Research Article

Nonviral transfection of human intervertebral disc cells with developmental transcription factor Brachyury induces reprogramming to a healthy pro-anabolic anti-catabolic/inflammatory phenotype: A proof of concept study

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ABSTRACT:

Intervertebral disc (IVD) degeneration is a major contributor to chronic low back pain and is characterized by decreases in cellularity and proteoglycan synthesis, upregulation of matrix degradation, and increases in pro-inflammatory factors with neurovascular invasion. Current treatments fail to target the underlying pathology or promote tissue repair and approaches such as viral transfection raise safety concerns due to mutagenesis and unwarranted immune responses. To avoid such concerns, nonviral transfection is a viable method of gene delivery into the host cell while bypassing the caveats of viral delivery. Brachyury is expressed in the developing notochord and is associated with an immature healthy nucleus pulposus (NP). We hypothesize that Brachyury can reprogram degenerate NP cells to a healthy pro-anabolic phenotype with increased proteoglycan synthesis and decreased expression of catabolic, inflammatory and neurovascular markers. NP cells obtained from human autopsy (non-painful) and surgical tissues (painful) were transfected with plasmids encoding for Brachyury or an empty vector control via bulk electroporation. Post transfection, cells were seeded in 3D agarose constructs cultured over 4 weeks and analyzed for viability, gene expression, and proteoglycan. Results demonstrated successful transfection of both non-painful and painful NP cells. We observed long term Brachyury expression, significant increased expression of NP phenotypic markers FOXF1, KRT19, and chondrogenic marker SOX9 with decreases in inflammatory cytokines IL1-β/IL6, NGF, and MMPs and significant increase in proteoglycan synthesis. These results highlight nonviral transfection with developmental transcription factors, such as Brachyury, as a promising method to reprogram degenerate disc cells towards a healthy NP phenotype.

Statement of Clinical Significance: This project proposes a novel translational approach to the treatment of intervertebral disc degeneration via direct reprogramming of diseased patient IVD cells to a healthy phenotype.

Keywords: Low Back Pain, Matrix degradation, Nucleus Pulposus, Therapeutics, Intervertebral Disc, Gene therapy

INTRODUCTION

Low back pain (LBP) is the leading cause of disability worldwide affecting 70-80% of the population in their lifetime with a socioeconomic burden over $100 billion in the United States alone[1,2]. Additionally, LBP is a large contributor to the growing opioid crisis due to their prescription for the treatment of back pain[3]. Intervertebral disc (IVD) degeneration is significantly associated with LBP and occurs due to complex mechanical and biological interplay, ultimately leading to structural failure[4,5]. The healthy IVD is an avascular aneural structure composed of concentric rings of collagen I that forms the annulus fibrosus (AF) surrounding a gelatinous hydrophilic proteoglycan core containing nucleus pulposus (NP) cells of notochordal origin enclosed cranially and caudally by the cartilage end plates (CEP)[6]. The NP is considered the "metabolic engine" of the IVD and is largely responsible for maintaining the hydrated core due to accumulation of aggregating proteoglycans[7]. However during disc degeneration, the NP is characterized by decreases in cellularity and proteoglycan synthesis, catabolism, and increases in pro-inflammatory factors with neurovascular invasion[8]. Specifically, there is decreased synthesis of aggrecan (ACAN), collagen type II (COL2), upregulation of matrix metalloproteinases (MMPs) such as MMP12 and MMP13, and increases in pro-inflammatory cytokines interleukin-1beta (IL-1β), interleukin-6 (IL-6), Tumor necrosis factor –alpha (TNFα), together with nerve growth factor (NGF)[9–12].

Current clinical strategies for treatment of LBP, including conservative non-surgical and surgical therapies, primarily target the symptoms of LBP while not treating the underlying disease pathology[13]. Furthermore, postoperative complications can include adjacent segment disease, nonunion, and potential nerve injury[14]. As such, there is a need to develop early stage interventions for IVD degeneration or treatments to reverse the degeneration process thereby

promoting tissue repair. Cell therapy, such as mesenchymal stem cell (MSCs) in matrix constructs, has been identified as a promising therapeutic strategy to promote regeneration, however limitations remain regarding cell retention and viability in the degenerate IVD[15–19]. Pro-anabolic gene therapy based approaches have been proposed including viral transfection of SOX9, which resulted in increases in matrix synthesis (collagen II) in degenerate NP cells [20–22]. However, the use of viral vectors for gene delivery poses concerns of insertional mutagenesis and unwarranted immune responses and is currently not FDA approved[23–25]. Nonviral, plasmid based vector systems eliminate much of the risk associated with viral vectors and have been used successfully in conjunction with novel tissue nanotransfection for rescue of skin tissue of mice under ischaemic conditions[26]. Therefore, transfection of a transcription factor that promotes a "healthy NP phenotype" via a nonviral vehicle is a promising therapeutic approach for treatment of IVD degeneration.

The transcription factor Brachyury (T) is expressed in the developing notochord and is associated with maintaining a healthy immature NP phenotype and essential for normal spine formation[27,28]. Recently, T has been transfected into human induced pluripotent stem cells (hiPSC) inducing successful differentiation into nucleus pulposus-like cells with similar morphology and NP surface marker expression[29]. We hypothesize that delivery of T encoding gene into degenerate human NP cells can reprogram degenerate NP cells into "healthy cells" with increases in proteoglycan and decreases in inflammatory, catabolic and pain associated factors. In this study, we aim to examine the effects of nonviral T transfection via bulk electroporation in non-painful autopsy and painful degenerate surgical primary human NP cells in an ex-vivo 3D agarose cell culture model.

METHODS

All reagents were obtained from Sigma Aldrich or Thermofisher Scientific unless otherwise stated.

Cell Isolation from Human Tissue

Autopsy lumbar spines were obtained through the Cooperative Human Tissue Network (CHTN, IRB exempt) within 24-hours post-mortem. IVDs were then isolated and graded according to the Thompson scale by 3 independent investigators and grades averaged (Table1). NP tissue was dissected out with ambiguous NP-AF interfaces removed to avoid contamination by AF cells. NP Cells were isolated using 0.03g/mL protease (Cat: P5147-1G) in digestion media (DMEM (4.5g/mL glucose), 1% penicillin/streptomycin (P/S), 0.5% Fungizone) for 1-hour at 37°C followed by 0.03g/15 mL collagenase II (Cat: 17101015) in digestion media for 4-hours at 37°C and strained through a 70μm cell strainer to remove debris and plated for expansion. Surgical NP tissue was obtained from patients undergoing microdiscectomy at The Ohio State University Wexner Medical Center (IRB: 2015H0385) with cells isolated as described above. Due to availability and age-related IVD degeneration, available autopsy samples with the lowest grade were selected to represent the “non-painful” experimental group while IVD tissues from patients represent the “painful” group.

Table 1: Human Autopsy and Surgical Specimen Demographics

Autopsy (Non-Painful)					Surgical (Painful)			
ID	**Sex**	**Age (years)**	**Level**	**Grade**	**ID**	**Sex**	**Age (years)**	**Level**
Hu-4	Female	49	L2-L3	2.5	Hs-2	Male	26	L5-S1
Hu-6	Male	45	L2-L3	3	Hs-11	Male	28	L5-S1
Hu-7	Female	56	L2-L3	2.5	Hs-29	Female	70	L5-S1
Hu-9	Female	58	L4-L5	2.5	Hs-34	Female	19	L5-S1
Hu-16	Female	19	L1-L2	1.5	Hs-39	Male	60	L5-S1

Cell Expansion

Non-painful and painful degenerate NP cells pre-transfection were expanded in disc cell media (DMEM (4.5g/mL glucose), 10% FBS, 1% P/S, 0.5% Fungizone, 50 µg/ml ascorbic acid (AA) fresh) in standard culture conditions (5% CO_2, 37°C) and fed every 3 days until 80% confluency for downstream transfection (Passage = P2). Post Transfection, cells were fed with antibiotic free disc cell media (DMEM (4.5g/mL glucose), 10% FBS, 50 µg/ml AA fresh) for 48-hours before feeding with disc cell media as described above until approximately 80% confluent.

Brachyury Transcription Factor Plasmid Generation

T, with pCMV6-XL5 vector and antibiotic selection marker, ampicillin, was obtained from OriGene Technologies (NM_003181, Cat: SC303281). The plasmids were transformed into DH5α E.coli bacteria via heat shock and incubated with S.O.C. Medium (2% Tryptone, 0.5% Yeast Extract, 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 10mM $MgSO_4$, 20mM glucose) for 1-hour (37°C, 225 rpm). Bacterial cells were then cultured on solid agar (4% Agar in Lysogeny

Broth, 24 hours) with ampicillin (25µg/mL) and then selectively cultured in liquid cultured for 24-hours. Plasmids were then isolated from bacterial culture using ZymoPURE II Plasmid Midiprep Kit (The Epigenetics Company #D4201) per manufacturer protocol and quantified on nanodrop.

Brachyury Transfection

Post expansion, cells were washed with 1X sterile PBS, trysinized, and a biological N=5 pooled for non-painful and painful cells respectively. Cells were centrifuged (4°C, 400rcf, 5 minutes) and the pellet washed with 1X Sterile PBS. Neon™ Transfection System MPK5000 from Invitrogen was used for this transfection protocol. Previously isolated T plasmid was mixed with R Buffer (0.05 ug DNA/uL Buffer) and transfected (V=1425 Volts, 30ms, 1 Pulse) with $1x10^6$ cells per transfection into groups: Non-painful T transfected (nPT) and painful T transfected (PT). As a SHAM control, empty vector pCMV6 was transfected into non-painful and painful NP cells using the same protocol. $1x10^6$ total cells from each experimental group were plated into 4-wells of a 12-well plate for quantitative-Polymerase chain reaction (RT-qPCR) and imaging at 48-hours in monolayer to ensure transfection while the remainder of cells was plated in large T-160 Flasks for expansion as described previously.

Seeding Cells in 3D Agarose Culture

Transfected cells at ~80% confluency were washed with 1x sterile PBS, trypsinized, and suspended at $40x10^6$ cells/mL in disc cell media[30]. To make 15 gels per experimental group (5 gels per time point for day 0, week 2 and 4), a 52mm x 32 mm rectangular construct was made using a 4mm thick sterile silicone sheet. Cells suspended in media were mixed with equal amounts of 4% biological grade agarose (Amresco, Cat: J234) at a 1:1 ratio at 50°C to create a 2%

agarose mixture of $20x10^6$ cells/mL. The mixture was pipetted up and down to ensure homogeneity and released onto the silicone mold sandwiched by 2 glass plates and let to solidify for 10-minutes at room temperature. Glass plates were removed and cell seeded agarose constructs were punched out with an 8mm diameter biopsy punch to create Ø=8mm, H=4mm cylindrical constructs. Individual constructs were cultured in 2mL of disc cell media in 24-well cell repellant culture plates to avoid leaching of cells outside the construct and fed 3 times a week (Figure1)

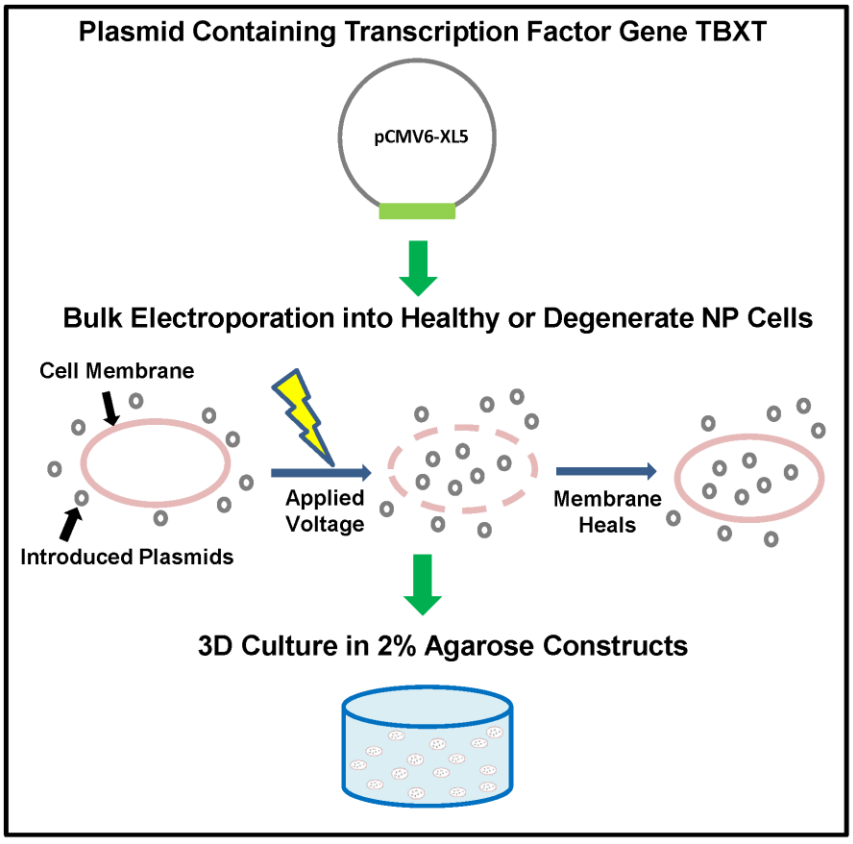

Figure 1: Schematic of bulk electroporation of isolated plasmids into cells and placement in 3D agarose culture

Dependent Variables

Cells in monolayer 48-hours post-transfection were lysed and stored in Trizol reagent for RT-qPCR. 3D agarose constructs (N=5/time point) from each experimental group were taken

down at day 0, week 2 and week 4 for the following dependent variable assessments. Each gel construct was divided: half of each construct was used for cell viability, a quarter used for gene expression, and a quarter used for assessment of proteoglycan/glycosaminoglycan (GAG) content.

Cell Viability in Agarose Constructs

Constructs were washed with 1X sterile PBS and incubated in 700 uL live/dead (Cat: L3224) solution for 18 minutes (2mM-Ethidium, 4mM-Calcein) prior to imaging on the Nikon TiE microscope using a fluorescence DS-Qi2 camera. 4x cross-sectional images and 10x images were taken of each gel. 10x images were automatically quantified via MIPAR Image Analysis Software by counting the number of live cells over the total cell count presented as % Live Cells.

Gene Expression

mRNA from constructs were isolated using the Trizol Plus RNA Purification Kit (Cat: 12183555). Briefly, constructs were digested in 1mL of Trizol and 0.2mL of chloroform added for phase separation. 70% Ethanol was added to the clear phase at a 1:1 ratio and binding, washing, and elution of RNA was completed per manufacture protocol. cDNA was synthesized with Maxima H Minus Mastermix (Cat: M1662) per manufacture protocol. RT-qPCR was ran on 384 well plate with 15ng of cDNA per well, Taqman Universal Master Mix II (Cat: 4440049) and respective Taqman primers detailed in Table2. Gene expression data was analyzed via the comparative $2^{-\Delta\Delta Ct}$ method normalized to the endogenous control, 18S, and experimental SHAM controls[31].

Table 2: Taqman Gene Expression Primer Details

Category	Target Gene	Assay ID
Control	18S	4333760F
Healthy NP Makers	Brachyury	Hs00610080_m1
	FOXF1	Hs00230962_m1
	KRT19	Hs00761767_s1
	SOX9	Hs01107818_m1
Inflammatory Cytokines	IL-1β	Hs00174097_m1
	IL6	Hs00174131_m1
	TNFα	Hs01113624_g1
Nerve Growth	NGF	Hs00171458_m1
Matrix Degrading Enzymes	MMP12	Hs00159178_m1
	MMP13	Hs00233992_m1
Matrix Genes	COL2	Hs00264051_m1
	ACAN	Hs00153936_m1

Proteoglycan/ Glycosaminoglycan (GAG) Content

Constructs were lyophilized overnight and digested in 1mL of ProteinaseK working solution (1:200 ProteniaseK (Roche, Cat: 03115828001) to ultra-pure distilled water, 10mM Tris-HCl) for 20-hours (250 rpm, 60°C). Proteoglycan content was measured via colorimetric Dimethylmethylene blue (DMMB, Cat: 341088) assay with chondroitin sulfate (Cat: c4384) as the standard curve and read at 530nm on an Enspire Plate Reader. GAG content from the assay was normalized to DNA content using the Sigma-Alrich DNA Quantification Kit (DNAQF).

Statistical Analysis

Due to the small biological sample size (N=5), the data cannot be assumed to be normally distributed. Thus, non-parametric, two-tailed, un-paired Mann Whitney tests were performed on the data to determine significant differences at α=0.05 for all viability, gene expression and GAG content.

RESULTS

48-Hour Post Transfection: Pre-3D Culture

Post transfection, cells imaged at 48-hours showed no observable morphological differences between groups (Figure2). To confirm successful transfection of T and upregulation in the cells, RT-qPCR was performed and normalized to respective SHAM controls (Figure3). T was highly upregulated in both nPT (9164-Fold) and PT (6383-Fold) cells relative to SHAM (N=1). The 48-hour time point monolayer culture provides an initial confirmation of successful transfection which was further confirmed and validated by 3D culture.

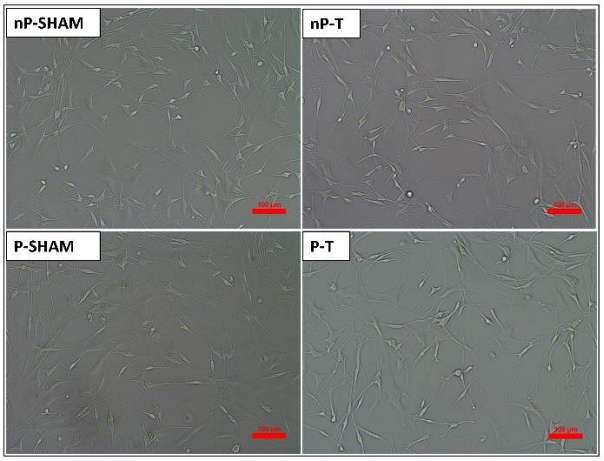

Figure 2: 10X objective microscopic images of nucleus pulposus cells 48 hours post transfection in monolayer. nP= Non-painful , P=Painful , SHAM =SHAM control, T= Brachyury transfection. Scale bars = 100 microns.

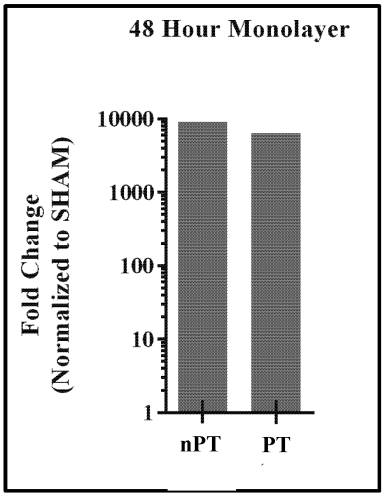

Figure 3: mRNA gene expression of Brachyury (T) for non-painful (NPT) and painful degenerate (PT) cells in fold change of T transfection normalized to SHAM after 48 hours.

Viability

Live/dead assay was used to access viability of cells in 3D culture where cells stained with Calcein (live cells =green) and ethidium represent (dead cells=red). Cells maintained high viability with no differences in cell viability between nPT and PT groups compared to their respective SHAM groups (Figure4).

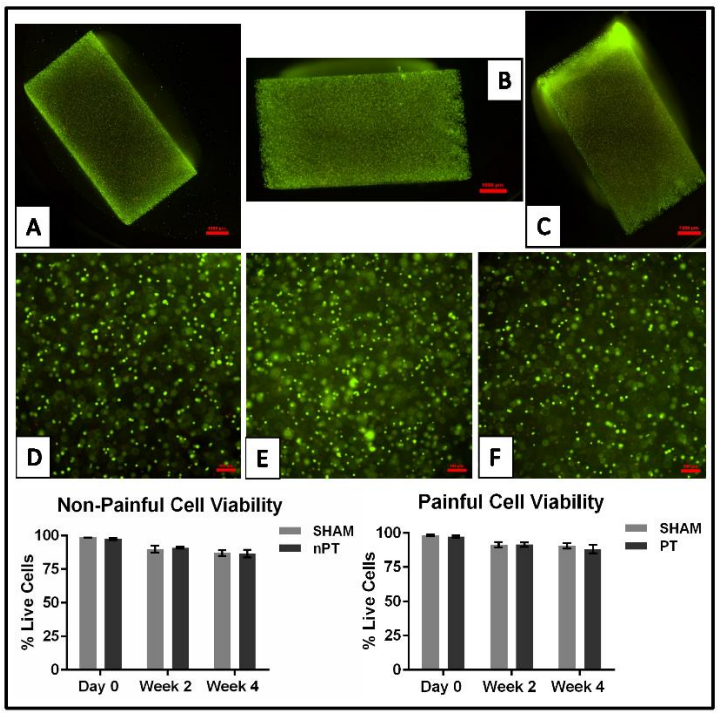

Figure 4: Representative 4x objective stitched images of gels at (A) day 0, (B) Week 2, and (C) Week 4 with respective 10x images for (D) day0, (E) Week 2, and (F) Week 4) for live/dead staining where green=live and red=dead. Red scale bars = 1000um for A, B, C and 100um for D, E, F. Histograms represent cell viability (% Live Cells) in non-painful and painful cells.

Gene Expression

Gene expression of phenotypic markers, inflammatory cytokines, NGF, and matrix related genes were analyzed via RT-qPCR and quantified via normalizing T transfected cells to their respective SHAM controls at day 0, week 2, and week 4.

Phenotypic Markers (Figure5)

Notochordal marker T demonstrated significantly increased expression relative to SHAM over 4 weeks ($P<0.05$) in both nPT (Day 0= 27.77, Week 2 = 107.37, Week 4= 387.49-Fold) and PT cells (Day 0= 5.67, Week 2 = 357.24, Week 4= 123.617-Fold) . In nPT cells, NP marker Forkhead Box F1 (FOXF1) demonstrated increased expression at day 0 (2.29-Fold) but no difference at week 2 and 4 with a significant decrease at week 4 (-1.27-Fold) compared to day 0. In PT cells, there were no differences in FOXF1 expression at day 0 or week 2 with a significant decrease at week 4 (-3.75-Fold). In nPT cells, Cytokeratin-19 (KRT19) expression was significantly increased at all time-points (2.29, 1.76, 2.49-Fold respectively) relative to SHAM. However in PT cells, there was an initial decrease in KRT19 (-1.71-Fold) for day 0 and a significant increase at Week 2 (1.87-Fold) with no significant differences at week 4. Chondrogenic marker, SRY-Box-9 (SOX9) showed an initial significant increase in nPT cells (2.52-Fold) but significant decrease in expression at week 4 for PT cells (-3.69-Fold).

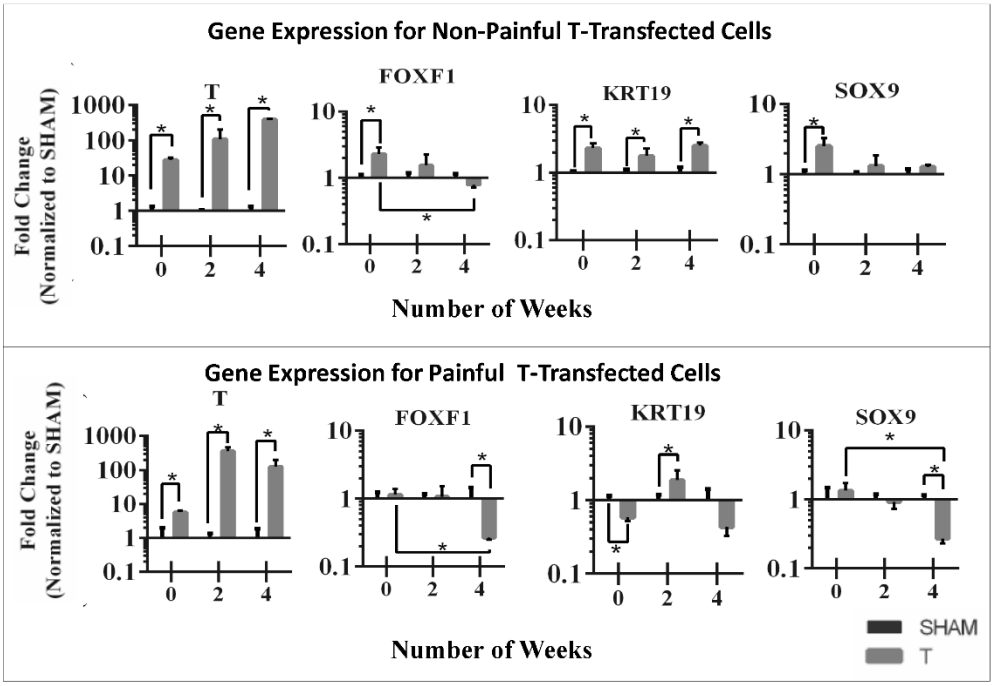

Figure 5: Relative gene expression of T transfected cells in 3D culture (N=5) relative to SHAM control for healthy nucleus pulposus phenotypic markers T, FOXF1, KRT19, and chondrogenic marker SOX9 respectively. Top= transfection in non-painful cells. Bottom = transfection in painful cells. (* indicates $p<0.05$)

Inflammatory Cytokines (Figure6)

IL-1β expression was significantly down-regulated at day 0 (-1.93-Fold) and week 2 (-4.00-Fold) with no difference at week 4 for nPT cells. In PT cells, IL-1β was increased at day 0 (1.97-Fold) with no significant difference at weeks 2 and 4. IL6 was significantly upregulated for both nPT and PT cells (1.55-Fold, 1.68-Fold respectively) with significant decreases at weeks 2 (2.06-Fold) and 4 (1.35-Fold) for nPT cells but no differences for PT cells at the corresponding time-points. TNFα was upregulated at week 2 for nPT cells (1.73-Fold) but showed no significant difference for the remaining time points relative to SHAM.

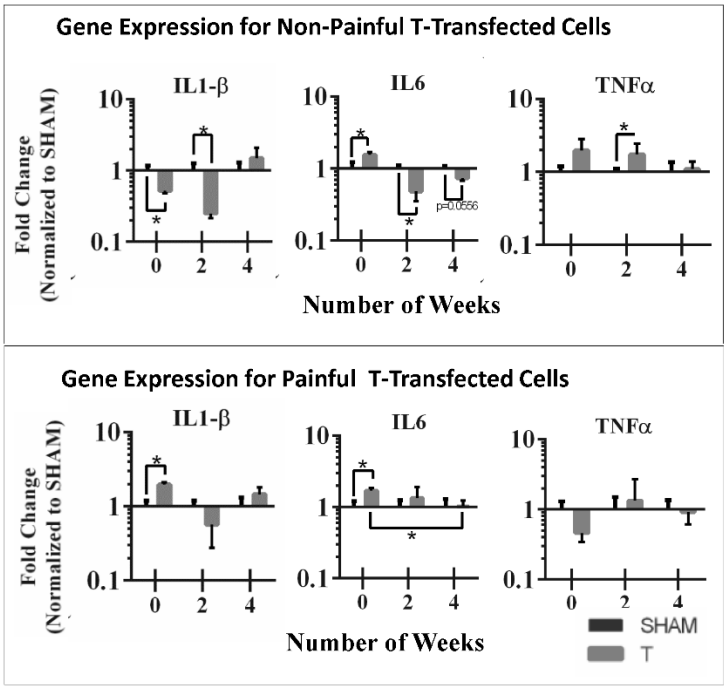

Figure 6: Relative gene expression of T transfected cells in 3D culture (N=5) relative to SHAM for inflammatory cytokines IL-1B, IL6, and TNFa respectively. Top= transfection in non-painful cells. Bottom = transfection in painful cells. (* indicates $p<0.05$)

Nerve Growth Factor (Figure7)

NGF was significantly down-regulated (1.13, -1.93, -4.23-Fold respectively) over time in nPT cells while being significantly down-regulated at week 2 (-2.13-Fold) for PT cells relative to SHAM.

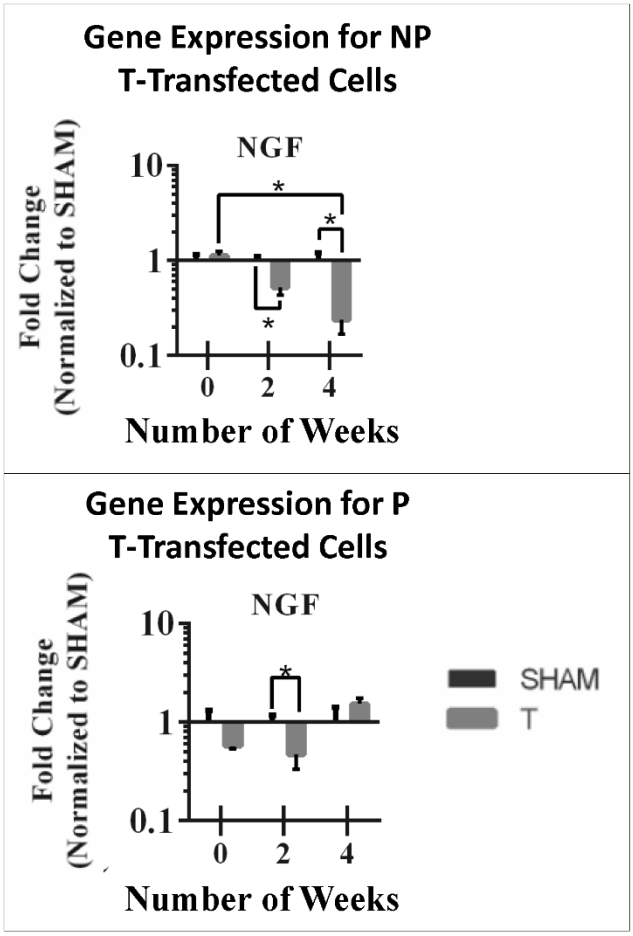

Figure 7: Relative gene expression of T transfected cells in 3D culture (N=5) relative to SHAM for inflammatory nerve growth factor (NGF). Top= transfection in non-painful cells. Bottom = transfection in painful cells. (* indicates $p<0.05$)

Matrix Genes (Figure8)

No significant changes in ACAN or COL2 were exhibited between transfection and SHAM groups in the first two weeks for nPT cells while there was a significant decrease in ACAN at week 4 (-2.27-Fold) and a significant increase in COL2 (1.98-Fold). In PT cells,

ACAN was significantly increased at week 2 (1.85-Fold) but significantly decreased at week 4 (-2.60-Fold) similar to the effects in nPT cells. In PT cells, COL2 was initially down-regulated (-2.30-Fold) but significantly increased at week 2 (3.44-Fold) while down-regulated again at week 4 (-9. 63-Fold)

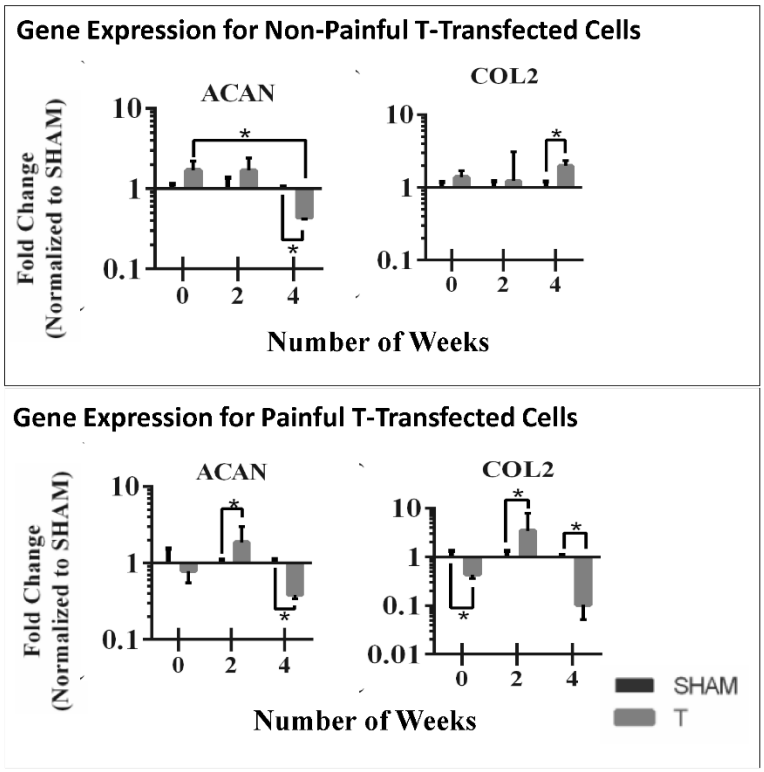

Figure 8: Relative gene expression of T transfected cells in 3D culture (N=5) relative to SHAM for ACAN and COL2 respectively. Top= transfection in non-painful cells. Bottom = transfection in painful cells. (* indicates $p<0.05$)

Matrix Metalloproteinases (Figure9)

In nPT cells, MMP12 was increased initially (8.91-Fold) with no significant differences at weeks 2 and 4 relative to SHAM but was significantly decreased at week 4 compared to day 0. MMP13 showed significant downregulation of T transfected cells compared to SHAM at all time

points (-4.25, -2.33, -2.69-Fold respectively). In PT cells, there were no significance differences in MMP12. MMP13 was significantly decreased at week 4 (-3.91-Fold).

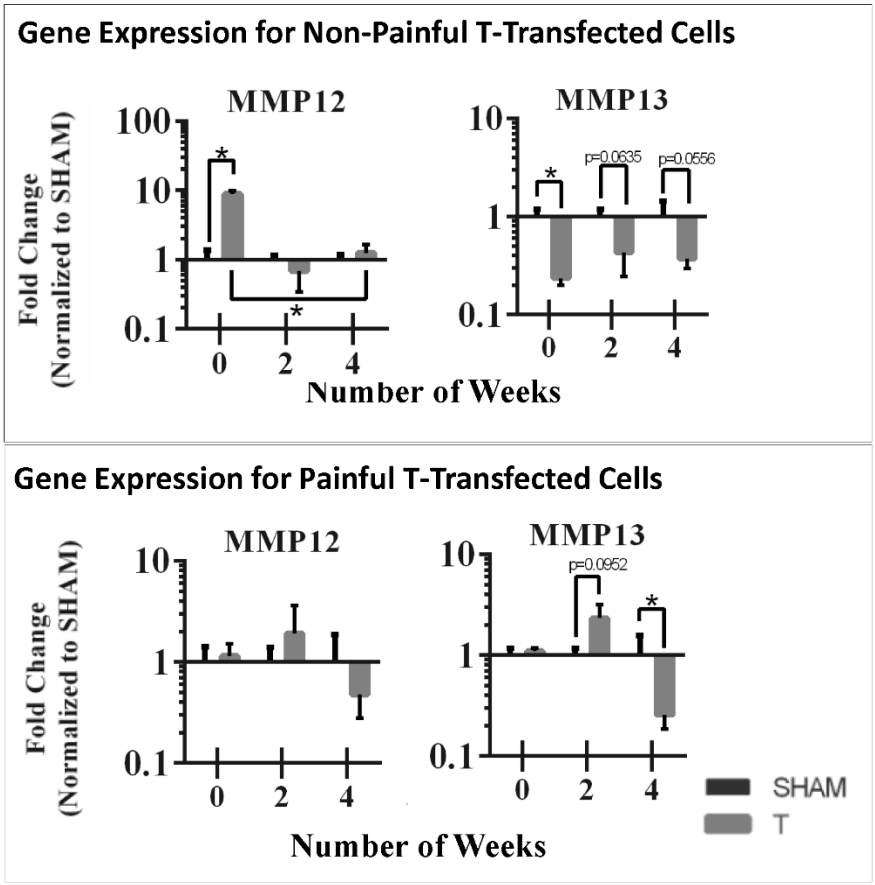

Figure 9: Relative gene expression of T transfected cells in 3D culture (N=5) relative to SHAM for Matrix degrading enzymes MMP12 and MMP13 respectively. Top= transfection in non-painful cells. Bottom = transfection in painful- cells. (* indicates $p<0.05$)

Proteoglycan/Glycosaminoglycan (GAG) Content

GAG content in 3D constructs was quantified using a DMMB assay and normalized to DNA to approximate amount of GAG/per cell (see supplemental figure 1 for raw GAG and DNA data) . For nPT cells, there was no significant difference in GAG between transfected cells and SHAM however there were significant differences with time in the SHAM group - week 4

(P<0.05) compared to Day 0 and week 2. However, in PT cells, there was a significant increase in GAG at weeks 2 (8.39 ± 1.91 ug GAG/µgDNA) and trend at week 4 (5.05 ± 1.59 ug GAG/ug DNA, p=0.0625) relative to day 0 and respective SHAMs. There were no differences observed in SHAM groups across the culture period.

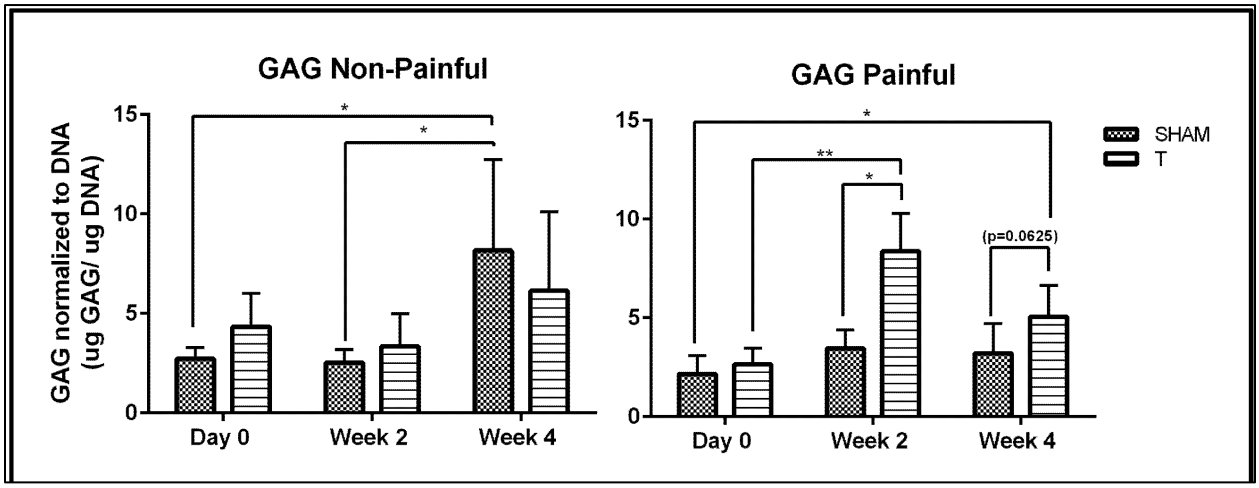

Figure 10: GAG content of gels at day 0, Week 2, and week 4 normalized to respective DNA for T-transfected cells and SHAM in 3D agarose culture. (* indicates p<0.05, ** indicates p<0.005)

DISCUSSION

The results from this novel proof of concept study demonstrate that: 1) T can be successfully transfected into non-painful and painful degenerate human NP cells with high expression maintained for 4 weeks in culture, 2) transfection with T can significantly modulate the genetic phenotype of NP cells with general decreases in catabolic, inflammatory and pain markers which were transient and more pronounced in the nPT group compared to the PT group and 3) T induced significant changes in the expression of ECM proteins and enzymes with up-regulation of GAG accumulation at weeks 2 and 4 in PT cells compared to SHAM controls. These results suggest that nonviral transfection of human NP cells with developmental

transcription factors such as T has the ability to promote and enhance a "healthy" pro-anabolic anti-catabolic/inflammatory phenotype and demonstrates potential as a novel therapeutic strategy to reprogram degenerate painful human NP cells to reverse LBP.

1) Successful transfection of Brachyury

Transfected cells imaged at 48-hours showed no differences in cell morphology and exhibited a fibroblast-like phenotype similar to non-transfected human NP cells. This is expected as NP cells exhibit a fibroblast-like phenotype in monolayer compared to physiologically relevant 3D environments[32]. Live/Dead staining of human NP cells suggested that bulk electroporation of T or SHAM plasmids had no detrimental effects on NP cell viability up to 4 weeks in high density 3D agarose cultures for both non-painful and painful specimens.

Interestingly, despite nonviral gene delivery being considered temporal with shorter periods of transgene expression, T expression was significantly increased throughout the 4 weeks compared to SHAM[33]. Tang et.al demonstrated similar levels of T gene expression when T was delivered to hiPSCs via lentivirus transfection for differentiation into NP-like cells and maintained for 12 days, however this enhanced expression of T may also be attributed to additional growth factors present in the media[29]. Bucher et al. showed that transfecting growth/differentiation factor-5 (GDF5) into MSCs via nonviral electroporation upregulated GDF5 up to 1,000-Fold after 21 day alginate culture[34]. Studies using nonviral gene delivery with microbubbles for human IVD cells found only 11-fold increase in the expression of green fluorescent protein (GFP) tagged vectors after 24 days[35,36]. Comparable studies to our own using the same bulk electroporation method on primary human NP cells have demonstrated less than a 100 fold increase in growth/differentiation factor-6 (GDF6) expression over 14 days[37]. This

implies that the initial electroporation of T into NP cells from degenerate human IVD tissue can upregulate and maintain high levels of T mRNA expression for at least 4 weeks in 3D culture which is comparable to similar studies that have transfected bioactive ligands/transcription factors. This suggests plasmid-driven expression of endogenous gene expression and would require further validation of plasmid vs endogenous gene expression in future studies. Growth factors have been widely studied for IVD cell transfection and have shown promise, however their effects are limited to transient changes in cell function while transcription factors can act up-stream and affect actual DNA transcription, thus in the case of T, have the potential to reprogram cell phenotype also. There are currently no studies directly comparing the efficiency of growth factors versus transcription factors[38].

2) NP genetic phenotype and the painful degenerate phenotype

FOXF1 and KRT19 are both considered "healthy NP phenotypic" markers representative of the immature NP while SOX9 is a chondrogenic marker previously shown increase matrix production in human IVD[20,39,40]. Thus, these are excellent markers to evaluate successful reprogramming of degenerate NP cells into a healthy phenotype relevant to T. FOXF1's initial increased expression in nPT indicates upregulation of FOXF1 due to transfection with T but only transiently. On the contrary, there were no changes in FOXF1 expression and a decrease at 4 weeks for PT cells. We suspect that these findings are due to either the transfection quantity or method of delivery which require further optimization as T has been shown to upregulate FOXF1 in cells such as mesodermal cells[41]. KRT19 was significantly increased at all time-points for nPT cells and increased at week 2 for PT cells indicative of a potential push towards an immature NP phenotype. There was an initial increase in SOX9 for nPT groups while the effect was not seen in PT cells, suggesting a need to optimize T delivery method and quantity. Thus, for healthy

phenotypic markers, we observed a general upregulation of important markers FOXF1, KRT19, and SOX9 in nPT cells but these effects were either transient or reduced in PT cells indicating a differential response between non-painful (less degenerate) compared to painful (more degenerate) human NP cells. This implies that the degree of degeneration and pathological nature of the tissue may affect the ability of transcription factors to reprogram cells and such strategies may have more benefit at an earlier stage of disease.

IVD degeneration is characterized by elevated levels of inflammatory cytokines such as IL-1β, TNFα and IL6 and neurotrophic factors such as NGF[42–44]. Thus, down-regulation of these factors would be important when determining the success of T transfection and reprogramming to a healthy anabolic anti-catabolic/anti-inflammatory phenotype. IL-1β and IL6 were both decreased in nPT cells at Day 0/week 2 and weeks 2/4 respectively. Although not significant, IL-1β and IL6 showed gradual decreases in expression in PT cells over time. For TNFα, there was an increase in expression for nPT cells at week 2 with no significant differences in PT cells. Of significance, NGF was increasingly downregulated over the 4 week culture period in nPT cells and down-regulation was observed at 2 weeks for PT cells. Thus, for cytokines and painful markers, we show a down-regulation of IL-1β, IL6, and NGF. These results suggest that transfection with T may have the potential to inhibit both inflammation and neuronal invasion and associated pain which has significant clinical implications for the treatment of LBP. Similar to the effects of T on healthy phenotypic markers, these effects were more pronounced in nPT cells versus PT, highlighting a need for further optimization of experimental conditions and delivery protocol while taking into account the pathological state of tissue. In addition previous studies by the authors have demonstrated that multiple transcription factors are often necessary

to reprogram somatic cells therefore warranting the potential need for a cocktail of transcription factors for improved reprogramming[26].

3) Extracellular matrix synthesis and degradation

The healthy NP is hydrated and gelatinous with an abundance of aggrecan and collagen II. However in degeneration, there is a significant increase in ECM degradation and decrease in synthesis, causing mechanical imbalance and alterations in overall structure and function[6]. Therefore, reducing catabolic gene expression and increasing the ability of NP cells to synthesize aggrecan and collagen II is critical for regeneration and repair and a major factor to evaluate when assessing the success of degeneration reversal and reprogramming to a healthy phenotype. MMPs are catabolic enzymes elevated in degenerate IVDs that degrade matrix components such as collagen II (MMP13) and chondroitin sulfates (MMP12)[11,45]. Assessing catabolic genes, MMP12 and MMP13, showed decreasing trends though more significant in MMP13. ACAN gene expression showed an initial increase while COL2 showed a delayed increase in nPT cells. In PT cells, ACAN and COL2 were both increased at week 2 highlighting another temporal peak. Interestingly, transfection of GDF6 in human IVD cells exhibited minimal to no significant changes in ACAN and COL2 gene expression[37]. Notably, in the 3D constructs, we observed significant increases in proteoglycan accumulation in PT NP cells compared to SHAM controls at 2 and 4 weeks however no significant differences compared to SHAM in nPT NP cells. When performing Hydroxyproline for collagen, we did not observe any detectable amounts of protein (data not shown). The differences between aggrecan gene expression and proteoglycan protein measurements could be explained by the temporal nature of RT-qPCR as a snapshot of the gene profile and thus may not correlate directly with protein expression. Similarly, a study using SOX9 transfection via adenovirus in IVDs have found successful upregulation of collagen II in

human and rabbit IVDs [20]. Taken together these observed decreases in catabolic MMPs with significant increases in proteoglycan accumulation especially in PT NP cells are indicative of successful cellular reprogramming of human NP cells to a "healthy" pro-anabolic and anti-catabolic phenotype.

Potential limitations and Future work

It is important to note potential limitations in this proof of concept study. First, this study utilized bulk electroporation to deliver plasmids into the human NP cells and there can be limitations associated with this technique namely high voltage pulses to allow plasmids to permeate through the lipid bilayer[46]. Outcomes include potential apoptosis, necrosis or cellular disfunction[47]. Our results demonstrated high cell viability up to 4 weeks and cellular morphology characteristic of the cell type. However, this does not exclude the possibility of other unwanted cellular effects and future cytotoxicity studies would be required. Because of potential problems associated with bulk electroporation and the route toward clinical translation, we propose using novel nanotransfection or engineered vesicles as a means to deliver nonviral plasmids into tissue/animal models, which has shown great promise in cellular reprogramming [26]. Second, but related to bulk transfection, we observed temporal effects in expression of phenotypic, inflammatory/neurotrophic and ECM related genes and thus plasmid quantity and transfection parameters require further refinement and would be likely improved by nano-transfection. Third, the use of a large cell density should be noted and was warranted in this study to determine feasibility and effects of transfection, however, the cell density is much less in the NP region of human IVDs in vivo and must be taken into consideration in future studies. Fourth, T is highly up-regulated in tumors of the notochord (i.e., chordomas) and is used as defining marker of these carcinomas[27]. Therefore, other transcription factors may need to be explored in addition to T.

Finally, as the biologic response of human IVD cells can be highly variable, cells from human patients were pooled for each patient source (autopsy vs surgery). This reduced the inter-human variability and was consistent with a "proof of concept" study focusing on potential of transfection of T to reprogram human NP cells. We will validate on individual samples/patients and increase the cohort to include gender, age and level in our analysis to determine whether demographics can influence the treatment response. Furthermore, the safety and efficacy of such gene therapy strategies should be validated in clinically relevant in-vivo animal models of LBP to determine whether this treatment can restore structure/function to the IVD while reducing the symptoms of pain.

CONCLUSION

This novel "proof of concept" study is the first to demonstrate the potential of Brachyury transfection to reprogram non-painful and painful human NP cells to a healthy NP-like phenotype with upregulation of key phenotypic markers, down-regulation of inflammatory, catabolic, and pain related markers at the gene level as well as increased proteoglycan synthesis on the protein level. Nonviral reprogramming of autologous human cells has significant clinical relevance and impact. While the delivery method requires further optimization and in vivo testing, this approach shows significant promise for treating painful IVD degeneration associated with LBP.

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