Utilizing Synthetic Gene Circuits in Mouse Mesenchymal Stem Cell Derived Chondrocytes to Verify Cartilage Regeneration Outcomes In Vitro

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Introduction

Articular cartilage, the avascular connective tissue present at the end of long bones, has limited healing capability when injured and its injury commonly leads to post traumatic osteoarthritis (PTOA) [1]. The current treatment strategies include microfracture, osteochondral transplantation, and autologous chondrocyte implantation. While these methods can improve cartilage quality, they are not sufficient to fully heal and restore its function [2]. Mesenchymal stem cell (MSC)-based cartilage repair is an appealing treatment strategy since these cells can differentiate into chondrocytes (cartilage producing cells) and produce cartilage macromolecules. However, clinical application of MSC-based cartilage repair requires significant improvements in stability of the chondrogenic phenotype and matrix accumulation by MSC derived chondrocytes (MdChs) under the hostile inflammatory environment of an injured joint [3].

Structure of Cartilage

There are many components of the extracellular matrix (ECM) of chondrocytes that are good markers for determining the quality and quantity of cartilage. Markers that are focused on in our lab include sGAG, collagen type II (COLII), collagen type X (COLX), and aggrecan. Sulfated glycosaminoglycans (sGAG) plays an important role in in maintaining homeostasis in cartilage, as it has a negative charge that attracts water molecules. This creates a high osmotic pressure contributing to the mechanical properties of cartilage. COLII and aggrecan contribute to the compressive and tensile strength of cartilage, while COLX plays a role in the hypertrophic maturation of chondrocytes [4].

Endochondral Ossification and RUNX2

The instability of the chondrogenic phenotype stems from the endochondral ossification pathway. While MSCs have the ability to differentiate into chondrocytes, once they do, they continue down the endochondral ossification pathway and undergo hypertrophic maturation [5]. Figure 1 outlines the pathway and shows pre-hypertrophic and hypertrophic chondrocytes. During hypertrophic maturation, SOX9 gets downregulated and the transcription factor RUNX2 is upregulated. This in turn downregulates collagen II and aggrecan and initiates the production of collagen type X [4]. In addition to this, hypertrophic MdChs start producing matrix degrading enzymes leading to lower compressive and tensile strengths of the tissue and causing a loss of function [6]. RUNX2 is the main transcription factor that drives chondrocyte hypertrophy, as it directly binds to the promotor regions of COL10a1 (collagen type X) and matrix degrading enzyme MMP13 (matrix metalloproteinase 13).

Inflammation and progression of PTOA is caused by the activation of synovial macrophages into a pro-inflammatory state following traumatic joint injury. This activation leads to an increase in inflammatory cytokines that inhibit MSC chondrogenesis through the suppression of SOX9, along with upregulating RUNX2 thereby inducing hypertrophic maturation. This response leads to the degradation of the cartilage matrix [7].

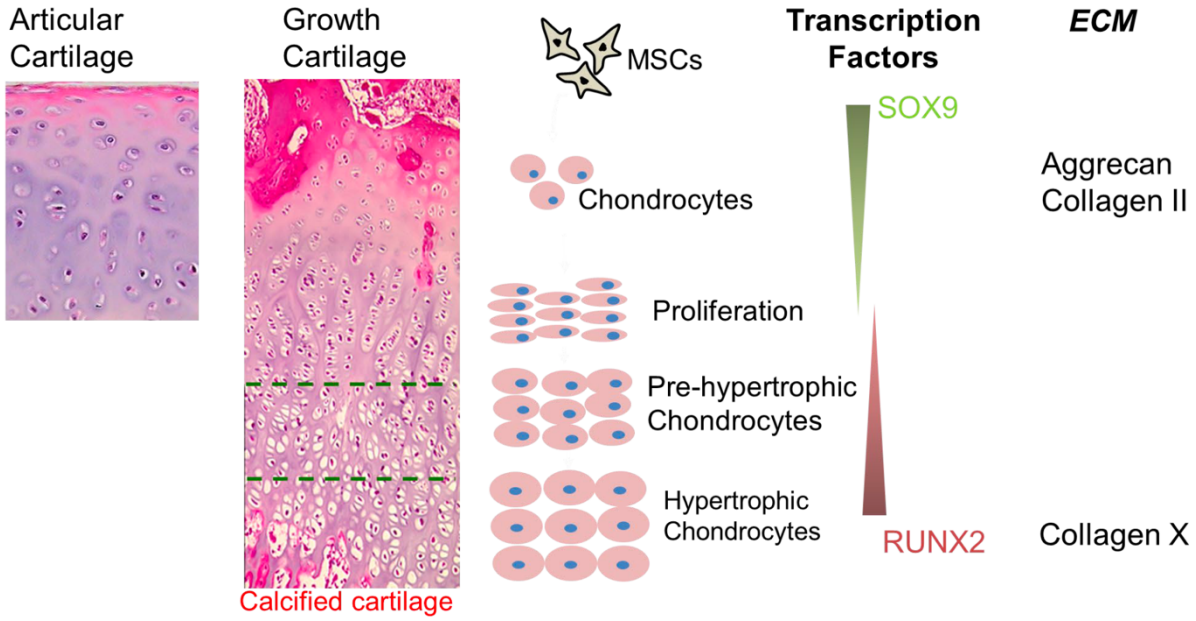

Figure 1. MSC chondrogenesis follows the endochondral ossification pathway.

In summary, RUNX2 is a transcription factor that is upregulated in chondrocytes during PTOA, inducing chondrocyte hypertrophy and leading to degradation of the cartilage extracellular matrix. In human MdChs, previous work in our lab has shown that in vitro suppression of RUNX2 has the ability to induce anti-inflammatory factors under inflammation, thus promoting extracellular matrix accumulation and maintenance of the articular cartilage phenotype [8].

Auto-regulatory RUNX2 Suppressing Gene Circuit

Suppression of RUNX2 has been achieved through the engineered auto-regulatory gene circuit shown in Figure 2. The circuit either contains one or three cis enhancer RUNX2 binding sites, allowing for low level (20%) and high level (70%) RUNX2 suppression. When RUNX2 is present it binds to the RUNX2 binding sites that are upstream of shRUNX2. From here the shRUNX2 sequence is transcribed to shRNA that goes and binds to the mRNA being transcribed for RUNX2 and degrades it, preventing it from creating the protein RUNX2. This inhibition creates an intracellular negative feedback loop. In addition to 1cis and 3cis shRUNX2 circuits, 1cis and 3cis scrambles are also used. The structure of the scrambles is the same as the shRUNX2 circuit, except instead of shRUNX2 being downstream of the RUNX2 binding sites there is a random sequence that does not target anything. The main purpose of these scramble circuits is to serve as a control. If unexpected results are obtained, having the scrambles helps pinpoint whether it was an issue with the RUNX2 suppression or an issue with the lentivirus and transduction of the gene circuit into the cells.

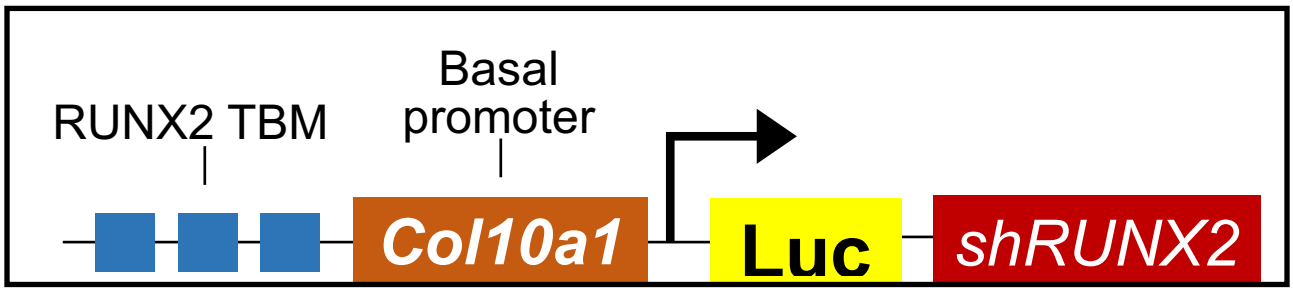

Figure 2. Auto-regulatory RUNX2 suppressing gene circuit has shown the ability to induce anti-inflammatory factors under inflammation and promote extracellular matrix accumulation in human MdChs.

Our lab has shown that the shRUNX2 circuit stabilizes the chondrogenic phenotype and protects human MdChs from inflammation-induced matrix catabolism in vitro [4]. To move forward and test the efficacy of the circuit using in vivo mouse models of PTOA, it needs to be confirmed that the gene circuit is equally successful in mouse MSC derived chondrocytes. We hypothesize that the suppression of RUNX2 activity, via the engineered gene circuit used in vitro in human MdChs, will be equally successful at inhibiting RUNX2 and preventing hypertrophic maturation in vitro in mouse MdChs. To confirm this hypothesis, we will be answering the questions: 1) do the engineered RUNX2 gene circuit modified mouse MdChs successfully inhibit RUNX2? and 2) can the articular cartilage-specific phenotype be achieved from mouse MSCs while preventing chondrocyte hypertrophy?

Methods

When performing any kind of cell culture, sterile technique is extremely important as the cells are very susceptible to bacterial and fungal contamination. Any form of contamination can impact the cells health, and even kill them. To do this the designated cell culture hood should always be wiped down with ethanol from the back of the hood to the front, since work is done at the front of the hood it has the highest possibility of being contaminated. Every bottle or tube should be wiped from top to bottom with ethanol before entering the hood, so we don't bring contaminants to the opening of the bottle. New packages of tips or other supplies should be wiped down and not opened until they are inside the hood. Other good practices for keeping your workspace as sterile as possible are frequently spraying and wiping your hands with ethanol and discarding pipettes if they accidentally touch something in the hood.

Cell Culture Procedure

The first step following the revival of the purchased mMSCs (Cyagen) is transduction to genetically manipulate the grown cells to express our engineered gene circuit. Transduction is a permanent method where the cells are infected with a virus containing the genes to be expressed and they become a part of the cells' DNA. It is permanent because the new gene gets passed down from cell to cell, therefore, it can be performed when we have a small number of cells and as these cells grow and divide the new gene will be maintained. Our cells were modified with four different

circuits: 1cis shRUNX2, 3cis shRUNX2, 1cis scramble, and 3cis scramble. In addition to these four sample types, a wild type (unmodified) sample that underwent the same culture to serve as a control. The mMSCs were treated with a combination of chondrogenic growth factors bone morphogenic protein 2 (BMP-2) and transforming growth factor-β3 (TGF-β3) to initiate the differentiation of the MSCs into chondrocytes and throughout chondrogenesis. From here the cells either underwent a short-term culture (28 days) or a long-term culture (56 days). Chondrogenic differentiation medium consisted of DMEM (high-glucose) supplemented with 0.1 μM dexamethasone, 50 μg/mL ascorbate 2-phosphate, 40 μg/mL L-proline, 100 μg/mL sodium pyruvate, 1% ITS+, 50 ng/mL BMP-2, and 10 ng/mL TGF-β3. When cells reached 85% confluence (D0), they were trypsinized and centrifuged into pellets containing 2.0×105 cells in round-bottomed polypropylene 96-well-plate. Throughout the culture the media is changed every 3 days so that the cells continue to grow and produce extracellular matrix. On day 28 and day 56 takedowns were performed for all 5 experimental groups: wild type, 1cis shRUNX2, 3cis shRUNX2, 1cis scramble, and 3cis scramble. During takedowns each cell type is split into two different groups, samples for histology and samples for dimethylmethylene blue (DMMB) analysis. The procedure for the two takedowns is slightly different due the differences in how they will be analyzed. For DMMB analysis 4 samples are taken for each cell type, with 3-4 pellets in each sample. Following the takedown, the samples are stored at -80°C. For the histology takedowns, 4 samples are taken for each cell type and they are stored at 4°C.

Processing

After the completion of the cell culture the samples must be processed before they can be used. The biochemical analysis samples were digested in papain buffer (papain, EDTA, and sodium phosphate) overnight at 65.5°C. The histological analysis samples were dehydrated with a series of ethanol (70-100%) and then cleared with xylene.

DMMB and DNA Assay

One important method used for quantifying the quality of cartilage produced is the dimethylmethylene blue (DMMB) dye assay. The DMMB dye binds to sulfated glycosaminoglycans (sGAG) in the extracellular matrix. In order to run the assay, it is important that the pellets are fully dissolved. The samples are vortexed and centrifuged. If this procedure does not dissolve the pellets, they are heated at 65.5°C, for approximately 15-30 minutes. Both the D28 and D56 samples were heated under those conditions to ensure they dissolved. A standard curve is created by performing serial dilutions with the chondroitin sulfate standards in papain buffer (PB) from 125 μg/ml - 3.9 μg/ml. It is important that the readings for the samples fall within those of the standard in order for the assay results to be accurate. If the readings are too high, the samples are diluted and run again. In this project the D28 samples were not diluted and the D56 samples were diluted 1:4 in PB. The samples are mixed with dye and read at two wavelengths: 525nm and 595nm. These wavelengths give the ratio of purple to blue dye and blue to purple dye respectively.

Following the DMMB assay the readings need to be standardized. They are standardized by the DNA content of the samples so that any differences seen from sample to sample are due to how much sGAG each cell is producing and not the number of cells in the sample. Papain-digested

samples are mixed with PicoGreen dye and read at both 355nm and 460nm. A standard curve is made using a serial dilution with the DNA.

Embedding and Sectioning

Following the processing of the histology samples, they are embedded in paraffin to then be sectioned onto slides for staining. Sectioning is done with a microtome, and 7μm thin sections are taken through the block, capturing the profile and contents of the pellets. Three to four sections are taken at a time.

Alcian Blue and Immunofluorescence Staining

At days 28 and 56, samples were stained with Alcian blue, which binds to sGAGs. The slides are first deparaffinized with Xylene, then they get rehydrated through a series of ethanol through distilled water. At this point they get stained with the alcian blue dye and then nuclear red fast dye. The nuclear red fast dyes the nuclei of the cells red so that the individual cells can be identified. After both dyes it goes through a dehydration series before being cleared with xylene and coverslipped.

Immunofluorescence staining starts off with the same xylene and hydration series as alcian blue staining. At this point the slides can be stored in distilled water at 4°C until ready to move onto the next step. Then the slides go through a series of steps to prepare the samples and allow for the dye to bind to the protein of interest. The antigen retrieval step enables the antibody to access and bind to the protein. Following this the slides are washed and sit in blocking solution for an hour at room temperature. The blocking step prevents non-specific binding of the antibodies to molecules other than the protein of interest. This is crucial so that there isn't overstaining or the appearance of higher concentration of the target protein. The slides then sit in the primary antibody overnight at 4°C for 16-20 hours for the initial binding. On day 2, the slides are washed and then sit in a secondary antibody for an hour to ensure accurate binding. Lastly, DAPI is used as a counterstain to stain the nuclei of the cells. Both the secondary antibody and DAPI are light sensitive so all steps following washing off the primary antibody need to be performed in the dark.

Imaging

Following alcian blue and IF staining, the slides need to be imaged with a microscope in order to analyze the results. For each slide, alcian blue images are taken at 10x and 20x magnification. For this staining no light is filtered out and white light is used. For IF, images are taken at 10x magnification with both purple and green light. Part of the light is filtered out and the purple light captures the DAPI staining, while the green light captures whatever protein is being tested. In this case that is RUNX2 and COLX.

Results

At day 28, the shRUNX2 and scramble gene circuit successfully underwent chondrogenesis and produced comparable sGAG amounts to the WT by day 28. When looking at the DMMB results

in Figure 3, you can see that there was no significant difference between any of the samples and the WT, so we can conclude that the transduction of the gene circuit and the inhibition of RUNX2 did not impact the cells normal sGAG production.

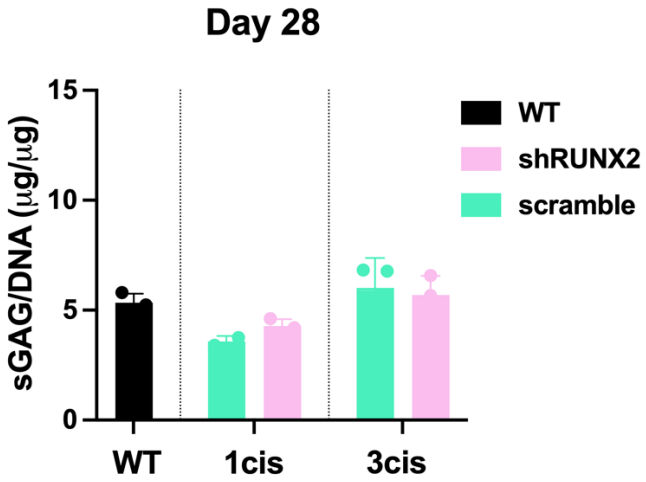

Figure 3. Quantification of sulfated glycosaminoglycan (sGAG) via dimethylmethylene blue assay at day 28.

The alcian blue imaging results agree with that of the DMMB at day 28. Figure 4 shows similar levels of sGAG in the ECM of the modified MdChs when compared to the WT, as they share similar amounts of blue staining.

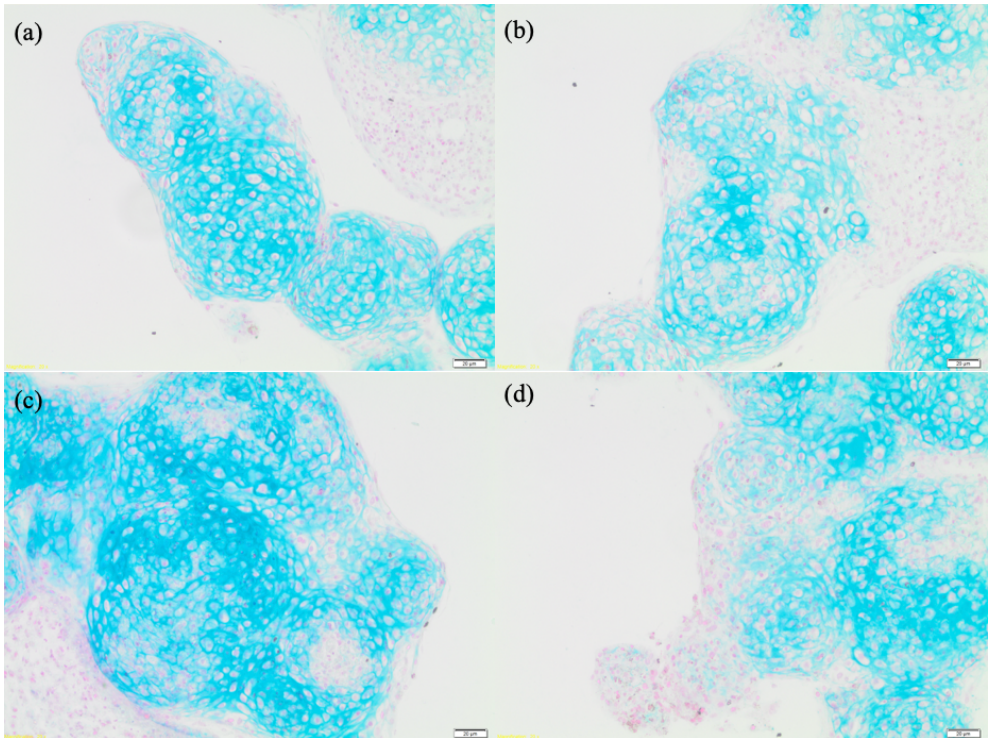

Figure 4. Alcian blue visualization of sulfated glycosaminoglycan (sGAG) levels at day 28. (a) WT (b) 3cis scramble (c) 1cis shRUNX2 (d) 3cis shRUNX2, scale bar: 100 µm.

An increase in sGAG accumulation by the WT, scramble and shRUNX2 modified mMdChs was observed from D28 to D56. Comparing the DMMB results in Figure 3 and Figure 5, one can see a significant increase in sGAG concentration across all samples. At D28 they are in the range of 5 μg/μg, and at D56 they are in the range of 10 μg/μg. Additionally, the 3cis shRUNX2 modified mMdChs produced a significantly higher amount of sGAG compared to the WT by day 56 (Figure 5).

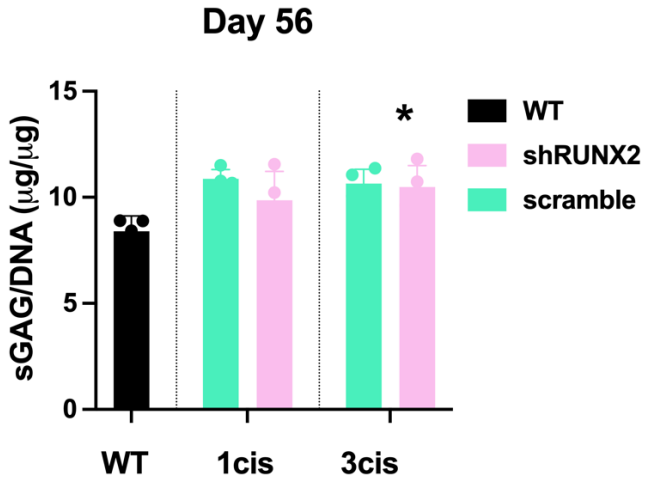

Figure 5. Quantification of sulfated glycosaminoglycan (sGAG) via dimethylmethylene blue assay at day 56. Significance was determined using one-way ANOVA where * represents $p<0.05$.

The 3cis shRUNX2 modified mMdChs exhibited more intense sGAG staining compared to the WT in the long-term culture (Figure 6). The staining for the 3cis shRUNX2 appears to be a much deeper or brighter blue than that of the WT, demonstrating an ECM more densely populated with sGAG.

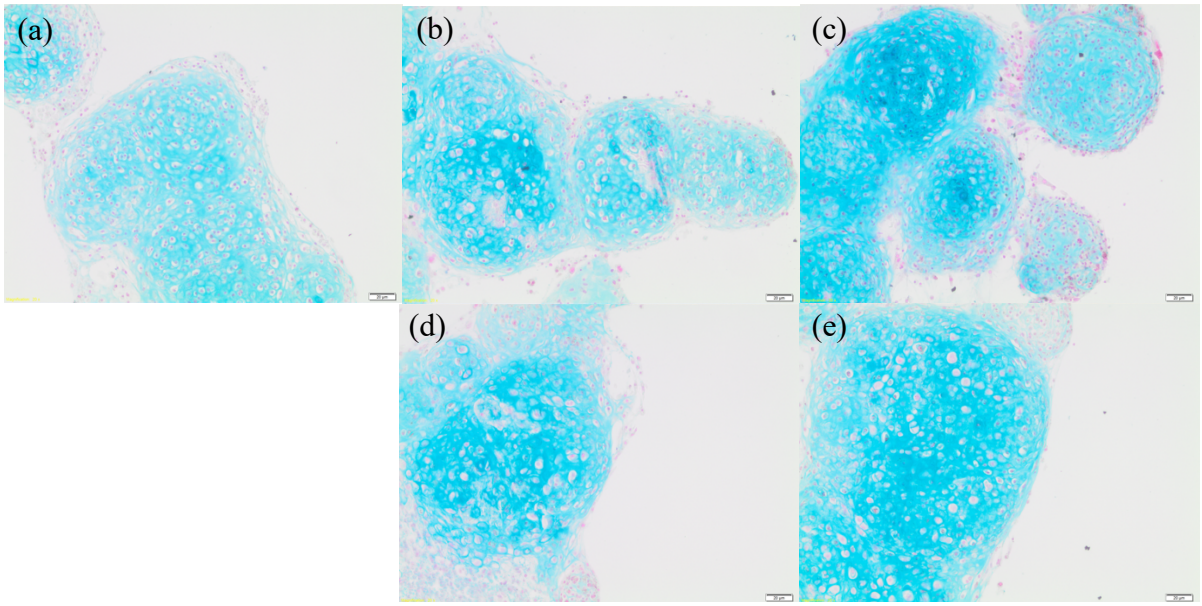

Figure 6. Alcian blue visualization of sulfated glycosaminoglycan (sGAG) levels at day 28. (a) WT (b) 1cis scramble (c) 3cis scramble (d) 1cis shRUNX2 (e) 3cis shRUNX2, scale bar: 100 μm.

To analyze whether our gene circuit actually suppressed RUNX2 expression in mMdChs, we performed immunofluorescence (IF) analysis. IF staining for RUNX2 protein expression demonstrated successful suppression of RUNX2 by both the 1cis and 3cis shRUNX2 circuits when compared to the WT (Figure 7). The 1cis and 3cis shRUNX2 samples show a much lighter green staining, meaning that there is less RUNX2 present in those samples.

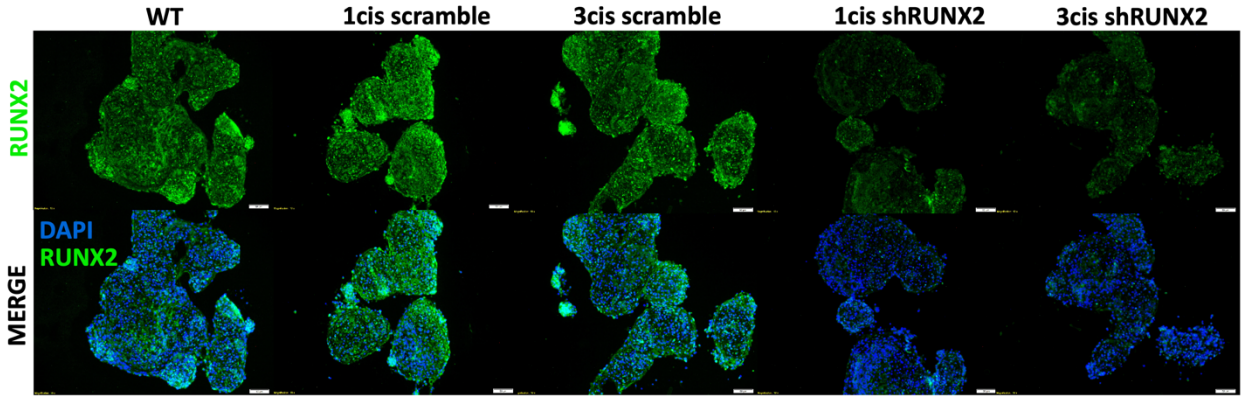

Figure 7. Successful suppression of RUNX2 modified mouse MdCh pellets. Immunofluorescence staining of RUNX2(green) in mMdCh pellets. Merge images are superimposed RUNX2 (green) and nuclear stain DAPI (blue), scale bar: 50 μm.

Next, immunofluorescence for protein expression of hypertrophic marker COLX was performed as it's expression is directly driven by RUNX2 and is known to be upregulated during matrix degradation and inflammation. We observed lower COLX expression in both 1cis and 3cis shRUNX2 modified mMdCh pellets compared to WT controls (Figure 8).

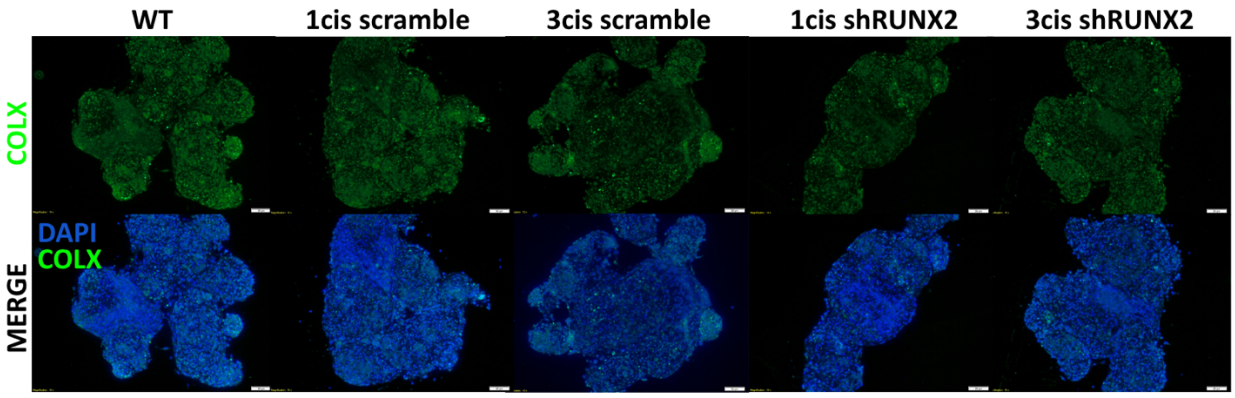

Figure 8. Decreased levels of COLX was not seen in modified mouse MdCh pellets. Immunofluorescence staining of COLX (green) in mMdCh pellets. Merge images are superimposed COLX (green) and nuclear stain DAPI (blue), scale bar: 50 μm.

Discussion and Conclusions

The instability of the chondrogenic phenotype has been a big obstacle for the cartilage regeneration community, when it comes to the clinical application of MSC derived, engineered cartilage. While RUNX2 is the main driver in hypertrophic maturation, we have previously shown that it is necessary for early chondrogenesis and cannot be constitutively suppressed [9]. In addition to this, we have previously shown the success of the auto-regulatory RUNX2 suppressing circuit at stabilizing the chondrogenic phenotype and protecting human MdChs from inflammation-induced matrix catabolism in vitro [4]. In this study we confirmed successful suppression of RUNX2 expression in mouse MSCs in vitro. Our results demonstrated that modifying mMSCs with the RUNX2 suppressing gene circuit did not negatively impact chondrogenesis and matrix accrual. Fifty-six days of culture showed that modifying mMSCs with the RUNX2 suppressing gene circuit increased sGAG accumulation, indicating an increase in the stability of mMdChs chondrogenic phenotype. The immunofluorescence staining also demonstrated that the shRUNX2 gene circuit successfully suppresses RUNX2, by showing decreased levels of RUNX2 along with decreased levels of the hypertrophic marker COLX. Collectively this data suggests that the engineered RUNX2 gene circuit modified mouse MdChs successfully inhibits RUNX2 and that the cartilage-specific phenotype can be achieved from mouse MSCs while preventing chondrocyte hypertrophy.

The auto-regulatory RUNX2 suppressing circuit allows for cells to self-regulate RUNX2 expression based on the intracellular concentration of RUNX2. This establishes a negative feedback loop that allows for low concentrations of RUNX2 and initiates the suppression of RUNX2 after the cells have successfully undergone chondrogenesis. This study shows that the negative feedback loop created by the gene circuit was equally successful in mouse MSC derived chondrocytes at suppressing RUNX2 without interfering with chondrogenesis, as it was in human MSC derived chondrocytes.

As the endochondral ossification pathway continues and chondrocytes progress to hypertrophic maturation, chondrogenic markers are downregulated and hypertrophic markers, including COLX, are upregulated by RUNX2. This study showed that with the inhibition of RUNX2 there is a

decrease in the levels of hypertrophic markers, thus contributing to stabilizing the chondrogenic phenotype.

The clinical application of MSC derived cartilage is highly dependent on the ability of human MSC derived chondrocytes to resist hypertrophic maturation. In this study we showed that the auto-regulatory RUNX2 suppressing gene circuit is successful at achieving this in mouse MSC derived chondrocytes. With this we are able to accept our hypothesis that the suppression of RUNX2 activity, via the engineered gene circuit used in vitro in human MdChs, will be equally successful at inhibiting RUNX2 and prevent hypertrophic maturation in vitro in mouse MdChs. From here we can move forward and test the efficacy of the circuit using in vivo mouse models of post traumatic osteoarthritis.

References

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