N-Cadherin-Mediated Signaling Regulates Cell Phenotype for Nucleus Pulposus Cells of the Intervertebral Disc

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Abstract—Juvenile nucleus pulposus (NP) cells of the intervertebral disc (IVD) are large, vacuolated cells that form cell clusters with strong cell–cell interactions. With maturation and aging, NP cells lose their ability to form these cell clusters, with aging-associated changes in NP cell phenotype, morphology, and proteoglycan synthesis that may contribute to IVD degeneration. Therefore, it is important to understand the mechanisms governing juvenile NP cell cluster behavior towards the goal of revealing factors that can promote juvenile, healthy NP cell phenotypes. N-cadherin has been identified as a cell–cell adhesion marker that is present in juvenile NP cells, but disappears with age. The goal of this study was to reveal the importance of N-cadherin in regulating cell–cell interactions in juvenile NP cell cluster formation and test for a regulatory role in maintaining a juvenile NP phenotype *in vitro*. Juvenile porcine IVD cells, of notochordal origin, were promoted to form cell clusters *in vitro*, and analyzed for preservation of the juvenile NP phenotype. Additionally, cadherin-blocking experiments were performed to prevent cluster formation in order to study the importance of cluster formation in NP cell signaling. Findings reveal N-cadherin-mediated cell–cell contacts promote cell clustering behavior and regulate NP cell matrix production and preservation of NP-specific markers. Inhibition of N-cadherin-mediated contacts resulted in loss of all features of the juvenile NP cell. These results establish a regulatory role for N-cadherin in juvenile NP cells, and suggest that preservation of the N-cadherin mediated cell–cell contact is important for preserving juvenile NP cell phenotype and morphology.

Keywords—N-cadherin, Intervertebral disc, Nucleus pulposus, Cell–cell interactions.

INTRODUCTION

Degenerative disc disease contributes to disability and pain pathology in the cervical and lumbar spine in over 80% of the US population.[35] Intervertebral disc (IVD) degeneration is believed to arise from aging-related changes in density and phenotype of the nucleus pulposus (NP) cells in the disc.[5] Healthy, juvenile NP cells, derived from the notochord, are characterized by large, vacuolated cells capable of forming clusters, producing functional extracellular matrix and secreting soluble factors that mediate and regulate proteoglycan synthesis to maintain a healthy IVD.[3,9,16,22,24,45] During disc degeneration or aging, NP cells transition to a sparse population of small, chondrocyte-like cells that lose their ability to produce functional matrix,[30,31,39] in a process that coincides with observed clinical findings of decreased IVD height, increased matrix stiffness, and decreased proteoglycan and water contents.[2–4] Unfortunately not much is known of these biological factors that contribute to the onset of changes in NP cell phenotype and morphology.[40,43]

Much work has been done in the field to study NP cell characteristics in IVD tissue of various species, specifically how NP cell characteristics change with age (with matrix elasticity values from 0.3 up to 5 kPa[11,26,27]). Juvenile NP cells have been characterized *via* the presence of brachyury-T[44] and production of extracellular matrix molecules (aggrecan, type II collagen, and laminin β1).[32,33,45] One of the most striking features of juvenile, young NP cells is their ability to remain rounded and form cell clusters, containing three or more cells.[7,16,21] Studies that quantify percentage of NP cells that appear in cell clusters indicate significantly greater number of NP cell clusters present in juvenile tissue compared to mature or degenerated tissue in rat[7] and human.[21] However, not much is known regarding the importance of these cell clusters. Prior work in our lab has demonstrated increased glycosaminoglycan production for juvenile NP cells that form cell clusters on soft, laminin-rich

surfaces.[14,16,26] Culturing juvenile porcine, notochordally-derived NP cells on soft (<700 Pa), laminin-rich surfaces[13,14,16,26] promotes NP cells to assemble into multi-cell aggregates, similar to those observed in situ, with increased glycosaminoglycan production[16,26] and preservation of NP markers.[26] In contrast, culturing juvenile porcine NP cells on stiff (>700 Pa), laminin- or collagen-rich surfaces promote NP cells to form spindle-like morphologies with long, thin processes extending away from or between cells.[16,26] These findings suggest a relationship between cell clustering behavior and matrix production, and provide a motivation for understanding what cell–cell interactions are involved in regulations of biosynthesis under conditions following multi-cell cluster formation.

Cell–cell adhesion molecules, specifically cadherins, are essential for the organization of cells into tissues during development,[1,19] and have been shown to regulate cellular signaling pathways in many cell types.[19,48,49] Cadherins function as membrane-spanning macromolecular complexes: the extracellular regions are responsible for adhesive recognition by binding to the ectodomains of other cadherins on neighboring cells[29] while the cadherin cytoplasmic tails interact with a range of proteins that link the cadherin receptor to a variety of intracellular processes, such as the actin cytoskeleton, cell signaling, and trafficking.[42,50] One subtype of cadherins, the classical cadherins (specifically N- and E-), have been identified as the main contributors to cell–cell interactions in many different cell types.[20,48] Recently, microarray analysis of IVD tissue from various species has revealed high expression of N-cadherin in juvenile or non-degenerate NP tissue compared to degenerate NP, anulus fibrosus (AF, outer region of the IVD) or even articular cartilage (AC) tissue (Table 1).[32–34,44] Despite the observation of high N-cadherin expression in juvenile NP tissue, there is very limited understanding of the importance of N-cadherin in regulating NP cell behavior or the biological mechanisms of N-cadherin-mediated cell–cell interactions in juvenile NP cells.

The objective of this work was to reveal the role of N-cadherin in regulating NP cell clustering behavior and the importance of these cell–cell interactions in maintaining the juvenile NP phenotype and morphology. As high N-cadherin expression has been observed in juvenile NP cells, we hypothesize that N-cadherin is the main cell–cell adhesion molecule that regulates NP cell cluster formation and without N-cadherin-mediated cell–cell interactions, a decrease in juvenile NP cell features will be observed. Juvenile NP cells were cultured under conditions that promote cell clustering and the presence of N- and E-cadherins was analyzed. Additionally, the ability to preserve a juvenile NP phenotype was further characterized and confirmed.

To confirm the importance of N-cadherin in regulating NP cell behavior, loss-of-function studies were performed to reveal changes in NP cell phenotype and morphology when cadherin (N- or E-) function was blocked. Results indicate NP cells form cell clusters *via* N-cadherin-mediated cell–cell contacts, and preservation of the juvenile NP phenotype was observed only when NP cells were able to form these cell clusters. Anulus fibrosus (AF) cells, which were used as a comparator cell group in this study, did not have high expression of N-cadherin, and cell matrix production was not affected by cadherin-blocking studies. These findings present strong evidence that N-cadherin-mediated cell–cell contacts are necessary for successful NP cell cluster formation and preservation of the juvenile NP phenotype and morphology.

METHODS

IVD Tissue and Cell Isolation

All tissue and cell samples used for this study were obtained according to institutional review board-approved protocols.

Pathologic human IVD tissue was obtained from different patients as to-be-discarded surgical waste, undergoing surgery for treatment of degeneration or adult scoliosis ($n = 15$, ages 6–42) at Duke University Medical Center. Regions corresponding to AF and NP tissue were embedded in cryoembedding medium (TissueTek, OCT), flash frozen in liquid nitrogen and stored in −80 °C for cryosectioning and immunostaining.

Porcine IVD tissue was obtained from lumbar spines of young pigs from an abattoir (4–5 months, Nahunta Pork Outlet, Raleigh NC, $n = 9$ separate isolation pools). Porcine tissue was processed in the same manner as human tissue: regions corresponding to AF and NP tissue were embedded in OCT, flash frozen in liquid nitrogen and stored in −80 °C.

Porcine NP and AF cells from lumbar spines of young pigs (4–5 months, Nahunta Pork Outlet, Raleigh NC, $n = 9$ separate isolation pools) were isolated *via* enzymatic digestion (as described in Gilchrist *et al.*[16]). Briefly, cells were isolated from NP tissues *via* pronase-collagenase enzymatic digestion, then resuspended in culture media (Ham's F-12 media (Gibco, Invitrogen) supplemented with 5–10% FBS (Hyclone, Thermo Scientific), 100 U/mL penicillin (Gibco) and 100 mg/mL streptomycin (Gibco)). Resuspended NP cells were cultured in sub-confluent monolayers on conditioned media (collected from rat carcinoma cell line, 804G[17,37]) tissue culture flasks for 2 days before use. Resuspended AF cells were cultured in sub-confluent

TABLE 1. Compilation of N- and E-cadherin expression in bovine, rat, and human IVD tissue.

	Species	1 mo NP/1 mo AC	1 mo NP/12 mo NP	NP/AC
N-cadherin	Rat	4.3	2.128	
	Bovine			2.02
	Human			2.0
E-cadherin	Rat	0.242	0.79	
	Bovine			0.96
	Human			1.2

Higher N-cadherin expression is present in NP tissue compared to AF or AC tissue in all species displayed in the table. Additionally lower levels of E-cadherin expression is observed for NP tissue compared to AF or AC tissue in rat, bovine and human tissue. Data obtained from publically available microarray databases (MIAME). Adapted from [Tang *et al.* (Rat),[44] and Minogue *et al.* (bovine[34] and human[33])] with additional analysis performed using BRB-Array Tools.[41] (mo = month, AC = articular cartilage, AF = annulus fibrosus, NP = nucleus pulposus).

monolayers on 0.1% gelatin-coated tissue culture flasks for 5 days before use.

Tissue Immunohistochemistry: N- and E-Cadherin

Frozen blocks of NP and AF tissue from human and porcine IVD tissue were cryosectioned into 5 μm thick sections, and used for immunostaining of N- and E-cadherins. Tissue sections were fixed in 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield PA) for 20 min, washed with DPBS, blocked for 1 h with serum blocking solution (3.75% BSA and 5% non-immune goat serum), and immunolabeled for N-cadherin (Abcam 12221, 150× dilution in serum blocking solution overnight) or E-cadherin (Abcam 15148, 150× dilution in serum blocking solution overnight). An appropriate isotype matched rabbit antibody was used as control (rabbit IGg, Abcam). Tissue sections were treated with goat-anti-rabbit Alexa-Fluor 488 secondary antibody (Invitrogen) for 30 min at room temperature, shielded from light.

Cell nuclei were stained using propidium iodide (Sigma, 0.33 mg/mL) for 20 min at room temperature, shielded from light. Immediately after each stain, tissue sections were mounted, coverslipped, and imaged *via* confocal microscopy (Zeiss LSM 510, 40× magnification).

Laminin-Rich Substrate Synthesis

Two substrates using basement membrane extract (BME, Matrigel®, growth-factor reduced, 13.8 mg/mL, Trevigen Inc) were created: a soft gel and a ligand-coated stiff glass substrate. To make soft gels, 40 μL of BME (13.8 mg/mL) was added to 8-well glass-bottom chamber slides (Lab-tek Nunc II, Sigma-Aldrich) and allowed to self-polymerize at 37 °C for 30 min. Oscillatory shear testing (Rheometer, AR-G2 Instruments) was used to determine gel stiffness ($E = 300$ Pa). The ligand-coated stiff glass substrate ($E > 50$ GPa[6]) was created by coating culture wells with BME (0.2 μg/mL in PBS) for 1 h at 37 °C.

Porcine NP Cell Culture on Substrate System

Juvenile porcine cells in monolayer culture were detached from the culture surface using 0.025% trypsin/EDTA (Cambrex) and immediately re-suspended in culture media. NP or AF cells (45,000 cells/well, $n = 3$ per measured variable) were cultured upon each substrate for up to 96 h (normoxic conditions: 37 °C, 5% CO_2). In parallel, two additional sets of cells (45,000 cells/well, $n = 3$ per measured variable) cultured upon the same substrates were treated with 40 μg/mL N-cadherin blocking antibody (C3865, Sigma-Aldrich) or 20 μg/mL E-cadherin blocking antibody (DECMA-1, Sigma-Aldrich). Blocking antibody was added to culture media for their respective treatment groups every 24 h for the duration of the culture period.

IVD Cell Immunohistochemistry

Twenty-four hours after culturing on various substrates and treatment conditions, NP and AF cells were fixed (4% paraformaldehyde, Electron Microscopy Sciences, Hatfield PA) for 20 min, washed with DPBS and immunolabeled for polymerized F-actin, N- and E-cadherin. The staining and imaging methods used for NP and AF cells is the same protocol as described in the section titled "Tissue Immunohistochemistry". Nine images across nine different porcine spines were taken for each sample.

Biochemical Analysis

NP and AF cell matrix production of sulfated glycosaminoglycans (sGAG) was analyzed after 96 h in culture using the dimethymethylene blue (DMMB) spectrophotometric method as previously described.[16]

Media overlay from culture samples was collected while cells and substrates remaining in corresponding wells after media removal were digested in papain solution (125 μg/mL in DPBS with 5 mM EDTA and 5 mM cysteine, 2 h, 65 °C). Control wells were also used (substrates only, $n = 3$), and processed in parallel. sGAG content was measured by mixing samples with DMMB dye, and absorbance (535 nm) was measured on a plate reader (Perkin-Elmer Enspire Multimode Reader). sGAG concentrations were determined from a standard curve prepared from chondroitin-4-sulfate (Sigma-Aldrich). For all samples, DNA content was also measured using picogreen assay (Quant-iT, Invitrogen). Total concentration of sGAG (media overlay plus cell digest) was normalized to total DNA content. Differences in sGAG production (sGAG/DNA) were tested using a two-way ANOVA (treatment, substrate) with Tukey's *post hoc* analysis (*$p < 0.05$).

mRNA Extraction

After 96 h of culture on substrates with each treatment group, mRNA was extracted from NP and AF cells. Cells were pooled from two wells to collect sufficient mRNA for one sample, and a total of three RNA samples ($n = 3$, across different spines and substrates) for each group was analyzed. Cells on soft substrates were separated from their corresponding soft substrate using a cell scraper and TRIzol reagent (Life Technologies) before mRNA extraction was performed using the RNeasy mini kit plus DNase I digestion (Qiagen). Cells on stiff substrates were separated from the substrate using a cell scraper and QIAshredder (Qiagen) before mRNA extraction was performed also using the RNeasy mini kit plus DNase I digestion (Qiagen). mRNA integrity and concentration was checked on the ND-1000 Spectrophotometer (NanoDrop). mRNA was reverse transcribed into cDNA using the iScript cDNA synthesis kit (Biorad). cDNA samples were diluted to a final concentration of 10 ng/μL using RNAse-free DNAse-free water.

Quantitative Real-Time PCR (qRT-PCR)

qRT-PCR, using Taqman primer probes (Life Technologies) of porcine NP-specific and NP-matrix related gene-specific primers, was performed on cDNA obtained from NP and AF cells after 96 h culture upon each of the substrates for all treatment conditions. The genes analyzed *via* qRT-PCR were: N-cadherin (custom-designed by Life Technologies), T-brachyury (Ss03374654_g1), type II collagen (Ss03373344_g1), laminin β1 (Ss03375563_u1), and aggrecan (Ss03374823_m1). The housekeeping gene, 18 s (4308329) was used as an inter-

nal control. qRT-PCR reactions were performed on the StepOnePlus real-time PCR system (Applied Biosystems) in duplicate in 96-well plates in a final volume of 25 μL using standard conditions. The PCR reactions contained 12.5 μL 2× universal master mix (Applied Biosystems), 1.25 μL Taqman primer probes, 9.25 μL ddH$_2$O, and 2 μL 10 ng/μL cDNA. Fold-differences were calculated using the delta–delta Ct method ($2^{-\Delta\Delta Ct}$), where the first Δ accounted for fold change over housekeeping gene and the second Δ accounted for fold change over stiff BME substrates. Differences in expression level for each gene was tested using a two-way ANOVA (treatment, substrate) with Tukey's *post hoc* analysis (*$p < 0.05$).

RESULTS

N-Cadherin is Expressed in Juvenile NP but not AF Tissue

Immunostaining for classical cadherins, N- and E-, was performed in human tissue to determine the presence of N- or E-cadherin in the IVD. Immunostaining results reveal positive N-cadherin staining in juvenile human NP tissue only (Fig. 1a). As seen in Fig. 1a, N-cadherin staining is positive (green) in NP tissue for a 6-year-old patient but is absent in patient samples older than 12-years of age, indicating loss of N-cadherin with aging. Additionally, E-cadherin expression is absent in all NP tissue, regardless of age (not shown). However, positive E-cadherin expression was observed in AF tissue for all human tissue, regardless of age (Fig. 1a for human, Fig. 1b for porcine). These findings indicate the presence of N-cadherin only in juvenile or young NP tissue, with loss of N-cadherin expression observed with aging. The loss of N-cadherin with age in human tissue could correlate with the observed decrease in cell density and clustering ability of NP cells with aging.

In addition to analyzing cadherin expression in human tissue, classical cadherin immunostaining was also performed on porcine IVD tissue in order to identify similarities or differences between human IVD tissue and porcine IVD tissue. Similar expression patterns observed in juvenile human tissue for N- and E-cadherin were also observed in porcine tissues. High N-cadherin expression was observed in porcine NP tissue only, and high E-cadherin expression was observed in porcine AF tissue only (Fig. 1b). Quantitative real-time PCR was performed on porcine NP, AF, and articular cartilage (AC) tissue to test for expression of an array of molecules associated with the juvenile NP cell, including laminins, brachyury and collagen. Gene expression analysis reveals significantly higher expression of N-cadherin, and juvenile NP
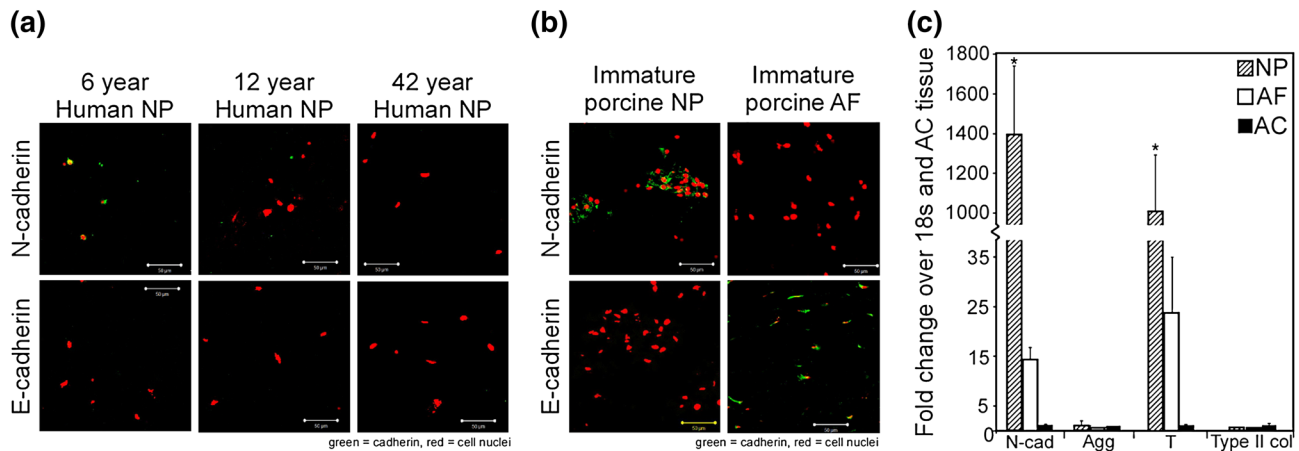
FIGURE 1. Presence of classical cadherins, N- and E-, in IVD. (a) Representative images for positive N-cadherin immunostaining observed in the NP region of 6-year old tissue, which disappears with age; E-cadherin is absent in these representative human NP tissue samples (red = cell nuclei, green = cadherin; scale bar = 50 μm) **(b)** Positive N-cadherin immunostaining is observed in the NP region of juvenile porcine tissue, and positive E-cadherin immunostaining is observed in the AF region of juvenile porcine AF tissue. (red = cell nuclei, green = cadherin; scale bar = 50 μm). **(c)** Higher gene expression levels of N-cadherin and Brachyury-T observed in porcine NP tissue compared to porcine AF and AC tissue (*$p < 0.05$, 1-way ANOVA with Tukey's *post hoc* analysis NP = nucleus pulposus, AF = anulus fibrosus, AC = articular cartilage).

markers, brachyury, laminin, type II collagen and aggreecan, in NP tissue compared to AF and AC tissue (Fig. 1c, *$p < 0.05$).

These findings confirm the presence of N-cadherin protein and mRNA expression in juvenile porcine and juvenile human NP tissue, which correlate with microarray analysis that also identifies N-cadherin mRNA expression in juvenile NP tissue of various species (rat, bovine, human), that decreases with age (Table 1).

Soft, Laminin-Containing Substrates Promote Cell Clustering and N-Cadherin-Mediated Cell–Cell Contacts in Porcine NP but not AF Cells

NP Cells

Prior work has indicated microenvironment cues can be useful in modulating juvenile porcine NP cell behavior, specifically microenvironments composed of soft extracellular matrix with high concentrations of laminin-111.[16] As used in the previously described study, soft BME substrates were also used for this work since BME is mainly composed of laminin-111[28] and BME has the ability to polymerize into a gel (E = 300 Pa, determined *via* oscillatory shear testing) at 37 °C. As demonstrated earlier by Gilchrist *et al.*,[16] NP cells cultured on soft BME naturally formed into large, 3-dimensional cell clusters whereas NP cells cultured on stiff BME attached as individual cells onto the substrate. Phalloidin immunostaining for F-actin in porcine NP cells on soft BME substrates reveal an interconnected network of cells without formation of obvious actin stress fibers (Fig. 2a) and intracellular

vacuoles are still observed (Figure not shown). This cellular organization is similar to *in situ* NP cell morphology of juvenile NP tissue.[7,25] In contrast, NP cells cultured upon stiff BME substrates formed a uniform monolayer with high expression of actin stress fibers observed throughout the cell (Fig. 2a).

Cadherin immunostaining was also performed for NP cells cultured upon the various BME substrates to observe any changes in N- or E-cadherin expression. N-cadherin staining intensity was higher for NP cells on soft BME compared to stiff BME (Fig. 2a) and N-cadherin staining expression and localization appears to be localized to cell edges. Some N-cadherin expression was present for NP cells on stiff BME with expression localized to regions right next to cell nuclei (Fig. 2a). As expected, E-cadherin expression was absent in porcine NP cells on all BME substrates, regardless of cell morphology and organization (Fig. 2a).

AF Cells

Similar to the cell organization observed in porcine NP cells, porcine AF cells also formed cell clusters on soft BME (Fig. 2b), as has been observed previously for human AF cells on soft BME.[18] Phalloidin immunostaining was observed at cell edges without observation of distinct actin stress fibers. Just like NP cells on stiff BME, AF cells on stiff BME formed a uniform monolayer with high distribution of actin stress fibers observed throughout the cell (Fig. 2b).

N- and E-cadherin immunostaining was also performed for AF cells on BME substrates to identify presence of cadherin expression. N-cadherin expression was absent from AF cells regardless of which
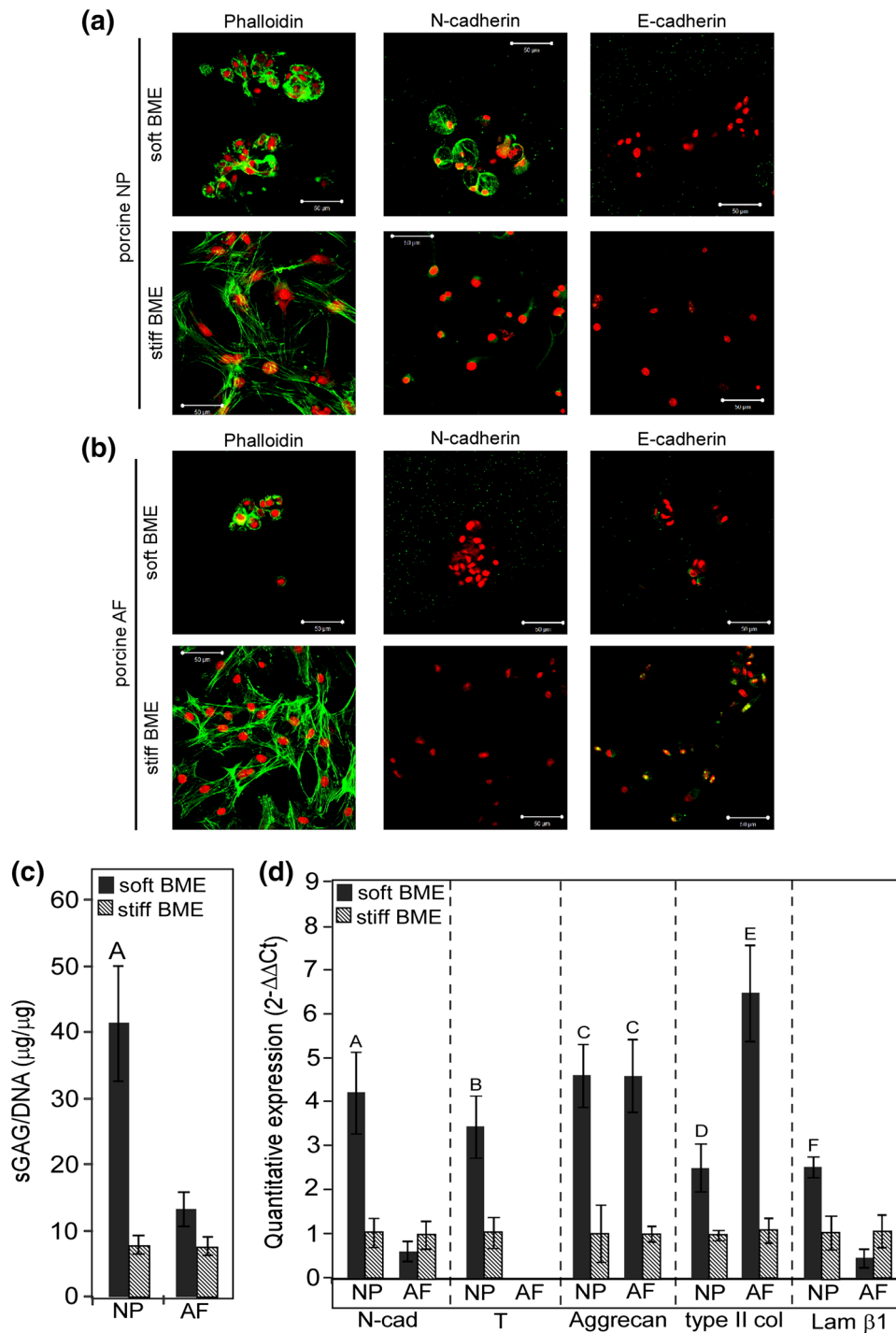
FIGURE 2. Soft, laminin-containing substrates promote N-cadherin-mediated porcine NP cell–cell contacts and regulate cell phenotype. (a–b) Porcine NP and AF cells form cell clusters on soft BME; positive N-cadherin, but negative E-cadherin expression is observed in porcine NP cells on soft, laminin-containing substrates. E-cadherin expression is observed in porcine AF cells on soft and stiff BME. (red = cell nuclei, green = protein (phalloidin or cadherin), scale bar = 50 μm) (c–d) Soft, laminin-containing substrates promote higher cellular biosynthesis in porcine NP cells compared to AF cells (ANOVA with Turkey's *post hoc* analysis, letters indicate significance compared to all other conditions, $p < 0.05$). Significantly higher gene level expression of NP-specific markers, N-cadherin and brachyury-T (T), and NP-matrix-related markers, aggrecan, type II collagen, and laminin, are observed when NP cells form clusters compared to other culture conditions or AF cells (different letters indicate significance within each marker, $p < 0.05$).

BME substrate cells were cultured upon (Fig. 2b); this finding was not surprising as our immunostaining of porcine AF tissue was also negative for N-cadherin. On the other hand, E-cadherin expression was present in AF cells regardless of which substrate the cells were cultured upon (Fig. 2b). Again, these findings are consistent with results of tissue immunostaining that revealed E-cadherin expression in porcine AF tissue.

N-Cadherin Regulates NP Cell Cluster Formation and Downstream Cellular Biosynthesis on Soft BME Substrates

To determine if N-cadherin is necessary for cell biosynthesis and preservation of an juvenile NP cell phenotype, studies in which cadherin function was blocked were performed. N- or E-cadherin blocking antibodies were used to understand the role that N- or E-cadherins may play in regulating cell phenotype and morphology. When NP cells were treated with an N-cadherin blocking antibody, the number of cell–cell clusters was greatly reduced on soft BME (Fig. 3a). Instead, many NP cells remained as individual cells, and a loss of N-cadherin protein immunostaining was observed (Fig. 3c). E-cadherin immunostaining appears to be unaffected by N-cadherin blocking antibody, as E-cadherin protein expression was still absent in NP cells (Fig. 3c). Conversely, blocking E-cadherin function did not appear to influence NP cell cluster formation on soft BME (Fig. 3a). NP cells were still able to form cell clusters with similar patterns of N-cadherin protein immunostaining when cells were cultured on soft BME (Fig. 3c). NP cell behavior did not appear to change on stiff BME substrates, indicating that microenvironment cues can be important for mediating formation of cadherin-mediated cell contacts. Also, these findings indicate a potential role for N-cadherin, but not E-cadherin, in the formation of NP cell–cell contacts on soft BME.

To further understand if N-cadherin helps regulate cellular biosynthesis or preservation of the cell phenotype, biochemical and gene expression analysis was performed for NP cells under the various cadherin blocking antibody treatment conditions. Treatment of NP cells with N-cadherin blocking antibody resulted in significantly decreased sGAG production (Fig. 3b, $*p < 0.05$) on soft BME when compared to no treatment (no blocking antibody) conditions; and no differences in sGAG production were observed for the same NP cells when cultured on stiff BME as compared to corresponding no treatment conditions. Furthermore, treatment of NP cells with E-cadherin blocking antibody did not influence sGAG production (Fig. 3b) on soft or stiff BME.
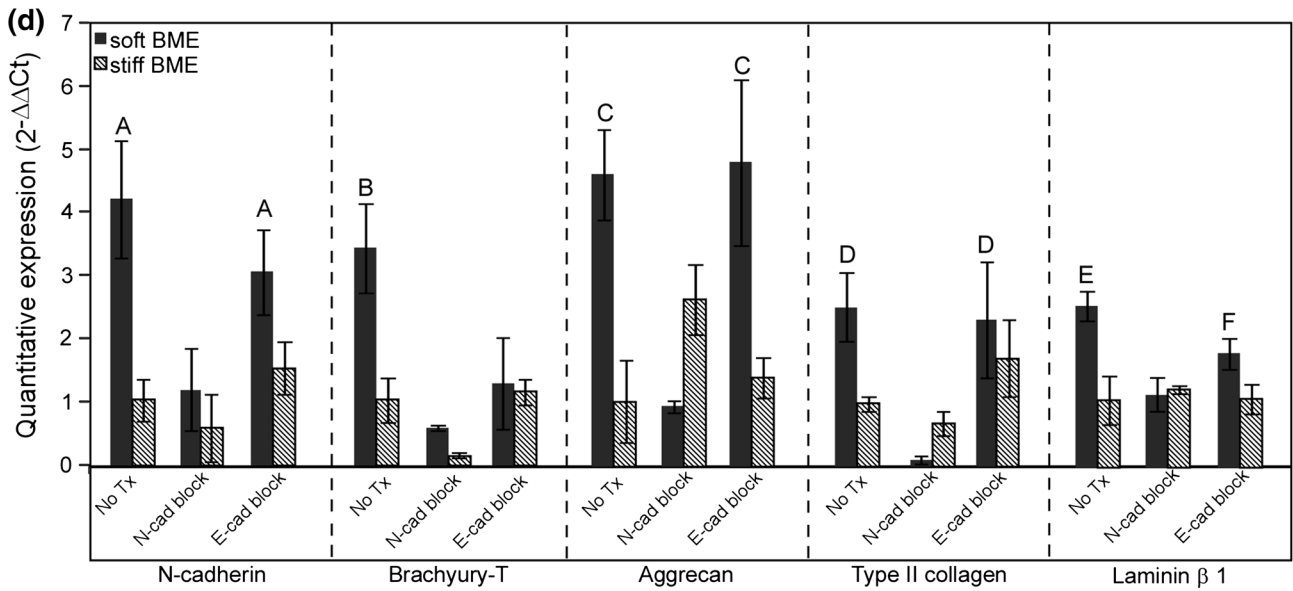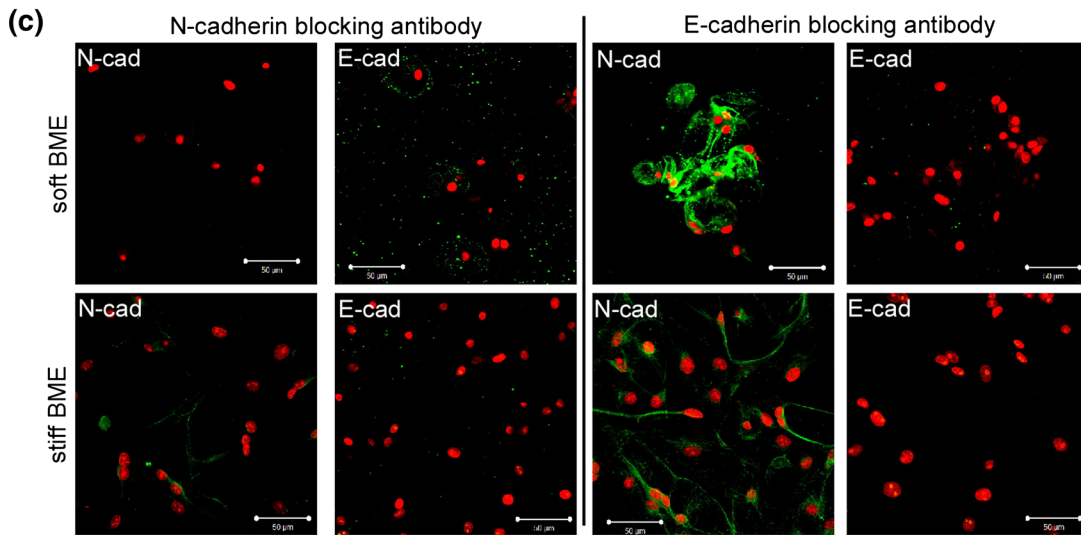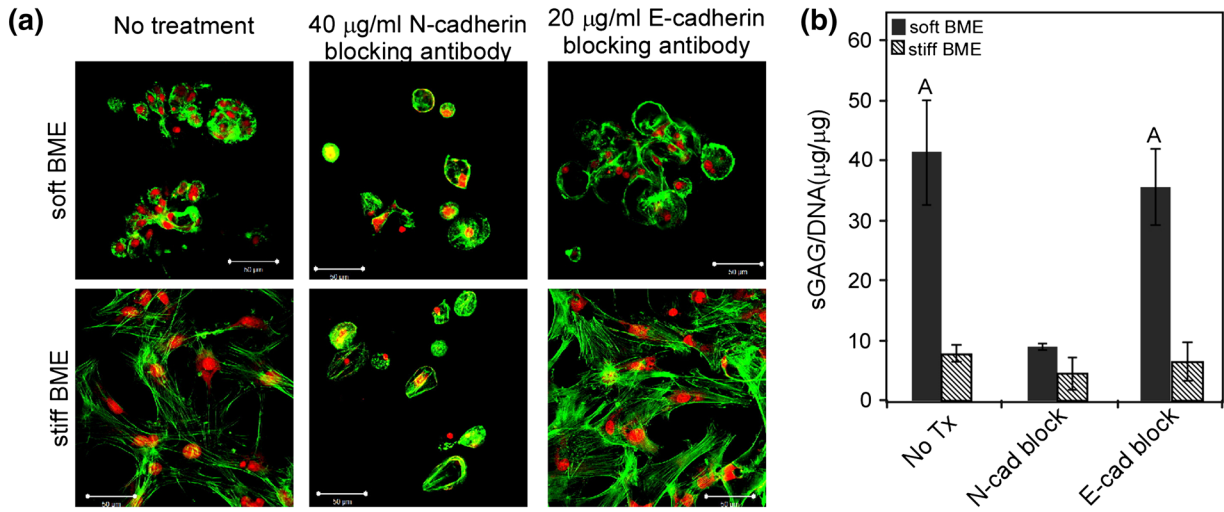
Similar to findings for sGAG production, N-cadherin blocking antibody treatment resulted in decreased mRNA expression for all NP-specific markers (N-cadherin, Brachyury-T, Aggrecan, type II collagen) in NP cells on soft BME (Fig. 3d). Additionally, treatment with E-cadherin blocking antibody did not influence mRNA levels for NP-specific markers in NP cells on soft BME, as gene expression levels were not significantly different from "no treatment" conditions (Fig. 3d). Together, these findings indicate a role for N-cadherin mediated cell–cell contacts in regulating cell biosynthesis at both mRNA and protein levels, and suggests a trend towards N-cadherin cell–cell contacts playing a role in preserving juvenile NP-specific markers.

E-Cadherin is Involved in AF Cell Cluster Formation but Does not Regulate AF Cellular Biosynthesis

Similar to NP cells, AF cells were treated with N- or E-cadherin blocking antibodies to study whether or not cell cluster formation on soft BME was regulated by cadherins. As seen in Fig. 4a (phalloidin), treatment with N-cadherin blocking antibody did not prevent cell cluster formation for AF cells; in contrast, treatment with E-cadherin blocking antibody did prevent AF cell cluster formation on soft BME. Additionally, cadherin blocking antibody treatment did not appear to regulate AF cell attachment on stiff BME (Fig. 4a). Thus, E-cadherin appears to regulate cell cluster formation for AF cells on soft, but not stiff BME.

Cellular biosynthesis and gene expression studies were also performed on AF cells after cadherin blocking antibody treatment to determine whether or not cadherins regulate sGAG production or gene expression of matrix markers in AF cells. Unlike NP cells, treatment of AF cells with N- or E-cadherin blocking antibody did not change the levels of sGAG for AF cells on soft or stiff BME substrates (Fig. 4b). Levels of sGAG for treatment conditions were not significantly different from those for "no treatment" conditions. A similar finding was observed for gene expression of the selected NP-specific and matrix-related markers in AF cells; regardless of blocking antibody treatment, gene expression levels for all markers (N-cadherin, Aggrecan, Type II collagen, laminin $\beta1$) were not significantly different from "no treatment" conditions (Fig. 4c). Brachyury-T was absent in porcine AF cells, which is why gene expression is not displayed for that specific marker.

Together, these findings indicate that while E-cadherin may be involved in AF cell cluster formation on soft BME, E-cadherin does not appear to mediate AF cell regulation for the subset of markers analyzed in this study.

(a) No treatment | 40 μg/ml N-cadherin blocking antibody | 20 μg/ml E-cadherin blocking antibody — soft BME / stiff BME

(b) sGAG/DNA (μg/μg) vs No Tx, N-cad block, E-cad block — soft BME, stiff BME

(c) N-cadherin blocking antibody / E-cadherin blocking antibody — soft BME / stiff BME — N-cad, E-cad

(d) Quantitative expression (2^{-ΔΔCt}) — soft BME, stiff BME — N-cadherin, Brachyury-T, Aggrecan, Type II collagen, Laminin β 1

◀FIGURE 3. **N-cadherin is necessary for maintenance of the juvenile NP cell phenotype and morphology. (a) Phalloidin immunostaining: treatment with N-cadherin blocking antibody reduces formation of cell–cell contacts in NP cells whereas treatment with E-cadherin blocking antibody still allows cell–cell contact formation in NP cells on soft BME. (red = cell nuclei, green = phalloidin, scale bar = 50 μm) (b) Cellular biosynthesis: significantly higher matrix production was observed in porcine NP cells on soft BME compared to NP cells on stiff BME or AF cells (*$p < 0.05$). Treatment with N-cadherin blocking antibody resulted in significantly less matrix production while treatment with E-cadherin blocking antibody did not influence matrix production in porcine NP cells on soft BME (*$p < 0.05$). (c) N- and E-cadherin immunostaining: loss of N-cadherin expression is observed when NP cells are treated with N-cadherin blocking antibody whereas positive N-cadherin expression is still observed after E-cadherin blocking antibody treatment. E-cadherin expression is absent regardless of blocking antibody treatment (red = cell nuclei, green = cadherin, scale bar = 50 μm). (d) High levels of N-cadherin, Brachyury-T, Aggrecan and Type II collagen are observed when juvenile porcine cells form cell clusters on soft BME substrates. Treatment with N-cadherin blocking antibody results in decreased gene expression levels of all markers in porcine NP cells on soft BME. Treatment with E-cadherin blocking antibody does not influence gene expression of N-cadherin or any of the matrix markers. (Different letters indicate significance within each analyzed protein, $p < 0.05$) (No Tx = no treatment condition, N-cad block = N-cadherin blocking antibody condition, E-cad block = E-cadherin blocking antibody condition).**

DISCUSSION

This work establishes the importance of N-cadherin in regulating cell–cell contacts that are observed *in situ* for juvenile NP cells, and reveals the importance of N-cadherin-mediated cell–cell contacts in regulating NP cell phenotype and morphology on soft, laminin-rich substrates. Findings indicate porcine NP cells, but not AF cells, maintain their juvenile phenotype when N-cadherin cell–cell contacts are engaged, which was demonstrated *via* cellular biosynthesis and gene expression analysis. Blocking N-cadherin function with blocking antibodies resulted in prevention of cell–cell contact formation and loss of the juvenile NP phenotype. By contrast, porcine AF cell cluster formation was influenced by E-cadherin blocking antibody but cell biosynthesis and gene expression were not influenced by any cadherin blocking antibody. These findings indicate N-cadherin is essential in regulating juvenile NP cell morphology and phenotype whereas E-cadherin may be involved in juvenile AF cell organization but not downstream signaling that affects biosynthesis.

While other groups have identified N-cadherin expression in NP tissues at the transcriptional and genomic level, this study is the first to demonstrate positive N-cadherin expression in NP tissue at the protein level. Findings in this study regarding the presence of N-cadherin but not E-cadherin in porcine and human NP tissue have been confirmed by high

N-cadherin expression levels also observed in NP tissue of many other species at the transcriptional and genomic level.[32,34,38,44] A compilation of various markers that have been identified as NP-specific markers in many different species demonstrated N-cadherin as one of two markers that are unique to juvenile NP tissue, regardless of species.[32] Importantly, these studies confirm the observation that N-cadherin expression is lost with aging and degeneration.[32] These findings coincide with observations of decreased cell density and an ability to form cell clusters in degenerated NP tissue.[45] Since N-cadherin expression is mediated by cell–cell contact formation, a decrease in cell–cell contact formations with aging or degeneration is most likely the reason for the observed decrease in N-cadherin expression. This hypothesis is confirmed in this study: when NP cells are unable to form cell–cell contacts, levels of N-cadherin mRNA and protein expression are observed to be decreased at the protein and genomic level (immunostaining and qRT-PCR).

While cadherin expression in AF tissue has not been studied as thoroughly as cadherin expression in NP tissue, based on publically available microarray data, we demonstrated that E-cadherin expression is higher in AF tissue compared to NP tissue for bovine and rat species. Additionally, in this study, immunostaining of porcine and human AF tissue revealed positive E-cadherin expression that was not present in NP tissue. Therefore, we conclude that N-cadherin expression is higher in NP tissue compared to AF tissue, and E-cadherin expression is higher in AF tissue compared to NP tissue. With these results in mind, it was hypothesized that the cell–cell contacts formed in NP cells would be mediated by N-cadherin, and formation of any cell–cell contacts in AF cells would be mediated by E-cadherin. When NP or AF cells were cultured on soft, laminin-containing substrates, both cell types formed cell clusters, and immunostaining of these cell clusters confirmed the presence of N- or E-cadherin in NP or AF cells, respectively.

In this study, an important finding reveals that juvenile NP phenotype is preserved when NP cells are able to form cell clusters. Prior work established the phenomenon of NP cell clustering on soft, laminin-rich substrates with initial studies identifying higher sGAG concentration on soft, laminin-rich substrates compared to stiff substrates or even soft, type II collagen substrates.[16] However, preservation of the juvenile NP phenotype during cell clustering has never been verified. Here, in addition to analyzing sGAG concentrations, qRT-PCR was also performed to characterize changes in the genomic levels for NP-specific (N-cadherin, Brachyury-T[38,44]) and NP-matrix-related markers (aggrecan, type II collagen, laminin $\beta 1$[10,15,17,31,38]). Higher expression of these markers was observed only
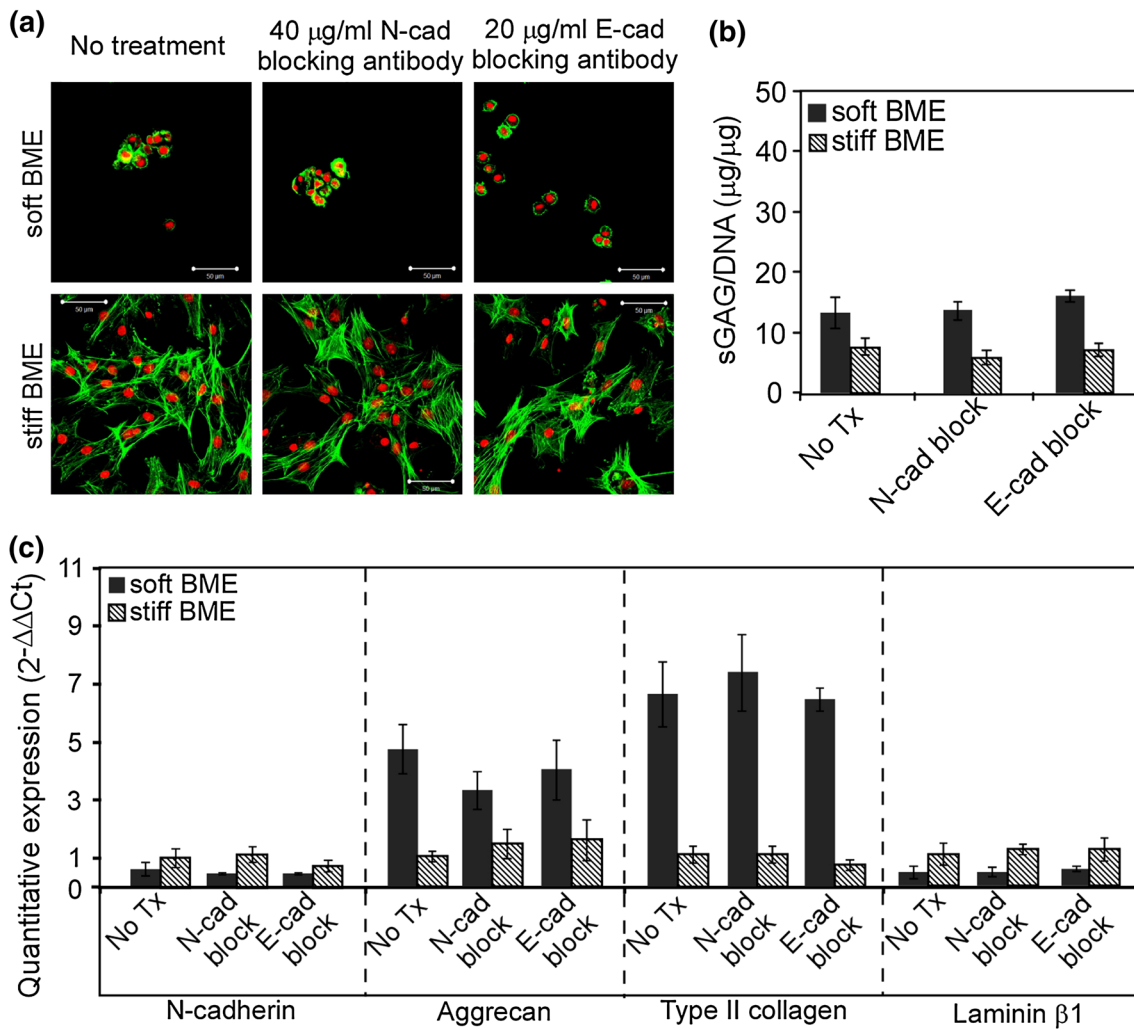
FIGURE 4. Response of porcine AF cells to blocking antibody treatment: blocking E-cadherin resulted in loss of AF cell–cell contacts but did not influence cell biosynthesis or gene expression levels (a) Phalloidin immunostaining: porcine AF cell morphology is unaffected by N-cadherin blocking antibody on soft, laminin-containing substrates. Treatment with E-cadherin blocking antibody results in loss of cell–cell contact formation. (red = cell nuclei, green = phalloidin, scale bar = 50 μm). (b) sGAG/DNA: cellular biosynthesis for porcine AF cells are not affected by N- or E-cadherin blocking antibody, despite changes observed in cellular morphology. (c) Quantitative gene expression: regardless of blocking antibody treatment method, there is no change in any of the markers analyzed for porcine AF cells on any BME substrate. Brachyury-T expression was absent in AF cells. (No Tx = no treatment condition, N-cad block = N-cadherin blocking antibody condition, E-cad block = E-cadherin blocking antibody condition).

in NP cells that were able to form cell clusters, demonstrating the importance of cell clustering in regulating the juvenile NP cell phenotype.

In addition to verifying the importance of NP cell clustering in regulating cell phenotype, this study also establishes the importance of N-cadherin-mediated NP cell–cell contacts in regulating the juvenile NP phenotype. Prevention of N-cadherin mediated NP cell cluster formation on soft, laminin-rich substrates resulted in significantly decreased sGAG production, and decreased expression of various NP-specific markers. These findings correspond with prior work analyzing the role of cadherins in regulating cell phe-

notype and behavior in many other cell types.[49] Various studies analyzing disruption of N- or E-cadherin engagement, either by altering ECM conditions to prevent formation of adherens junctions or by knocking down cadherin protein expression, have shown deviations in expected cell behavior and phenotype.[8,12,23,36,46] For example, perturbation of N-cadherin via blocking antibodies to myoblasts resulted in inhibition of myotube formation,[8] and knockdown of N-cadherin in chick limb mesenchymal stem cells prevented chondrogenesis.[12] Now we have demonstrated a functional role of N-cadherin in regulating NP cell behavior, phenotype, morphology, and matrix synthesis, which contributes to

the growing body of literature emphasizing the importance of cadherins and downstream cellular signaling.

One of the limitations of this study is that the mechanisms linking N-cadherin to cellular biosynthesis were not investigated. Prior work has shown ectopic expression of E-cadherin in IVD cells under control of a CMV promoter drives increased synthesis of aggrecan and type II collagen, that coincides with upregulation of BMP-Smad1/2 signaling pathways.[47] In the current study, changes in actin filament organization during N-cadherin blocking points to actin-mediated signaling pathways as contributing to the cadherin-mediated regulation of biosynthesis. Indeed, there is increased interest in understanding whether or not WNT/β-catenin, Rho GTPases,[26] or myocardin-related transcription factors (MRTF)/serum response factor (SRF) signaling are involved in regulating cellular biosynthesis of NP cells. Future work will focus on identifying cellular signaling pathways that regulate NP cell biosynthesis during N-cadherin-mediated signaling.

In conclusion, juvenile NP cell cluster behavior on soft, laminin-rich substrates are mediated by the cell–cell adhesion molecule, N-cadherin, with associated preservation of key features of a juvenile NP cell phenotype. Positive expression of N-cadherin is observed in juvenile NP cells that are able to form cell clusters on soft, laminin-rich substrates, that disappears when NP cells are cultured on stiff, laminin substrates. Additionally, prevention of N-cadherin-mediated cell–cell contacts results in decreased cell clustering behavior on soft, laminin-rich substrates and associated decrease in unique features associated with an juvenile NP cell. On the other hand, juvenile AF cells form clusters *via* E-cadherin, but cell matrix synthesis does not appear to be altered. Together, these findings demonstrate juvenile NP cell organization and phenotype are regulated by N-cadherin-mediated cell–cell interactions, and that alterations in cell–cell interactions can affect NP cell organization, function, or fate, which has been demonstrated in many other cell types.[23,29,46,48–50] This work is significant as it is the first step in understanding how NP cell–cell interactions regulate NP cell behavior, and is important knowledge for development of novel biological treatment methods for disc degeneration.

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CONFLICT OF INTEREST

P.Y. Hwang, L. Jing, K.W. Michael, W.J. Richardson, J. Chen, and L.A. Setton all declare they have no conflicts of interest.

ETHICAL STANDARDS

All tissue and cell samples used for this study were obtained according to institutional review board-approved protocols.

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