Notochordal cell rich nucleus pulposus tissue increases proteoglycan accumulation and promotes a healthy nucleus pulposus phenotype in human MSCs

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INTRODUCTION
Mesenchymal stem cells (MSCs) are an attractive cell source for IVD repair, yet the means of differentiating these cells to a nucleus pulposus (NP) phenotype is not clear and an area of high priority. Notochordal cells (NCs) play an influential role in early development of the intervertebral disc (IVD). NCs populating the nucleus pulposus (NP) disappear during growth in humans, but certain species that do not experience IVD degeneration retain NCs into maturity [10]. Conditioned media from NCs seeded in alginate stimulated significant proteoglycan biosynthesis of human MSCs after 7 days in culture suggesting NCs may promote MSC differentiation toward a young NP phenotype [32]. However this study on NC conditioned media was of short duration, has not been replicated, and did not include soluble factors from notochordal cell rich NP tissues. The aim of this study was to compare effects of conditioned media derived from NCs both seeded in alginate and derived from notochordal NP tissue on MSC differentiation toward an NP phenotype in a 3 week study.

METHOD AND MATERIALS
Isolation of tissue and generation of conditioned media: NP tissue was isolated from porcine spines (n=8). To produce conditioned media (CM) from alginate beads, tissue was digested and seeded in beads at a density of 2x10^5 cells per ml of 1.2% low viscosity alginate. 10 beads per well in a 12 well plate were cultured for 4 days in hypoxia (5% O2, 5% CO2, 37°C) with 2ml of CM (low glucose DMEM+ITS). For generation of CM from NP tissue, 3 discs were soaked in 30mls of CM without ITS for 4 days in hypoxia. Media from alginate and tissue were filtered (3K Amicon Ultra-15, Millipore) and re-suspended in Basal media (CM) (without fungizone with ITS) to create NC CM from NP Tissue (NCT) and NC CM from Alginate beads (NCA).

MSC culture with Basal (B), NCA and NCT media: Human bone marrow derived MSCs samples (age range 22-37yrs, n=3) were obtained from Texas A&M (Temple, TX) and expanded in culture. Cell pellets were formed in 15ml polypropylene tubes at a density of 250,000, seeded in CM without fungizone, with 50ug ascorbate, 0.1uM dexamethasone, 40ug L-proline and 10ng/ml TGFβ3 and maintained in hypoxia for 24 hours to ensure adequate pellet formation. 500 μL of B, NCA and NCT were added to each tube the next day and cultured for 21days, with media changes each 3-4 days.

Dependent variables; qRT-PCR gene profiling: RNA was isolated, cDNA synthesized and a custom RT profiler PCR array (SA Biosciences: CAPH-0817A) was run. Relative gene expression was calculated using the comparative Ct method normalized to undifferentiated MSCs from the same patients (Day 0) and 3 housekeeping genes (HKs). For normalization purposes, undetermined values for Day 0 were given an arbitrary value of 40 as undifferentiated MSCs did not express all these genes (leading to some catabolic genes with artificially high fold increases). Student’s t-tests were performed, with p<0.05 significant. Cell viability was analyzed with the Live/dead kit (Invitrogen). Glycosaminoglycan (GAG) in the cell pellet was assessed using DMB and normalized to DNA content using the picogreen assay (Invitrogen). Alcian blue staining of intact pellets evaluated GAG content.

RESULTS
NCT conditioning of human MSCs resulted in a significant up-regulation in SOX9 and COL2A1 compared to Day 0 and B (Figure 1A). The NCA group demonstrated limited effects on anabolic gene expression after 3 weeks culture, however caused significant down-regulation of the fibrotic genes COL1A1 and COL3A1. NCA had no change in hypertrophic COL10A1 gene expression unlike NCT which showed significant up-regulation. A general up-regulation was observed for catabolic genes and inhibitor (TIMP1) for all media conditions. NCA and NCT demonstrated similar effects on growth factor gene expression, but it was notable that NCT significantly up-regulated both TGFb1 and TGFb3 and CTGF was down-regulated for all media groups but most notably for NCA. Changes in inflammatory cytokine gene expression were also observed for NCA and NCT with significant up-regulation of IL1B and TNF gene expression with NCA and for NCT up-regulation of IL1B but down-regulation of NGF (data not shown). IVD cells appeared viable with very few dead cells observed and little difference between media conditions, and DAPI staining further demonstrated homogeneous cellularity throughout the pellet (not shown). Significantly more GAG associated with the cell pellet was observed with NCT media compared to all other groups (Figure 1B). This was corroborated with Aelastic staining demonstrating greater intensity of staining throughout the pellet for the NCT group (Figure 1C).

DISCUSSION
This study evaluated the potential of soluble factors from NCs in alginate and notochordal NP tissue to differentiate MSCs to a NP phenotype. A novel feature of this study involved the use of a custom PCR array capable of measuring expression of 42 genes important in NP differentiation. The NCT media condition showed most promise for human MSCs differentiation to a NP phenotype with maximum GAG production and up-regulation of several anabolic genes. These findings highlight that maximal GAG production likely requires cell-matrix interactions or matrix cellular proteins such as CTGF, yet there was also up-regulation of collagen X which may be associated with hypertrophy. NCA conditioning did not up-regulate collagen X expression and also had the lowest levels of gene expression for collagen I & III. The significant down-regulation of CTGF (compared to B) seems to correlate with this inhibition and suggests that NC cells in alginate alone may produce soluble factors capable of inhibiting hypertrophy and synthesis of certain fibrous proteins. The generally high expression of catabolic genes and inflammatory genes observed in this pellet culture system are likely associated with cell differentiation rather than catabolism as matrix enzymes are important for cell differentiation and chondrogenesis [10]. This study provides further evidence that soluble factors from NC cells can stimulate MSC differentiation to a healthy NP phenotype and demonstrates that matrix associated proteins are important for maximum GAG stimulation. Results also underscore the complex interactions between NCs and MSCs, depending on soluble factors present and cell-matrix environment.


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