig. 3(A)]. Both doses of NCCM significantly decreased the length of tubules and increased the fragmentation of tubular networks compared to Basal control (P = 0.0244) [Fig. 4(A)].

Angiogenesis and inflammation occur simultaneously in the painful degenerate IVD. We therefore investigated whether NCCM, CS and Noggin could inhibit cell invasion and tubular invasion in...
the presence of TNFα. TNFα induced a pro-inflammatory environment involving the up-regulation of multiple pro-inflammatory cytokines (IL-1β, TNFα, IL-6 and IL-8) in HUVECs (Supplemental data 3), as would be expected in the degenerated IVD microenvironment. NGF protein expression was not detected in any of the Basal of TNFα treated media samples. There were no significant differences in cell invasion between Basal and TNFα controls. NCCM in the presence of TNFα at both 10% and 100% suppressed cell invasion compared to TNFα controls (P = 0.0002). Similar inhibitory effects were observed for CS at 10% and 100 μg and Noggin at 10 and 100 ng also in the presence of TNFα (Fig. 6) (P = 0.0002 and P = 0.0003, respectively). When assessing effects on tubular formation, HUVECs in Basal or TNFα conditions demonstrated intact tubular networks with no differences between these two groups (Fig. 7). However, NCCM, CS and Noggin at doses of 10% and 10 μg and 10 ng, respectively, significantly inhibited tubular formation compared to TNFα controls (P = 0.0009, P = 0.0190 and P = 0.0149, respectively).

NCCM inhibits the expression of pro-angiogenic markers VEGF, MMP-7, IL-6 and IL-8

To examine the mechanisms through which NCCM might be inducing its inhibitory effects, we investigated the gene expression of VEGF, MMP-7, IL-6 and IL-8. Under Basal conditions, NCCM (100%) significantly down-regulated all genes apart from IL-8 compared to Basal control (P = 0.0256, P = 0.0201 and P = 0.0283, respectively) (Table 1). Noggin and CS had no effects on pro-angiogenic genes under Basal or TNFα conditions. In the presence of TNFα, NCCM demonstrated significant decreases in VEGF, MMP-7, IL-6 and IL-8 expression compared to the TNFα control (P = 0.0140, P = 0.0162, P = 0.0346 and P = 0.0346, respectively) (Table 1 and Supplemental data 4).

Discussion

This is the first study to demonstrate that soluble factors from the immature NC-rich porcine NP (NCCM) can inhibit angiogenic processes in vitro, suggesting that NC-derived factors may be harnessed and used to treat vascular ingrowth. We investigated candidates CS and Noggin, previously identified in the notochord and observed inhibition of endothelial invasion and tubular formation at similar magnitudes to that of NCCM. NCCM itself as a potential therapeutic intervention has limitations as the immature NC-rich human IVD transitions into a chondrocyte-like NP-rich tissue at adolescence25; so isolation of candidates CS and Noggin offer a more targeted approach. Vascular ingrowth occurs in painful degenerate IVDs in the presence of pro-inflammatory cytokines20 and we demonstrated that NCCM could also inhibit angiogenesis in the presence of TNFα further highlighting the therapeutic potential of these ligands. NCCM decreased the expression IL-6, IL-8, MMP-7, and VEGF in endothelial cells both in the presence and absence of TNFα, suggesting that NCCM may inhibit angiogenesis through suppression of these pro-angiogenic genes. These results support our hypothetical model in which soluble factors from the notochord can be utilized to inhibit vascularization suggesting they...
may be useful in treating innervation and painful processes in the degenerate IVD.

To assess the effects of NCCM on angiogenic mechanisms in vitro, we used the cell invasion and tubular formation assays. The cell invasion assays tests HUVECs ability to break down and invade the matrix towards a chemo-attractant and the tubular formation assay tests HUVECs ability to form tubules mimicking the formation of vessels in vivo. NCCM inhibited both cell invasion and tubular formation and these effects were largely dose independent for the invasion assay, however, dose effects were observed for the tubular assay; 100 μg CS and 10 ng Noggin exerted the greatest inhibitory effects in basal conditions. Interestingly dose effects were also observed for the tubular assay in the presence of TNFα, however, significant changes were observed for NCCM 10%, 10 μg CS including 10 ng Noggin. It is possible to speculate that dose effects of CS and Noggin for the tubular assay but not invasion assay including variations between basal and inflammatory conditions may be attributed to differences in chemotactic (cell invasion) vs cell–cell interactions (tubular formation) for these ligands, which are likely altered in an inflammatory microenvironment. We observed minimal effects on HUVEC cell viability in the presence of NCCM, CS and Noggin, with the exception of CS at 100 μg suggesting that NC-derived soluble factors are largely not cytotoxic. However, these factors warrant further investigation in 3D organ culture and animal models to confirm safety and efficacy.

The therapeutic potential of soluble factors from the notochord has been highlighted by several studies. NCS co-cultured with bovine NPCs demonstrate increased proteoglycan synthesis and CTGF/CCN2 was identified as the soluble factor responsible for the observed effects. NCCM can rescue degenerate human NP cells and differentiate human MSCs toward a healthy NP phenotype. Prior studies on NCCM focused primarily on the anabolic but not the symptom-modifying potential of NC-derived factors. NCs have anti-inflammatory and protective effects with the ability to decrease expression of IL-6, IL-8, and nitric oxide synthase as well as inhibit cell death and apoptosis in IVD cells in vitro. We observed that NCCM could inhibit endothelial cell invasion and tubular formation in the presence of TNFα confirming the anti-inflammatory effects of NC derived factors and their ability to function in the presence of TNFα which stimulated production of multiple pro-inflammatory cytokines, many of which have been detected in the degenerate human IVD. Our previous work has shown that NCCM inhibits neurite outgrowth from both neuroblastoma and DRG cells whilst maintaining cell viability and that CS is a factor in NCCM responsible for these inhibitory effects. Together, these results demonstrate that NCCM has both anti-neuronal and anti-angiogenic potential as well as structure-modifying properties which can be used to target the degenerate and painful IVD.

CS is a sulfated GAG that is an essential component of proteoglycans and is abundant in the healthy IVD but decreases with age and degeneration. CS derived from the notochord repulses axonal elongation from dorsal root ganglion cells during development. CS has been successfully used as an anti-inflammatory in osteoarthritis to prevent joint space narrowing and reduce joint swelling. However, the clinical potential of CS may be more mixed.

![Fig. 6. NCCM, CS and Noggin inhibit cell invasion in the presence of TNFs. HUVECs cultured with TNFs demonstrated no significant differences in cell invasion compared to basal controls. Addition of NCCM at 10% and 100% significantly decreased invasion compared to TNF controls, P = 0.0002 (A) (error bars represent CI: Basal [49.26, 77.17], B + TNF [84.93, 93.27], NC10 + TNF [36.58, 53.72] and NC100 + TNF [28.48, 50.90]). Addition of CS and Noggin at both doses demonstrated similar effects, with significant decreases in cell invasion compared to TNF controls, P = 0.0002, P = 0.0003, respectively (B, C) (for CS, CI: Basal [70.96, 79.91], B + TNF [80.38, 84.30], CS10 + TNF [39.78, 54.82] and CS100 + TNF [50.60, 53.06] and for Noggin CI: Basal [62.50, 72.68], B + TNF [81.59, 88.49], Nog10 + TNF [47.18, 60.16] and Nog100 + TNFs [39.15, 60.68]. All scale bars correspond to 200 μm.)

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in IVD degeneration, as glucosamine sulfate exhibited anti-anabolic effects in animal models of IVD degeneration\(^45,46\). This study demonstrated that CS was able to inhibit HUVEC invasion and tubular formation in the presence of TNF\(\alpha\). The ability of the proteoglycan aggrecan to inhibit endothelial cell adhesion/migration and nerve outgrowth depends on the degree of glycosylation\(^8,29\), suggesting that CS is largely responsible for the proteoglycan's anti-angiogenic effects. These studies also demonstrated that there were differences in the extent of inhibition induced by aggrecan derived from AF and NP cells suggesting that studies examining differences in the GAG profiles between NP cells and immature NCS are warranted. Blocking studies where NCCM was depleted of intact Noggin is known to have anti-angiogenic effects during development\(^47\). Noggin is a BMP antagonist that is produced by the notochord and is an essential mediator in patterning the spine during development\(^47\). Noggin and CS restored cell invasion and tubular length back to basal levels confirming that CS was necessary for NCCM's inhibitory effects. We have shown that CS can inhibit neural and vascular growth simultaneously, making it an attractive treatment option and suggesting that restoration of the matrix barrier rich in CS is likely to be very important to repel and inhibit neurovascular ingrowth in diseased IVDs.

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Table I

<table>
<thead>
<tr>
<th>Treatments</th>
<th>VEGF</th>
<th>MMP7</th>
<th>IL-6</th>
<th>IL-8</th>
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<td>Basal Control</td>
<td>1 (95.6, 93.3)</td>
<td>1 (13.3, 13.5)</td>
<td>1 (11.1, 11.3)</td>
<td>1 (7.4, 7.9)</td>
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<td>NCCM</td>
<td>-68.2 (-37.4, -103.9)*</td>
<td>-31.4 (-10.0, -57.7)*</td>
<td>-58.8 (-30.3, -92.1)*</td>
<td>-12.2 (-3.7, -22.9)</td>
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<td>Noggin</td>
<td>-7.4 (-7.5, -11.6)</td>
<td>-6.8 (-2.8, -11.7)</td>
<td>-1.5 (-0.8, -2.1)</td>
<td>-2.7 (-3.0, -3.7)</td>
</tr>
<tr>
<td>CS</td>
<td>-3.0 (-0.15, -6.7)</td>
<td>-1.3 (-2.3, -3.8)</td>
<td>-3.6 (6.2, 1.7)</td>
<td>-6.0 (20.1, 16)</td>
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<tr>
<td>TNF(\alpha)</td>
<td>1 (10.5, -10.3)</td>
<td>1 (11.3, -11.9)</td>
<td>1 (13.7, -14.2)</td>
<td>1 (17.5, -19.0)</td>
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<tr>
<td>TNF(\alpha) Control</td>
<td>-180.0 (-35.6, -372.7)*</td>
<td>-86.0 (-28.6, -158.4)*</td>
<td>-195 (-86.9, -327.5)*</td>
<td>-39.7 (-18.9, -64.2)*</td>
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<tr>
<td>NCCM</td>
<td>-3.0 (-7.0, -10.7)</td>
<td>-4.9 (1.5, -26.4)</td>
<td>-1.2 (4.0, -0.5)</td>
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<td>Noggin</td>
<td>-26.6 (-0.64, -61.5)</td>
<td>-14.9 (0.1, -36.6)</td>
<td>-4.5 (4.6, -1.5)</td>
<td>-4.5 (0.1, -10.4)</td>
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</table>

![Fig. 7. NCCM, CS and Noggin inhibit tubular formation in the presence of TNF\(\alpha\). HUVECs cultured in Basal conditions or with TNF\(\alpha\) demonstrated intact tubular networks. However, addition of NCCM at 10% significantly decreased tubular length compared to TNF\(\alpha\) controls, \(P = 0.0009\) (A) (error bars represent CI: Basal [224.9, 313.5], TNF\(\alpha\) [244.1, 324.9], NCCM10 + TNF\(\alpha\) [82.84, 141.1] and NCCM100 + TNF\(\alpha\) [1073, 1341]). Addition of CS at 10 \(\mu\)g and Noggin at 10 ng demonstrated similar effects, with significant decreases in tubular length compared to TNF\(\alpha\) controls, \(P = 0.0190, P = 0.0149\), respectively (B, C) (for CS: CI: Basal [224.9, 313.5], TNF\(\alpha\) [244.1, 324.9], C50 + TNF\(\alpha\) [94.56, 162.0] and C100 + TNF\(\alpha\) [77.28, 221.0] and for Noggin: CI: Basal [224.9, 313.5], TNF\(\alpha\) [364.1, 324.9], Nog75 + TNF\(\alpha\) [136.62, 126.1] and Nog100 + TNF\(\alpha\) [63.98, 259.7]). All scale bars correspond to 200 \(\mu\)m.]
inhibition of VEGF protein expression and promoter activity, or (2) down-regulation of E-cadherin via Lef1-mediated transcription. In our study we demonstrated that 10 ng/ml was the optimal dose to inhibit both invasion and tubular formation in basal and TNFα stimulated culture conditions. The fact that 100 ng did not induce similar effects was surprising as Noggin has been shown to have an inhibitory effect on endothelial tubular formation with increased doses. Differences may likely be attributed to slight variations in the 3D matrices utilized such as Matrigel vs Geltrex. Currently, there is no accepted method to directly block the function of Noggin in vitro, so only additive studies were performed.

We examined the expression of pro-angiogenic factors VEGF, MMP-7, IL-6 and IL-8 in HUVECs treated with NCCM, CS and Noggin to assess if each of these factors acted via similar pathways. NCCM inhibited the expression of all genes assessed with greatest suppression for VEGF, suggesting that NCCM may inhibit cell invasion and tubular formation through the modulation of all of these pro-angiogenic factors. VEGF can be described as the master regulator of angiogenesis and is a key growth factor that stimulates proliferation, migration, and tube formation through the modulation of all of these pro-angiogenic factors. VEGF expression for the first time that soluble factors from NC-rich NP tissue are capable of inhibiting angiogenesis in basal and pro-inflammatory conditions as might be present in the degenerate micro-environment. NCCM and candidate factors CS and Noggin demonstrated anti-angiogenic effects on HUVECs and NCCM appeared to act via the VEGF pathway. NCCM is not a feasible treatment option highlighting a need to identify isolated factors from NCCM. CS has the ability to inhibit both angiogenesis and innervation, and may be useful as a minimally invasive treatment option for discogenic back pain, although precise mechanisms of action warrants further investigation.

Author contributions

All authors have met the ICMJE's definition of authorship. MCC, SKC, CG, JC1 & DP contributed to (1) the conception and design of the study, or acquisition of data, or analysis and interpretation of data, (2) drafting the article or revising it critically for important intellectual content and (3) final approval of the version to be submitted. DP takes responsibility for the integrity of the work as a whole, from inception to finished article.

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Competing interest statement

The authors have no competing interests to declare.

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Supplementary data

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