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Synergistic CRISPRa-Regulated Chondrogenic Extracellular Matrix Deposition Without Exogenous Growth Factors

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Stem cell therapies have shown promise for regenerative treatment for musculoskeletal conditions, but their success is mixed. To enhance regenerative effects, growth factors are utilized to induce differentiation into native cell types, but uncontrollable *in vivo* conditions inhibit differentiation, and precise control of expressed matrix proteins is difficult to achieve. To address these issues, we investigated a novel method of enhancing regenerative phenotype through direct upregulation of major cartilaginous tissue proteins, aggrecan (*ACAN*), and collagen II (*COL2A1*) using dCas9-VPR CRISPR gene activation systems. We demonstrated increased expression and deposition of targeted proteins independent of exogenous growth factors in pellet culture. Singular upregulation of *COL2A1/ACAN* interestingly indicates that *COL2A1* upregulation mediates the highest sulfated glycosaminoglycan (sGAG) deposition, in addition to collagen II deposition. Through RNA-seq analysis, this was shown to occur by *COL2A1* upregulation mediating broader chondrogenic gene expression changes. Multiplex upregulation of *COL2A1* and *ACAN* together resulted in the highest sGAG, and collagen II deposition, with levels comparable to those in chondrogenic growth factor-differentiated pellets. Overall, this work indicates dCas9-VPR systems can robustly upregulate *COL2A1* and *ACAN* deposition without growth factors, to provide a novel, precise method of controlling stem cell phenotype for cartilage and intervertebral disc cell therapies and tissue engineering.

Keywords: stem cell, intervertebral disc, cartilage, CRISPRa, cell engineering

Impact Statement

Stem cell therapies have come about as a potential regenerative treatment for musculoskeletal disease, but clinically, they have mixed results. To improve stem cell therapies, growth factors are used to aid a regenerative cell phenotype, but their effects are inhibited by *in vivo* musculoskeletal disease environments. This article describes CRISPR gene activation-based cell engineering methods that provide a growth factor-free method of inducing chondrogenic extracellular matrix deposition. This method is demonstrated to be as/more potent as growth factors in inducing a chondrogenic phenotype in pellet culture, indicating potential utility as a method of enhancing stem cell therapies for musculoskeletal disease.

Introduction

MUSCULOSKELETAL DISEASE RANKS second in years lived with disability, making it a major health care concern.¹ The most common musculoskeletal diseases are osteoarthritis, and low back pain (LBP), and current treatments for these are palliative.^{2–4} One major characteristic that describes musculoskeletal tissue degeneration is a loss of tissue components that support tissue mechanical function. Major extracellular matrix (ECM) proteins that decrease include aggrecan and type II collagen.^{5,6} To restore and regenerate musculoskeletal tissue, cell and tissue engineering therapies are being developed.^{7,8}

Several cell therapy treatments have been developed for the treatment of osteoarthritis and LBP and are being used and tested clinically.^{7,9} Currently, cell phenotype for these cells is regulated *ex vivo* with growth factors and biomaterial cues, but the signaling environment becomes difficult to control once the cells are delivered *in vivo*, which leads to deleterious alterations in cell phenotype after delivery.^{8,10,11} A method to more reliably control and regulate cell phenotype regardless of external signaling cues would be advantageous to improving control over cell therapies and improving outcomes.

To make stem cell therapies more consistently regenerative, previous work has attempted to match stem cell

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phenotype to native musculoskeletal cells by promoting their differentiation, often using growth factors,¹² biomaterials with growth factors,¹³ or gene therapies that introduce growth factors.¹⁴ These approaches have shown some success, but have a number of challenges. One challenge is growth factor effects are inhibited by the hostile inflammatory microenvironments within pathological musculoskeletal tissues.^{10,11} In addition, these approaches do not provide precise control over levels of expressed matrix proteins, which is needed to best mimic native tissue. For example, articular cartilage has a type II collagen to aggrecan ratio of about 2:1, whereas the nucleus pulposus of the intervertebral disc (IVD) has a ratio of about 27:1.¹⁵

Previous work directly regulating ECM protein expression is limited. This is likely as the delivery of these genes is nontrivial due to their large size¹⁶ and endogenous gene activation techniques before the invention of CRISPR gene activation (CRISPRa) (i.e., TALENs) are difficult to design due to need for protein engineering.¹⁷ To our knowledge, only one previous publication has attempted to directly regulate the production of type II collagen and aggrecan.¹⁸ This work by Shi *et al.* introduced transcripts for type II collagen subunit, *COL2A1*, and the gene for aggrecan, *ACAN*, into bovine articular chondrocytes, but demonstrated no increase in these proteins. This may be due to these proteins having complex biosynthesis pathways that require numerous chaperone proteins and post-translational modifications.^{19,20}

In this study, we investigate a novel method of directly regulating the expression of *ACAN* and *COL2A1* in human adipose-derived stem cells (hADSCs) using a CRISPRa technique. These recently built CRISPRa systems provide the ability to target and upregulate endogenous genes acting as artificial transcription factors that provide robust control over gene expression.^{17,21,22} We utilized the dCas9-VPR CRISPRa system²² to activate genes of interest in hADSCs and demonstrated an ability to robustly upregulate both target proteins singularly and together without the addition of exogenous growth factors. Furthermore, our results indicated *COL2A1* upregulation more robustly drove a chondrogenic phenotype in pellet culture by driving broader gene expression changes.

Nonetheless, multiplex upregulation of *ACAN* and *COL2A1* induced a phenotype that most closely mimicked that induced by chondrogenic growth factors in pellet culture. Overall, this work indicates we can utilize the dCas9-VPR CRISPRa system as a method to robustly upregulate specific endogenous ECM proteins to regulate cell phenotype independent of growth factors, providing a platform to more precisely and directly regulate ECM deposition cell phenotype.

Materials and Methods

Experimental overview

Lentiviral vectors were built to upregulate genes *ACAN* and *COL2A1* using the dCas9-VPR CRISPRa system. These lentiviral vectors were transduced into hADSCs to test their ability to engineer stem cells with enhanced regenerative phenotype.

Ability to upregulate target genes was verified by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). Once gene upregulation was verified, the abil-

ity of these systems to upregulate relevant protein expression in three-dimensional (3D) pellet culture was measured by histology, immunohistochemistry (IHC), and biochemical assays. In addition, the broader gene expression changes these systems induce during 3D culture were assessed by RNA-seq. Both *COL2A1* and *ACAN* upregulation provided desired positive effects, but the effects of *COL2A1* upregulation were more robust, although it did not result in the desired increase in aggrecan deposition. Therefore, *COL2A1* and *ACAN* upregulation were multiplexed, and effects were tested in pellet culture to explore how co-deposition of these proteins can further enhance desired phenotypes.

Vector constructs, gRNA design, and cloning

Guide RNA design was performed using Genome Target Scan 2 (GT-Scan 2).²³ The 5'-UTR and the promoter region up to 500 base pairs upstream of *ACAN* and *COL2A1* were analyzed for gRNAs. Top gRNAs with the fewest off-target sites were further inspected using the BLAT tool of the UCSC genome browser,²⁴ to ensure gRNA sequences do not overlap and have at least 50 bp spacing. Two gRNAs per gene were selected, along with a gRNA that does not target the human genome (nontarget control/NTC) (Fig. 1A). These gRNAs were synthesized, annealed, phosphorylated, and ligated into a vector for gRNA expression (83919; Addgene). Insertion of gRNA sequences was verified by Sanger sequencing (Supplementary Data). For dCas9-VPR expression, a previously made lentiviral vector was utilized (Fig. 1A, 99373; Addgene).

Lentivirus production

Lentivirus production was performed as previously described.²⁵ For gRNA lentivirus, the supernatant containing lentivirus was directly diluted and utilized for transduction, as described below. For dCas9-VPR lentivirus, lentivirus containing supernatant was concentrated 100× by centrifuging at 20,000 *g* for 4 h at 4°C and then resuspending in an appropriate amount of phosphate-buffered saline (PBS), according to previous protocols.²⁶ All virus was aliquoted, and frozen at -80°C for later use.

Lentivirus transduction

Lentiviral constructs were sequentially transduced into immortalized hADSCs (SRCR-4000; ATCC) as follows: hADSCs were plated at a density of 10,000 cells/cm² in 24-well plates in 500 µL of manufacture-recommended expansion media (PCS-500-040; ATCC), and allowed to adhere overnight. The next day, concentrated dCas9-VPR virus was diluted 1:10 with expansion media supplemented with 2 µg/mL polybrene and placed on cells (250 µL virus/well). After 24 h, the virus was removed, and cells were washed 5× with PBS. Three days later, puromycin cell selection was performed using 1.25 µg/mL puromycin for 3 days, with medium changes each day. Untransduced cells were puromycin treated alongside as a negative control to ensure the selection of only transduced cells.

This process resulted in a population of hADSCs expressing dCas9-VPR. These cells were then transduced with gRNA virus using the same transduction protocol above, with the modification of diluting the virus 1:4 and using 4 µg/mL polybrene. Transduced cells were not sorted for as

FIG. 1. Design and verification of CRISPRa vectors. **(A)** Map of lentiviral vectors for dCas9-VPR expression cassette and gRNA expression cassette. **(B)** Sequence of gRNAs designed to target each gene and their respective PAM sequence. **(C)** Gene expression changes of *ACAN* in cells transduced with *ACAN* targeting gRNAs relative to NTC cells ($n=3$, $*p<0.05$ relative to NTC group). **(D)** Gene expression changes of *COL2A1* in cells transduced with *COL2A1* targeting gRNAs relative to NTC cells ($n=3$, $*p<0.05$ relative to NTC group). **(E)** Difference in normalized gene expression of *ACAN* or *COL2A1* in NTC cells or *ACAN* or *COL2A1* upregulated cells relative to *COL2A1* cells with gRNA inducing highest gene upregulation ($n=3$, $*p<0.05$ relative to *COL2A1* group). CRISPRa, CRISPR gene activation; NTC, nontarget control.

transduction efficiency was at nearly 100% (Supplementary Data). Cells were cultured for at least 7 days to allow for transgene expression and generation of a sufficient number of cells for experiments.

Verification of *ACAN* and *COL2A1* upregulation

Transduced cells were analyzed for *ACAN* or *COL2A1* gene expression by qRT-PCR as follows ($n=3$): RNA was harvested by the Purelink RNA Micro Kit (Ambion). Complementary DNA (cDNA) synthesis was performed with purified RNA using the high-capacity cDNA reverse transcription kit with RNase inhibitor (Applied Biosystems). This cDNA was then used for qPCR with TaqMan gene expression assays (ThermoFisher) for our genes of interest (*ACAN*: Hs00153936_m1, *COL2A1*: Hs00264051_m1) as well as a housekeeping gene (*GAPDH*: Hs02758991_g1). Changes in *ACAN* or *COL2A1* expression were normalized to *GAPDH* expression, and fold change in mRNA expression relative to the NTC or *COL2A1* upregulated cells was calculated using the $\Delta\Delta C_t$ method.

Pellet culture of human adipose-derived stem cells

hADSCs containing the best performing gRNAs for each gene were put through pellet culture to assess changes in ECM deposition induced by targeted gene upregulation. Pellet cultures were performed according to previous methods, but without any growth factor added to basal media.²⁵ Pellets were cultured for 21 days, with media changed every 2–3 days. As a positive control, NTC cells were cultured in basal media with chondrogenic growth factors (10 ng/mL TGF β 3 and BMP-6 [Peprotech]). Cell pellets were harvested after 21 days of culture for imaging, histology, collagen II IHC, and biochemical analysis as described below. The supernatant was collected and stored at -20°C at each medium change for future biochemical analysis.

Macroscopic pellet imaging

At the end of pellet culture, pellets were imaged (Pentax K5) in their respective wells for qualitative comparison of the gross pellet phenotype.

Histology and immunohistochemistry

To prepare pellets for staining, pellets ($n=4$) were fixed in 10% neutral-buffered formalin solution for 24 h. Pellets were then embedded in paraffin, and 5 μm sections were mounted on glass slides.

Sections for each sample were stained with Alcian blue to analyze sulfated glycosaminoglycan (sGAG) qualitatively. Sections were deparaffinized, hydrated in distilled water, and mordanted with 3% glacial acetic acid for 3 min. Alcian blue (pH 2.5; Newcomer Supply) was then applied for 30 min. Sections were then washed in running tap water for 10 min, rinsed in distilled water, and counterstained in Nuclear Fast Red (Newcomer Supply) for 5 min. Sections were washed in running tap water for 1 min before being dehydrated, cleared in xylene, and cover slipped.

For collagen II IHC staining, samples were processed using the following protocol: sections were air-dried and melted in a 60°C oven for 30 min. Slides were then loaded onto the Ventana ULTRA automated staining instrument for the following steps: slides were deparaffinized with EZ Prep solution (Ventana Medical Systems [VMS]), and antigen retrieval was performed with Protease 2 enzyme (VMS) for 12 min at 37°C . The primary antibody (Polyclonal rabbit anti-collagen II; Leica) was applied at a concentration of 1:50 for 2 h at room temperature. Subsequently, an Amplification kit (VMS) was applied to increase the antibody signal. Antibody staining was then visualized using the ultraView Universal DAB detection kit (VMS). Sections were counterstained with hematoxylin (VMS) for 8 min. The slides were gently washed in a mixture of 1% DAWN™ in

distilled water solution, and then just distilled water until all wash mixture was removed. Slides were dehydrated in graded ethanol solutions, cleared in xylene, and cover slipped.

Sulfated glycosaminoglycan quantification

The total amount of sGAG content contained within papain digested cell pellets and supernatant of pellet cultures ($n=5-10$) was analyzed using a previously described dimethylmethylene blue assay.²⁷

Hydroxyproline quantification

The total amount of collagen content contained within papain digested cell pellets and supernatant of pellet cultures ($n=5-10$) was analyzed using a collagen quantification assay based on measuring hydroxyproline content. The hydroxyproline assay protocol used was adapted from a previously described hydroxyproline assay.²⁸ Adaptations include increasing autoclaving for hydrolysis to 40 min and adjusting the pH of the oxidation buffer to 6.5 with glacial acetic acid.

RNA sequencing

hADSCs with or without *ACAN* or *COL2A1* upregulation were put through pellet culture (as described in the Pellet Culture of hADSCs section) for 1 week and harvested for subsequent RNA-seq analysis ($n=2$ pellets/group). Pellets were cultured for 6 days before being harvested for RNA-seq analysis. Total RNA was harvested using the Purelink RNA Micro kit (ThermoFisher). Total RNA (100–500 ng) was hybridized with Ribo-Zero Gold to substantially deplete cytoplasmic and mitochondrial rRNA from the samples. Stranded RNA sequencing libraries were prepared using the Illumina TruSeq Stranded Total RNA Library Prep Gold kit with TruSeq RNA UD Indexes. Sequencing libraries (1.3 nM) were chemically denatured and applied to an Illumina NovaSeq flow cell using the NovaSeq XP chemistry workflow, and a 2×51 cycle paired-end sequence run was performed using a NovaSeq S1 reagent Kit.

Sequencing reads were aligned to the hg19 build of the human genome using HISAT2.²⁹ Sam files were converted to bam format and sorted using SAMtools.³⁰ Reads mapping to UCSC known genes³¹ were counted using featureCounts from the SubRead package.³² Reads were normalized, and differential analysis was done in a pairwise manner using DESeq2.³³ Heatmaps were generated using the pheatmap package in R.³⁴ GO biological processes enriched were determined from genes significantly upregulated using Enrichr.^{35–37}

Multiplex aggrecan and collagen II upregulation

To investigate if multiplexing *COL2A1* and *ACAN* upregulation enhances desired phenotypic effects, hADSCs with collagen II upregulated were transduced with the gRNA that best upregulates *ACAN* (gRNA 2) using lentivirus transduction methods as described in the Lentivirus Transduction section. This generated hADSCs with simultaneous *COL2A1* and *ACAN* upregulation. To verify both genes were upregulated, qRT-PCR for *ACAN* and *COL2A1* was performed on cells as described in “Verification of *ACAN* and *COL2A1* Upregulation” section ($n=3$). Once simultaneous

upregulation was verified, cells were then put through pellet culture as previously described in Pellet Culture of hADSCs section. Pellets also underwent the same imaging (Macroscopic Pellet Imaging section), histological and IHC analysis (Histology and IHC section, $n=4$), and biochemical analysis (sGAG Quantification section and Hydroxyproline Quantification section, $n=5-6$).

Statistics

Statistical analysis of qRT-PCR and biochemical data was performed using JMP Pro 14 software (SAS). These data were analyzed by a one-way analysis of variance using Tukey's *post hoc* test, with the exception of qRT-PCR data for multiplex gene upregulation, which only had two experimental groups and was analyzed by a two-tailed Student's *t*-test ($\alpha=0.05$ for all tests). Statistical analysis of RNA-seq data was performed through data analysis tools utilized to analyze differential gene expression data (Enrichr, DESeq2, $\alpha=0.05$).

Results

*Singular *ACAN* and *COL2A1* upregulation*

CRISPRa is able to robustly upregulate *ACAN* and *COL2A1* expression. Lentivirally delivered dCas9-VPR systems and gRNAs targeting *ACAN* or *COL2A1* are able to significantly upregulate target genes. Increases of target genes relative to nontarget control (NTC) cells are $2^{2.61}$ - and $2^{4.75}$ -fold for *ACAN* ($p<0.0001$) and $2^{12.38}$ - and $2^{15.28}$ -fold for *COL2A1* ($p<0.0001$) (Fig. 1C, D). While gene expression increases for *ACAN* are lower, the gene expression level achieved is similar, although statistically different, to that of *COL2A1*, as seen when evaluating fold changes in expression relative to *COL2A1* upregulated cells (Fig. 1E). This effect is seen as baseline levels of *ACAN* are higher compared with *COL2A1* within the hADSCs.

CRISPRa based *ACAN* and *COL2A1* upregulation improves sGAG and collagen production. Gross morphological analysis of 3-week pellets indicates visible differences in pellet morphology between cell groups (Fig. 2A). Consistent with *ACAN*'s known structural characteristics, the cell pellet with *ACAN* upregulated cells has a less dense gel-like appearance, akin to the nucleus pulposus tissue of the intervertebral disc, which has high ratios of aggrecan to collagen content. The pellet with *COL2A1* upregulated cells has a more contracted and white opaque appearance, and looks most similar to the growth factor-differentiated pellet.

Histological analysis of sGAG deposition by Alcian blue staining qualitatively demonstrates that sGAG content is enhanced in *ACAN* and *COL2A1* upregulated cells (Fig. 2B). Surprisingly, it can be seen that the increase in sGAG deposition is increased in *COL2A1* upregulated cells, and staining appears similar to that of growth factor-differentiated cells. Examining IHC analysis of collagen II indicates collagen II deposition increases only in *COL2A1* upregulated cells, although these levels are still below what is seen in growth factor-differentiated cells (Fig. 2C).

Quantitative measurement of sGAG content within 3-week pellet cultures demonstrates that sGAG deposition is significantly increased by *ACAN* or *COL2A1* upregulation without

FIG. 2. Qualitative analysis of pellet cultures with singular gene upregulation. (A) Macroscopic images of cell pellets demonstrating their morphological similarities and differences (scale bar is 2 mm). (B) Alcian blue staining of cell pellets (scale bar is 40 μm). (C) Collagen II IHC of cell pellets (scale bar is 40 μm). IHC, immunohistochemistry.

exogenous growth factors (Fig. 3A). Total sGAG content (*ACAN*: $p=0.0121$, *COL2A1*: $p<0.0001$) significantly increased for both *ACAN* and *COL2A1* upregulated cells relative to NTC cells. The total sGAG content was surprisingly higher in *COL2A1* upregulated cells compared to *ACAN* ($p=0.0001$). Consistent with IHC data, the measurement of collagen content demonstrates that collagen deposition is only significantly increased in *COL2A1* upregulated cells ($p=0.0024$ relative to NTC group) (Fig. 3B). Total collagen content in *ACAN* upregulated cells does not significantly change compared to NTC cells ($p=0.354$). Overall, *COL2A1* demonstrated the highest deposition of sGAG and collagen, but these levels were still significantly below that of growth factor-differentiated cell pellets (sGAG: $p<0.0001$, collagen: $p=0.0002$).

RNA-seq analysis indicates *COL2A1* upregulation induces broad gene expression changes associated with a chondrogenic phenotype. To understand what signaling changes are occurring after induction of *ACAN* and *COL2A1*, RNAseq analysis was performed after 1 week of pellet culture. Analysis of cell pellets cultured for 1 week indicates that *COL2A1* upregulation more broadly changes gene expression and that these gene expression changes aid in

driving a chondrogenic phenotype. Looking at a heatmap of the different cell groups, we see that the *ACAN* upregulated cell expression profile is similar to NTC cells, while the *COL2A1* expression profile is orthogonal in expression to both *ACAN* and NTC cells (Fig. 4A). This is confirmed in differential expression plots where it can be seen that a larger number of genes are significantly differentially expressed in *COL2A1* upregulated cells relative to NTC cells (2532 genes), compared to *ACAN* upregulated cells relative to NTC cells (38 genes) (Fig. 4B, C).

ACAN was not upregulated during pellet culture when targeting *COL2A1*, despite evidence of increased sGAG deposition. Therefore, we examined genes associated with proteoglycans and GAG synthesis to investigate the potential for enhanced sGAG content in these cells. This revealed that proteoglycans Lumican and Biglycan are upregulated along with enzymes associated with GAG synthesis, potentially explaining why we saw an increase in sGAG content (Fig. 4D).

Gene enrichment analysis revealed several biological processes significantly associated with genes upregulated in *COL2A1* upregulated cells, whereas no biological processes were significantly associated with genes upregulated in *ACAN* upregulated cells (Fig. 4E). Aside from the expected biological processes associated with ECM organization,

FIG. 3. Biochemical assays quantifying ECM content in pellet cultures. (A) Total sGAG produced by pellet cultures. (B) Total collagen produced by pellet cultures ($n=5-10$, * $p<0.05$ relative to NTC, dotted line represents amounts produced by NTC + GF cell pellets). sGAG, sulfated glycosaminoglycan.

FIG. 4. RNA-seq analysis of cells cultured in pellet culture for 1 week. **(A)** Heat map of differentially expressed genes in all cell groups **(B, C)** Differential gene expression analysis of *ACAN* **(B)** or *COL2A1* **(C)** upregulated cells versus NTC cells. **(D)** Top five biological processes associated with genes differentially expressed after *COL2A1* upregulation. **(E)** Fold change and significance of gene expression changes in proteoglycans and enzymes associated with proteoglycans and chondroitin sulfate synthesis after *COL2A1* upregulation.

biological processes associated endoplasmic reticulum (ER) stress responses are also significantly associated with genes upregulated in *COL2A1* upregulated cells. This is consistent with previous research that demonstrates ER stress response has been associated with cell differentiation, including chondrogenic differentiation.^{38–40}

Multiplex *ACAN* and *COL2A1* upregulation

Multiplex upregulation of *ACAN* and *COL2A1* results in comparable target gene upregulation to singular gene upregulation of *ACAN* and *COL2A1*. Multiplex delivery of *ACAN* and *COL2A1* gRNAs along with dCas9-VPR systems is able to significantly upregulate both *COL2A1* and *ACAN* simultaneously. Increase of *ACAN* relative to NTC cells is 2⁴-fold ($p=0.0008$) (Fig. 5B). The increase of *COL2A1* relative to NTC cells is 2^{17.66}-fold ($p<0.0001$) (Fig. 5C).

This demonstrates multiplex gene upregulation can provide similar gene upregulation results to singular gene upregulation.

Multiplex upregulation of *ACAN* and *COL2A1* increases sGAG and collagen deposition up to levels induced by growth factor-based differentiation. Gross morphological analysis of pellets indicates visible differences in pellet morphology between cell groups (Fig. 6A). The cell pellet with multiplex *ACAN* and *COL2A1* upregulated cells has a contracted and white opaque appearance similar to that of the growth factor-differentiated pellet. Histological analysis of sGAG deposition by Alcian blue staining qualitatively demonstrates that sGAG content is increased dramatically in multiplex *ACAN* and *COL2A1* upregulated cells relative to NTC cells, and staining appears similar to that of growth

FIG. 5. Verification of multiplex gene upregulation by CRISPRa vectors. **(A)** Map of lentiviral vectors for dCas9-VPR expression cassette and gRNA expression cassettes expressed in hADSCs for multiplex gene upregulation. **(B)** Sequence of gRNAs targeting each gene and their respective PAM sequence. **(C)** Gene expression changes of *ACAN* in cells with multiplex *ACAN* & *COL2A1* upregulation relative to NTC cells ($n=3$, $*p<0.05$ relative to NTC group). **(D)** Gene expression changes of *COL2A1* in cells with multiplex *ACAN* & *COL2A1* upregulation relative to NTC cells ($n=3$, $*p<0.05$ relative to NTC group).

FIG. 6. Qualitative analysis of pellet cultures. **(A)** Macroscopic images of cell pellets demonstrating their morphological similarities and differences (scale bar is 2 mm). **(B)** Alcian blue staining of cell pellets (scale bar is 40 μ m). **(C)** Collagen II IHC of cell pellets (scale bar is 40 μ m).

factor-differentiated cells (Fig. 6B). Examining IHC analysis of collagen II indicates collagen II deposition is dramatically higher in multiplex *ACAN* and *COL2A1* upregulated cells compared to NTC cells and that levels are similar, if not higher, than that seen in growth factor-differentiated pellets (Fig. 6C).

Quantitative measurement of sGAG content within pellet cultures demonstrates that sGAG deposition is significantly increased by multiplex *ACAN* and *COL2A1* upregulation without exogenous growth factors relative to NTC cells ($p<0.0001$), and even exceeds that seen in growth factor-differentiated pellets ($p=0.0049$) (Fig. 7A). Measurement of collagen content demonstrates that collagen deposition is significantly increased in multiplex *ACAN* and *COL2A1* upregulated cells relative to NTC cells ($p<0.0001$) (Fig. 7B). In addition, it can be noted that total collagen levels are at those seen within growth factor-differentiated pellets ($p=0.834$). Overall, these data demonstrate that multiplex upregulation of *COL2A1* and *ACAN* allows for improved ECM production in comparison to singleplex upregulation of these genes by allowing for ECM production to achieve levels seen in growth factor-differentiated pellets.

Discussion

Stem cell therapies have significant potential for cell supplementation within degenerative musculoskeletal tissue, which have degenerated ECM and additionally lost many of their cells,^{6,41} although their regenerative results, even with the help of differentiation enhancing growth factors, are

FIG. 7. Biochemical assays quantifying ECM content in pellet cultures. **(A)** Total sGAG produced by pellet cultures. **(B)** Total collagen produced by pellet cultures ($n=5-6$, $*p<0.05$ relative to NTC, dotted line represents amounts produced by NTC + GF cell pellets).

mixed due to microenvironmental challenges that drive deleterious changes to regenerative cell phenotype.^{5,10,11,42} Control of stem cell phenotype through direct modulation of ECM expression can provide a method to more consistently enhance regenerative effects within pathological musculoskeletal tissue and allows for more precise control of phenotype depending on the desired application. In this study, we utilized the dCas9-VPR CRISPRa system to upregulate ECM proteins relevant to musculoskeletal tissue, aggrecan, and collagen II, to achieve this more direct control of cell phenotype.

CRISPRa systems were able to successfully singularly upregulate the expression of *ACAN* and *COL2A1* in hADSCs (Fig. 1). The overall level of expression reached is slightly higher for *COL2A1*, but similar between *COL2A1* and *ACAN*. Once these systems were verified to perform successful gene upregulation, we moved forward with analyzing their effects on ECM production in pellet culture without exogenous growth factors. This was to determine if this ECM-targeted gene upregulation could independently enhance the deposition of targeted ECM proteins.

From this experiment, we saw that we are indeed able to modulate targeted ECM expression, but we saw that *COL2A1* upregulation had broader effects on cell phenotype (Figs. 2 and 3). This was characterized by elevated sGAG deposition, in addition to expected elevated collagen II deposition seen both qualitatively and quantitatively. While collagen and sGAG deposition are enhanced in *COL2A1* upregulated cells, they do not reach levels seen with added growth factors.

To further investigate these effects of *COL2A1* upregulation, we performed RNA-seq analysis of pellets from all cell groups cultured for 1 week to capture active gene expression changes occurring during 3D pellet culture. From this analysis, we uncovered that *COL2A1* upregulation was creating much broader gene expression changes than *ACAN*, with 2532 genes differentially expressed in *COL2A1* upregulated cells compared to 38 genes in *ACAN* upregulated cells (Fig. 4). Gene enrichment analysis of differentially expressed genes indicates there are only biological processes enriched in *COL2A1* upregulated cells.

When we took a closer look at the top five biological processes enriched, we see processes associated with ECM organization, but we also see processes associated with ER stress. Enrichment of biological processes associated with ER stress is notable as ER stress has been previously im-

plicated in playing an important role in cell differentiation, including chondrogenic differentiation.^{38-40,43} To help explain increases in sGAG deposition, we also looked at genes associated with proteoglycans and enzymes involved in sGAG synthesis. *ACAN* was not upregulated in *COL2A1* cells in 3D culture, but other proteoglycans and enzymes associated with sGAG synthesis were, helping explain why sGAG deposition increased.

Overall these data further support that *COL2A1* upregulation is a powerful regulator of cell phenotype and has broader desired effects by inducing gene expression changes associated with differentiation in 3D pellet culture without growth factors, although these data also indicate that gene expression changes in *COL2A1* and *ACAN* upregulated cells are orthogonal, as we also see on the heat plot (Fig. 4) and by the fact that the major chondrogenic gene *ACAN*^{44,45} is not upregulated. The lack of this important gene helps explain why sGAG and collagen deposition levels do not reach that of growth factor-differentiated cells.

To better promote the desired chondrogenic phenotype, we took the approach of upregulating both *ACAN* and *COL2A1* in the hADSCs simultaneously. Before performing pellet cultures, we verified that we are able to successfully upregulate both genes to similar levels achieved singularly by qPCR. Once this was verified, we analyzed these cells in pellet culture and found that we are able to better promote a chondrogenic phenotype, defined by increased sGAG and Collagen II deposition, with this multiplex ECM gene upregulation. This was seen by synergistic increases in total sGAG and collagen deposition, increasing to levels achieved by growth factors as well as drastic increases in collagen II IHC staining (Figs. 6 and 7). These same levels in either collagen type II or sGAG were not observed when *COL2A1* and *ACAN* were targeted independently.

These results overall indicate that collagen II and aggrecan deposition together drive chondrogenic ECM deposition, at levels similar to growth factor-differentiated cells in pellet culture. By directly driving these genes to regulate cell phenotype, this has the potential to more directly control the desired cell phenotype after delivery *in vivo*, compared to traditional cell differentiation strategies.⁴⁶

It is interesting to note that *COL2A1* upregulation, even by itself, caused such drastic changes in gene expression, while *ACAN* did not. This may be due to structural and signaling changes in the environment *COL2A1* upregulation induces that *ACAN* upregulation does not. When you look at images

of the pellet culture morphology, you can see that the pellet with *COL2A1* upregulated cells has a more contracted and dense appearance, like that of the growth factor-treated pellet (Fig. 2A). It is known that the structural properties of a cell's environment, like tissue density and stiffness, do influence stem cell phenotype.^{47–49} The pellet contraction in *COL2A1* upregulated pellets creates an environment with a structure more like that of the growth factor-treated pellets. Therefore, it is possible that phenotypic changes more similar to growth factor-treated pellets occur.

It is also known that ECM content influences cell phenotype^{50–52} and that collagen II plays a major role in integrin signaling.⁵³ Therefore, the changes in the surrounding structure and ECM content likely work together to induce phenotypic changes in the hADSCs. This may explain why multiplexing *ACAN* upregulation with *COL2A1* upregulation most robustly induces cartilaginous tissue formation as it creates an environment closest to these cartilaginous tissues with increased amounts of both aggrecan and collagen II present.

It is still unclear why the differences in the mechanical environment and ECM content caused by *ACAN* upregulation did not induce phenotypic changes that more drastically increase sGAG content. Lack of collagen II could be the cause, as there was no detectable collagen II produced by these *ACAN* targeted cells (Fig. 2), despite collagen II being a prominent component of articular cartilage, fibrocartilage, and IVD.^{15,54,55} In summary, what is known from this study is that collagen II upregulation singularly induces broad phenotypic changes, but multiplex collagen II and aggrecan upregulation provide synergistic changes toward the desired phenotype.

Overall these studies provide a platform for future work in utilizing dCas9-VPR systems to upregulate major musculoskeletal ECM proteins Aggrecan and Collagen II to improve regenerative properties of stem cell therapies. This study was done in a pellet culture system, which, while highly useful for assessing the initial effectiveness of this approach and method optimization, does not fully capture the 3D environment in which these cells would be cultured in clinical cell therapy and tissue engineering approaches. Thus, future work will focus on assessing the effectiveness of these CRISPRa cell engineering techniques in inducing chondrogenic ECM deposition in 3D scaffolds and eventually *in vivo* to better capture the environment the cells would be exposed to when clinically used.

In addition, testing was only done in hADSCs, but the lentiviral CRISPR gene regulation systems used can be applied to other cell types, as has been demonstrated in work in the CRISPR gene regulation field.^{25,56–61} Also, in general, lentivirus has been shown to perform gene delivery successfully to a broad range of cell types.⁶² The delivery of the dCas9-VPR system can also potentially be done nonvirally to improve the safety profile of this approach. Nonetheless, either approach is clinically feasible in terms of safety, as lentivirus is extensively used in *ex vivo* engineered cell therapies currently being clinically tested.^{63,64}

Conclusion

In this study, we utilized CRISPRa systems to upregulate Aggrecan and Collagen II to effectively modulate stem cell phenotype without exogenous growth factors. We

demonstrated the ability to effectively upregulate both ECM genes at a transcriptional and translational level. In addition, we demonstrated that *COL2A1* upregulation has broader effects on cell phenotype in 3D pellet culture and that multiplexing *COL2A1* with *ACAN* upregulation induces stem cells to produce tissue that most closely resembles that produced by cells in chondrogenic growth factor-treated pellets. In summary, this study demonstrates the ability to use these systems to robustly upregulate these ECM genes in stem cells. Thereby, these systems provide a novel platform to regulate cell ECM phenotype, and cell phenotype more broadly, to improve the potential of cell therapies for musculoskeletal disease.

Disclaimer

The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH.

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Supplementary Material

Supplementary Data
Supplementary Figure S1

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