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Use of Antisense Oligonucleotides to Target Notch2 in Mouse Chondrocytes

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ABSTRACT

NOTCH2 is a transmembrane receptor that is part of the Notch receptor family, known for controlling cell differentiation and function. Notch receptors play a crucial role in skeletal development and bone homeostasis. Hajdu Cheney Syndrome (HCS) is a rare monogenic disorder affecting the skeleton caused by a gain-of-function mutation in NOTCH2. Antisense oligonucleotides (ASO) are sequence-specific single-stranded nucleic acids that bind to target mRNA and initiate mRNA degradation. While previous work has explored the role of Notch2 ASOs in osteoblasts and osteoclasts, this paper explores the role of *Notch2* and Notch2 ASOs in cells of cartilage tissue. The effect of Notch2 ASOs on *Notch2* were tested in chondrocytes of a murine model of HCS (*Notch2*$^{tm1.1Ecan}$) in vitro and in vivo. Notch2 ASO downregulated *Notch2* in chondrocytes of both *Notch2*$^{wt/wt}$ mice and *Notch2*$^{tm1.1Ecan}$ mice. Notch target genes *Hes1, Hey1, Hey2,* and *HeyL* were amplified in *Notch2*$^{tm1.1Ecan}$ mice, indicating the presence of the HCS gain-of-function *Notch2* mutation. Notch2 ASOs significantly downregulated *Notch2* and Notch target genes in chondrocytes from *Notch2*$^{tm1.1Ecan}$ mice. The mutant gene *Notch2*$^{6955C>T}$ was only detected in *Notch2*$^{tm1.1Ecan}$ mice and was downregulated by a mutant specific *Notch2*$^{6955C>T}$ ASO. In conclusion, Notch2 ASOs successfully downregulate *Notch2, Notch2*$^{6955C>T}$, and Notch target genes in mouse chondrocytes.

INTRODUCTION

Notch signaling is fundamental to many different tissues. Gain or loss of elements of Notch signaling may result in a number of human disorders, including developmental diseases, immune disorders, cancer, and skeletal diseases (1,2). In mammals, there are four Notch transmembrane receptors (Notch1-4), all expressed in skeletal cells to varying degrees. NOTCH1 and NOTCH2

are known to be expressed by cells of the osteoblast and osteoclast lineage, and NOTCH3 is expressed by osteoblasts and osteocytes, but not by cells of the osteoclast lineage. NOTCH4 is expressed in skeletal cells to a lesser extent and its functions are not as well known (3). NOTCH1 inhibits, while NOTCH2 induces, osteoclastogenesis and bone resorption (4). Osteoclastogenesis is the development of osteoclasts from macrophages, and bone resorption is the process in which osteoclasts break down the tissue in bones and release the minerals to blood (5). NOTCH2 also impairs osteoblast maturation, which are responsible for bone formation (3). NOTCH3 induces osteoclastogenesis by indirect mechanisms (6). Notch receptors are activated when interacting with one of the five transmembrane ligands on a neighboring cell (Jagged1, Jagged2, Delta-like1, Delta-like3, and Delta-like4). This paper focuses on NOTCH2 and uses JAGGED1 to induce Notch activation. JAGGED1, encoded by *JAG1*, is a Notch ligand that is prevalent in skeletal cells. JAGGED1 binds to and activates NOTCH2, among other Notch receptors (3, 7, 8).

Notch are transmembrane receptors with a complex structure. The extracellular domain is responsible for interacting with Jagged or Delta-like ligands. In between the extracellular domain and the transmembrane domain is the negative regulatory region (NRR), a site where cleavage occurs after activation. The Notch intracellular domain (NICD) contains nuclear localization sequences, required to regulate transcription (3). On the C-terminus of Notch lies the proline- (P), glutamic acid- (E), serine- (S) and threonine- (T)- rich (PEST) domain (13). The PEST domain is targeted by ubiquitin ligases for the proteasomal degradation of Notch (1). Each Notch receptor carries out distinct functions in skeletal and non-skeletal cells. This is due to different patterns of cellular expression, variation in the affinity of the Notch extracellular domain to the Delta-like and

Jagged ligands, and variation in the NICD and hence diversified interaction between the NICD and its nuclear targets.

Notch receptors are activated upon short-range cell-cell interaction with Jagged or Delta-like ligands. Upon activation, the Notch receptor undergoes two proteolytic cleavage events. The first is catalyzed by the ADAM-family of metalloproteases, whereas the second cleavage is catalyzed by γ-secretase, releasing the NICD (9). The NICD is then released into the cell where it translocates to the nucleus to associate with DNA-bound proteins (1). In the absence of a Notch signal and hence NICD, the recombination signal-binding protein for immunoglobulin kappa J (RBP-Jκ) represses Notch target genes through the recruitment of corepressor complexes (2). The NICD associates with RBP-Jκ and mastermind (MAML) to activate transcription of Notch target genes (10, 11). After transcription is activated, the NICD is released and degraded by cell proteosomes.

Hairy/Enhancer of split family genes (*Hes*) and *Hes*-related with YRPW motif (*Hey),* are well-established Notch target genes expressed in multiple cell types that encode transcription factors (12). There are seven *Hes* proteins (*Hes1-7*), and all of them except *Hes2* and *Hes3* are targets of the Notch canonical signaling pathway. There are three Hey transcription factors (*Hey1, Hey2,* and *HeyL*), all targets of Notch (4). The Notch target genes that are analyzed in this paper are *Hes1, Hey1, Hey2,* and *HeyL*. *Hes1* preserves the undifferentiated state of precursor cells during development and throughout cell life. *Hes1, Hey1, Hey2,* and *HeyL* have all been studied in the skeleton and cartilage and have been shown to be expressed during chondrogenesis and chondrocyte hypertrophy (13, 14).

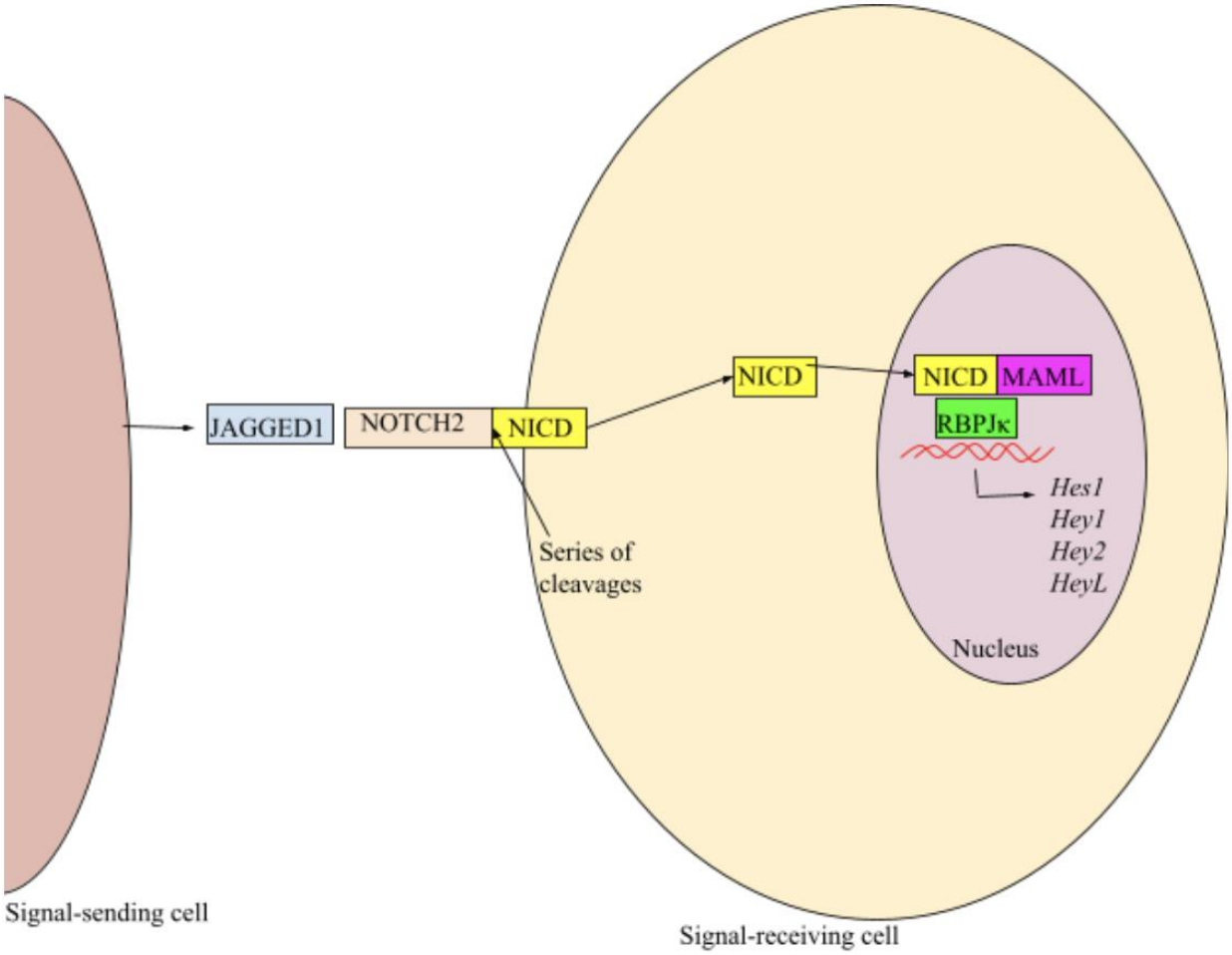

Figure 1. The Notch canonical signaling pathway, shown with NOTCH2. JAGGED1 binds to NOTCH2 receptor, releasing the NICD into the cell. The NICD interacts with RBPJ and MAML to enhance transcription of target genes *Hes1*, *Hes4-7*, and *Hes*-related family *Hey1, Hey2, and HeyL*. The Notch target genes shown in this figure and investigated in the paper are *Hes1, Hey1, Hey2, and HeyL*. *Note:* This is not a comprehensive diagram of all of the components involved in the Notch signaling pathway, but rather the key points as described in this paper.

Hajdu Cheney Syndrome (HCS) is a monogenic disorder caused by gain-of-function *NOTCH2* pathogenic variants. HCS is a disease in humans characterized by osteoporosis, bone fractures, acro-osteolysis, wormian bones, and craniofacial developmental defects (15). As it is caused by an overactive NOTCH2 protein, HCS causes increased osteoclastogenesis and bone resorption in

mice (16). HCS is inherited as autosomal dominant and is associated with a truncated NOTCH2 protein. The mutant NOTCH2 protein is truncated directly upstream of the sequences coding for the PEST domain, therefore creating a functional protein with a constitutively active NICD (17). Without the PEST domain, the NICD is not degraded by cell proteosomes and is stable within the cell, therefore gain-of-function. A mouse model heterozygous for this HCS mutation has been previously created and termed *Notch2*$^{tm1.1Ecan}$. The mouse line harbors a 6955C>T substitution in *Notch2* (7). This nucleotide substitution codes for a Q2319X amino acid change (a stop codon) in the sequence upstream of the PEST domain and resulting in a truncated protein of 2318 amino acids (17).

Antisense oligonucleotides (ASO) are short and single-stranded nucleotides that alter RNA by binding to RNA sequences directly and inducing RNA degradation by RNase H, therefore modifying protein expression (18, 19, 20). Notch2 antisense oligonucleotides have been shown to downregulate *Notch2* in cells of the spleen, kidney, and femur from *Notch2*$^{wt/wt}$ mice. In subsequent experiments, the Notch target genes *Hes1, Hey1, Hey2,* and *HeyL* were shown to be induced in bone extracts from mutant *Notch2*$^{tm1.1Ecan}$ mice and suppressed upon subcutaneous administration of Notch2 ASO (21).

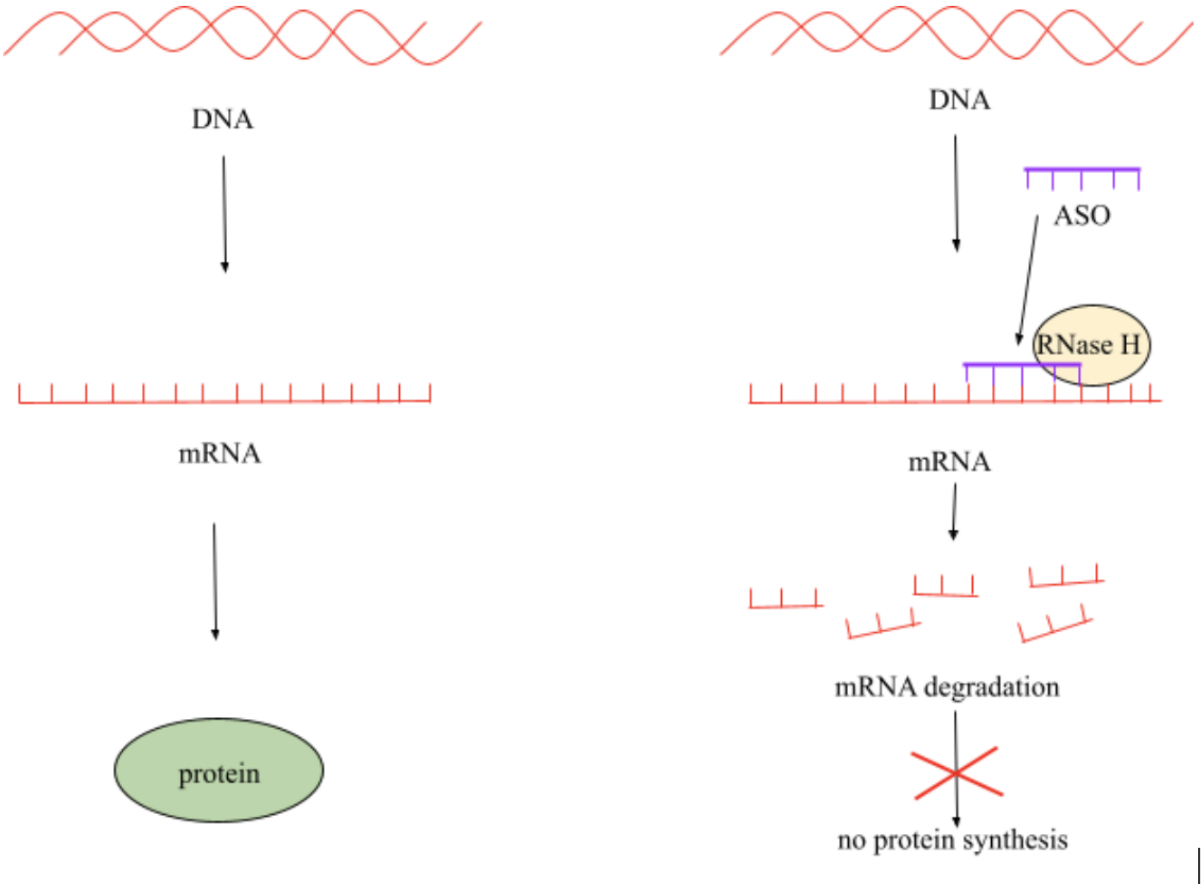

Figure 2. The process by which antisense oligonucleotides (ASO) facilitate mRNA degradation through recruitment of RNase H.

Chondrocytes are specialized cells that function to produce, sustain, and degrade the cartilage extracellular matrix (22). Chondrogenesis is the process by which cartilage is formed: mesenchymal cells, a type of multipotent stem cell, differentiate into chondrocytes. (23). Chondrocytes in the hyaline cartilage undergo proliferation, hypertrophy, and apoptosis to be gradually replaced by bone in a process known as endochondral ossification. The cartilaginous scaffold that results from this allows for osteoblasts to replace the cartilage with bone. This process forms the appendicular skeleton and parts of the axial skeleton. Bone is formed and remodeled throughout one's lifetime through coordination of osteoblasts and osteoclasts (24). Notch signaling

plays a key role in the life cycle of chondrocytes. Notch signaling has been shown to suppress chondrogenesis and promote the onset of chondrocyte hypertrophy, and it was shown that Notch2 controls the inhibitory effects of Notch on skeletal development (14, 25). Additionally, NOTCH2 gain-of-function has been documented in femoral heads of Notch*2*$^{tm1.1Ecan}$ mice and was shown to sensitize *Notch2*$^{tm1.1Ecan}$ mice to osteoarthritis, a disease characterized by cartilage degradation (26).

Because Notch signaling is involved in chondrocyte maturation and degradation, we asked the question as to whether Notch2 ASO would downregulate *Notch2* and Notch target genes in chondrocytes from *Notch2*$^{tm1.1Ecan}$ mice. Successful downregulation of *Notch2* in mice harboring HCS could help to correct the effects of the gain-of-function HCS mutation. The effects of Notch2 ASO treatment on *Notch2*, *Notch2*$^{6955C>T}$, and Notch target genes *Hes1*, *Hey1*, *Hey2*, and *HeyL*, were explored in chondrocytes from *Notch2*$^{tm1.1Ecan}$ mice and *Notch2*$^{wt/wt}$ mice in vitro and in vivo.

METHODS

Notch2$^{tm1.1Ecan}$ Mice

Notch2$^{tm1.1Ecan}$ mice were created previously in a C57BL/6 background to harbor a point 6955C>T mutation in *Notch2*. *Notch2*$^{tm1.1Ecan}$ mice were crossed with wild type C57BL/6 mice for a yield of 50% heterozygous mutant and 50% wild type newborns. Newborn mice were tattooed on feet for identification and their tails were snipped for genotyping. DNA was extracted from the tails using the HotSHOT DNA extraction method (27). Genotyping was conducted in tail DNA extracts by polymerase chain reaction (PCR) and the products were resolved through gel electrophoresis and visualized under UV light. For genotyping, forward primer *Notch2Lox* gtF 5’-

CCCTTCTCTCTGTGCCGTAG -3' and reverse primer *Notch2Lox* gtR 5'-CTCAGAGCCAAAGCCTCACTG -3' were used. (Integrated DNA Technologies; IDT, Coralville, IA).

Chondrocyte Cultures

The joints were dissected from the hind limbs of both male and female three- to four-day-old *Notch2*$^{tm1.1Ecan}$ mice and *Notch2*$^{wt/wt}$ mice in phosphate-buffered saline (PBS), and placed in Dulbecco's Modified Eagle Medium (DMEM, Life Technologies, Waltham, MA). The chondrocyte-enriched cells were trypsinized in 0.25% trypsin-EDTA at 37°C for 40 minutes, and then incubated while rotating in 200 units/mL of collagenase type 2-DMEM mix (Worthington Biochemical Corporation, Lakewood, NJ) for 2 hours at 37°C, as described (26). The digested cartilage was homogenized, strained through a 70 µM membrane, and centrifuged for 5 minutes. Cells were suspended in DMEM with 10% fetal bovine serum (FBS) and 100 µg/mL ascorbic acid (Sigma-Aldrich, St. Louis, MO) at 37°C and cultured in a 5% CO_2 atmosphere, as described (26). The culture was confirmed to consist of chondrocytes because gene markers characteristic of chondrocytes were present, such as Type 2 collagen, type 10 collagen, and aggrecan. Cells were then seeded on plates coated with JAGGED1 to activate Notch receptors. Either control ASO, Notch2 ASO, or *Notch2*$^{6955C>T}$ ASO (Ionis Pharmaceuticals, Carlsbad, California) were added to the culture medium in varying amounts of dose and time, as stated in figure legends.

***Notch2* Expression In Vivo**

Control and Notch2 ASOs were injected subcutaneously at 50 mg/kg into 1–2-month-old *Notch2*$^{wt/wt}$ mice and *Notch2*$^{tm1.1Ecan}$ mice. The animals were sacrificed 72 hours later, femoral

heads were isolated, and RNA was subsequently extracted and reverse-transcribed in the presence of specific primers for *Notch2* and *Notch2*$^{6955C>T}$, as described (21).

RNA Integrity and Real Time Reverse Transcription Polymerase Chain Reaction

RNA was extracted from cell cultures with the RNeasy kit (Qiagen, Valencia, CA) and RNA concentrations were measured via nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA). The RNA integrity of a random sample from each group was assessed with a Bio-Rad Experion microfluidic electrophoresis system (Bio-Rad, Hercules, CA). All RNA that was analyzed had a quality indicator number higher than 9.0. RNA was treated with DNase and subsequently reverse-transcribed using the iScript RT-PCR kit (Bio-Rad). Once converted to cDNA, the samples were amplified in the presence of specific primers (Table 1) and SYBR green in a Bio-Rad CFX96 thermal cycler. Copy number was estimated by comparison to a standard curve of a serial dilution of cDNA for *Notch2* (from Open Biosystems, Huntsville, AL), *Hes1* (from American Type Culture Collection; ATCC, Manassas, VA), *Hey1* and *Hey2* (from T. Iso, Los Angeles, CA), and *HeyL* (from D. Srivastava, Dallas, TX). Copy number was corrected for expression of *Rpl38*. *Rpl38* cDNA was obtained from ATCC. To determine the expression of the mutant transcript, DNase-treated RNA was reverse-transcribed in the presence of specific primers for the mutant transcript, with a 6955C>T substitution in *Notch2*, and with SYBR Universal Probe and custom TaqMan assay probe, as described (7). The *Notch2*$^{6955C>T}$ transcript copy number was measured and corrected for *Rpl38* expression. Fluorescence was monitored during every PCR cycle at the annealing step, as described (26).

Gene	Strand	Sequence 5'-3'	GenBank Accession Number
Notch2	Forward	5'-TGACGTTGATGAGTGTATCTCCAAGCC-3'	BQ186939.1
	Reverse	5'- GTAGCTGCCCTGAGTGTTGTGG -3'	
Rpl38	Forward	5'-AGAACAAGGATAATGTGAAGTTCAAGGTTC-3'	NM_001048057; M_001048058; NM_023372
	Reverse	5'-CTGCTTCAGCTTCTCTGCCTTT-3'	
Hey1	Forward	5'-ATCTCAACAACTACGCATCCCAGC -3'	NM_010423
	Reverse	5'-GTGTGGGTGATGTCCGAAGG-3'	
Hey2	Forward	5'-AGCGAGAACAATTACCCTGGGCAC-3'	NM_013904
	Reverse	5'-GGTAGTTGTCGGTGAATTGGACCT-3'	
HeyL	Forward	5'-CAGTAGCCTTTCTGAATTGCGAC-3'	NM_013905
	Reverse	5'-AGCTTGGAGGAGCCCTGTTTC -3'	
Hes1	Forward	5'-ACCAAAGACGGCCTCTGAGCACAGAAAGT-3'	NM_008235
	Reverse	5'-ATTCTTGCCCTTCGCCTCTT-3'	

Table 1. Primers used for qRT-PCR determinations.

Statistics

Data are expressed as means ± standard deviation (SD), and statistical differences were determined by unpaired t-test for Figures 3, 4, 5, 7, and 8, and by two-way ANOVA with Tukey's multiple comparisons test for Figure 6.

RESULTS

Effect of Notch2 ASOs on *Notch2* and *Notch2*$^{6955C>T}$ Expression in Chondrocytes

To determine the most effective duration of treatment, a time course experiment was conducted. In cells from *Notch2*$^{tm1.1Ecan}$ mice, 10 µM control or Notch2 ASO were administered, and cells cultured for 24 to 96 hours after the addition of the ASO. *Notch2* mRNA expression increased when cells were cultured for 72 hours in the presence of control ASO compared to cells cultured for 24 hours. Addition of Notch2 ASO prevented the increase in *Notch2* expression so that Notch2 ASO inhibited *Notch2* expression with an effect that was maximal after 72 hours, proven significant by unpaired t-test (Figure 3).

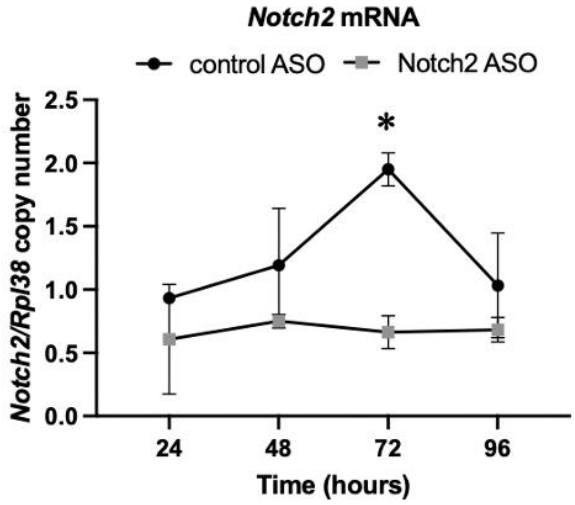

Figure 3. Time course experiment. Effect of 10 µM control or Notch2 ASO in chondrocytes from *Notch2*$^{tm1.1Ecan}$ mice cultured on JAGGED1-coated plates for varying time periods. Data are shown as *Notch2* copy number corrected for *Rpl38* expression. Values are means ± SD; n=3. * Significantly different between the same dose of control ASO and Notch2 ASO after a certain elapsed time, $p < 0.05$ by unpaired t-test.

To determine the effective dose of Notch2 ASOs, doses of 0, 5, 10, and 20 µM control and Notch2 ASO were added to chondrocyte cultures from *Notch2*$^{tm1.1Ecan}$ mice for 72 hours. Control ASO did not have an effect on *Notch2* expression. Administration of Notch2 ASO at doses of 5, 10, and 20

µM to cells of *Notch2*$^{tm1.1Ecan}$ mice significantly decreased *Notch2* expression, with a maximal decrease at a dose of 20 µM (Figure 4A). *Notch2*$^{tm1.1Ecan}$ chondrocytes, cultured with 20 µM of Notch2 ASO, showed significantly decreased expression of the HCS mutant transcript, *Notch2*$^{6955C>T}$, compared to cells cultured with equivalent dose of control ASO (Figure 4B).

A **B**

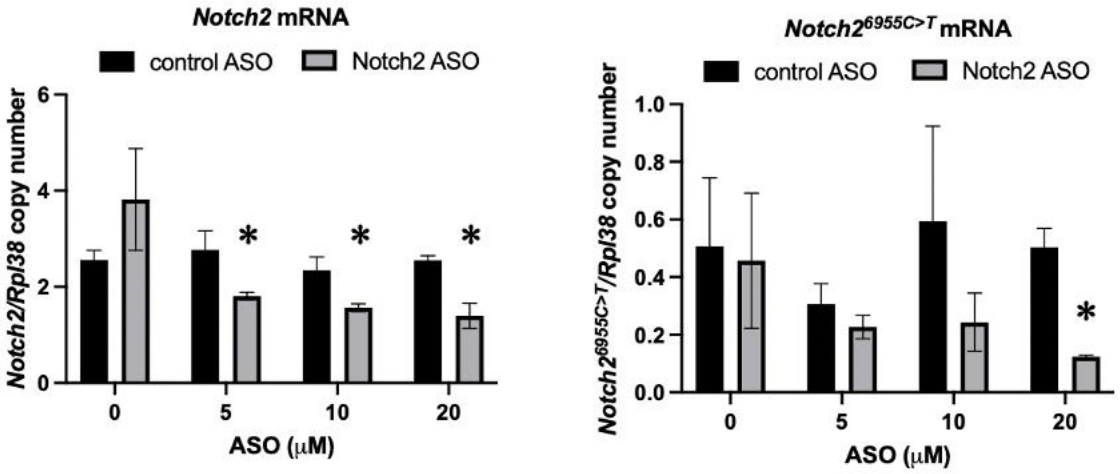

Figure 4. Effect of varying doses of control or Notch2 ASO in chondrocytes from *Notch2*$^{tm1.1Ecan}$ mice cultured on JAGGED1-coated plates for 72 hours. Panel A shows *Notch2* copy number corrected for *Rpl38* expression. Panel B shows expression of the HCS 6955C>T point mutation, *Notch2*$^{6955C>T}$, as copy number corrected for *Rpl38* expression. Values are means ± SD; n=3. * Significantly different between the same dose of control ASO and Notch2 ASO, $p < 0.05$ by unpaired t-test

To compare the effect of Notch2 ASOs on cells from wild type and mutant mice, expression of *Notch2* and *Notch2*$^{6955C>T}$ transcripts were measured in wild type and mutant cells. In chondrocytes from both *Notch2*$^{wt/wt}$ mice and *Notch2*$^{tm1.1Ecan}$ mice, Notch2 ASOs significantly decreased *Notch2* mRNA expression at 20 µM, decreasing by 80% in mutant cells (Figure 5A-B). Chondrocytes isolated from *Notch2*$^{wt/wt}$ mice did not express the HCS mutant transcript, *Notch2*$^{6955C>T}$. In cells from *Notch2*$^{tm1.1Ecan}$ mice, expression of *Notch2*$^{6955C>T}$ decreased by 50% when chondrocytes were treated with 10 µM and 20 µM of Notch2 ASO, compared to control ASO (Figure 5C).

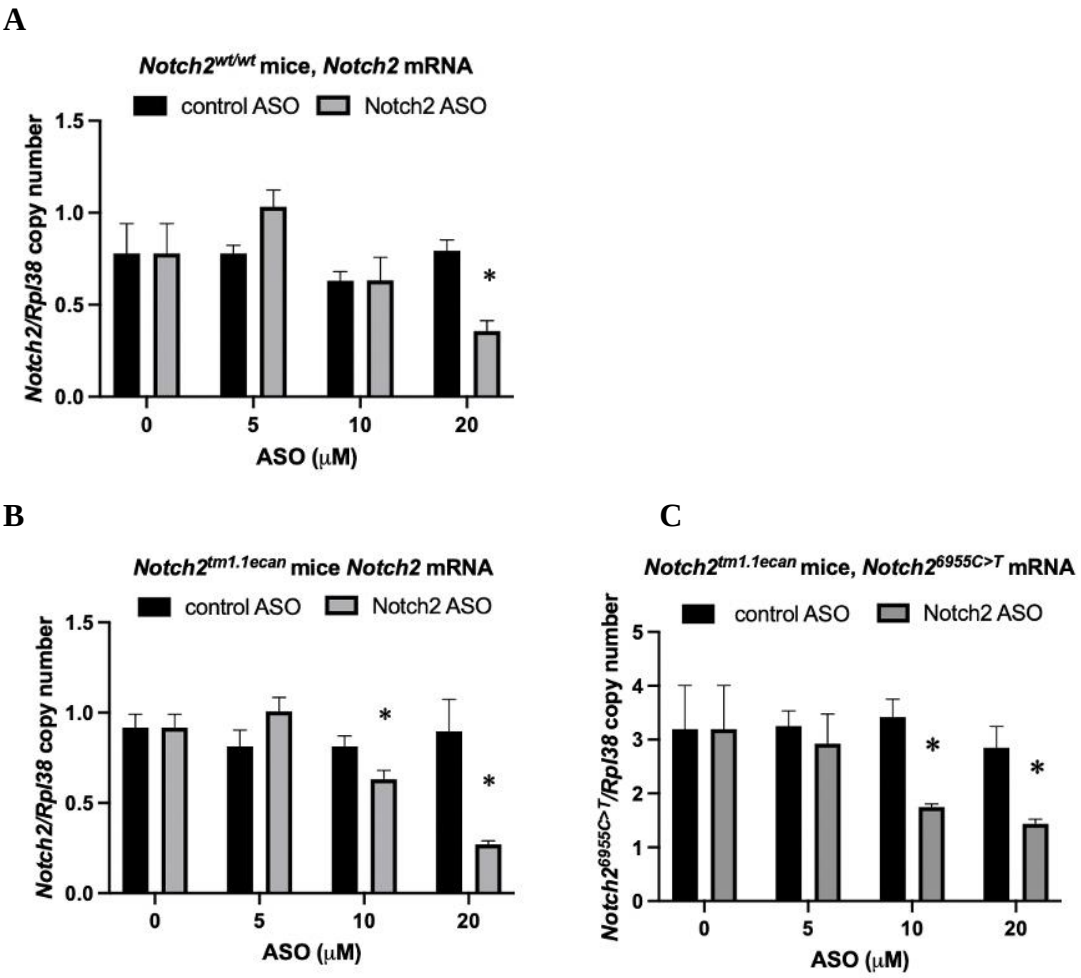

Figure 5. Effect of varying doses of control or Notch2 ASOs in chondrocytes from *Notch2*$^{wt/wt}$ mice and *Notch2*$^{tm1.1Ecan}$ mice cultured on JAGGED1-coated plates for 72 hours. Panel A shows expression of *Notch2* as copy number corrected for *Rpl38* expression in *Notch2*$^{wt/wt}$ mice, Panel B shows expression of *Notch2* as copy number corrected for *Rpl38* expression in *Notch2*$^{tm1.1Ecan}$ mice, and Panel C shows expression of *Notch2*$^{6955C>T}$ as copy number corrected for *Rpl38* expression in *Notch2*$^{tm1.1Ecan}$ mice. *Notch2*$^{6955C>T}$ was not detected in chondrocytes of *Notch2*$^{wt/wt}$ mice. Values are means ± SD; n=3. * Significantly different between the same dose of control ASO and Notch2 ASO, $p < 0.05$ by unpaired t-test.

Effect of Notch2 ASOs on the Expression of Notch Target Genes in Chondrocytes

We asked the question as to whether Notch signaling is present in chondrocytes. Expression of the Notch target genes *Hes1, Hey1, Hey2,* and *HeyL* was significantly increased in *Notch2*$^{tm1.1Ecan}$ chondrocytes, compared to chondrocytes from *Notch2*$^{wt/wt}$ mice when cultured on JAGGED1-coated plates. (Figure 6). Expression of *Hes1, Hey1, Hey2,* and *HeyL* in chondrocytes from

Notch2$^{tm1.1Ecan}$ mice decreased significantly when treated with 20 µM Notch2 ASO (Figures 6A-D).

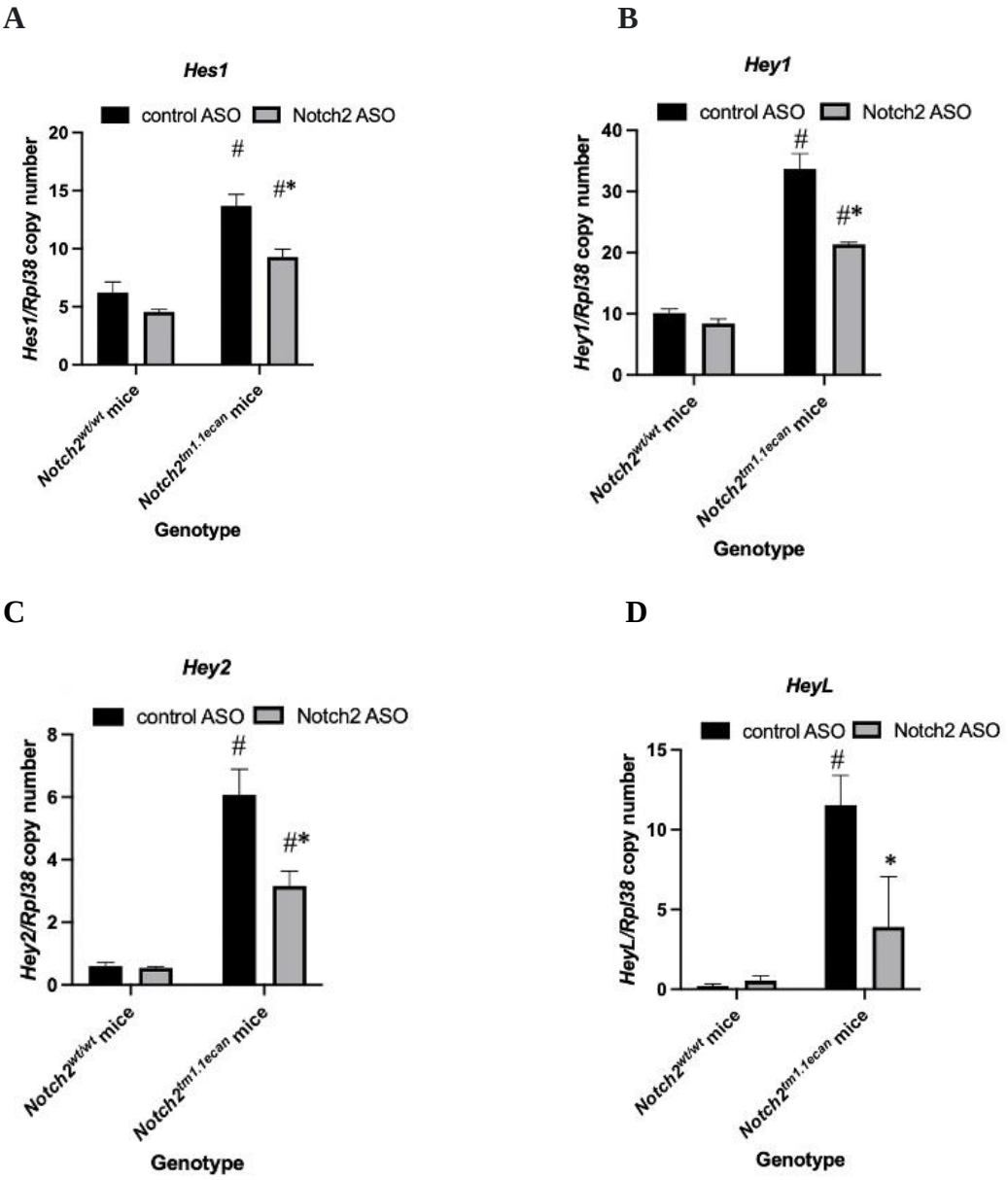

Figure 6. Effect of 20 µM control or Notch2 ASO on *Hes1*, *Hey1*, *Hey2*, and *HeyL* expression in chondrocytes from *Notch2*$^{wt/wt}$ mice and *Notch2*$^{tm1.1Ecan}$ mice cultured on JAGGED1-coated plates for 72 hours. Panels A-D show data expressed as copy number of *Hes1*, *Hey1*, *Hey2*, and *HeyL*, corrected for *Rpl38* expression. Values are means ± SD; n=3. * Significantly different between control ASO and Notch2 ASO, $p < 0.05$; # significantly different between *Notch2*$^{wt/wt}$ mice and *Notch2*$^{tm1.1Ecan}$ mice, $p < 0.05$ by two-way ANOVA with Tukey's multiple comparisons test.

Effect of Mutant-Specific Notch2$^{6955C>T}$ ASO on Mutant *Notch2$^{6955C>T}$* Transcript Expression

Expression of *Notch2* did not change in chondrocytes isolated from *Notch2$^{wt/wt}$* mice and *Notch2$^{tm1.1Ecan}$* mice treated with mutant specific Notch2$^{6955C>T}$ ASO (Figure 7A). In contrast, expression of the mutant transcript, *Notch2$^{6955C>T}$*, significantly decreased by 83% in chondrocytes from *Notch2$^{tm1.1Ecan}$* mice when treated with the mutant specific Notch2$^{6955C>T}$ ASO. As expected, expression of *Notch2$^{6955C>T}$* was not detected in chondrocytes from *Notch2$^{wt/wt}$* mice treated with control or Notch2$^{6955C>T}$ ASO (Figure 7B).

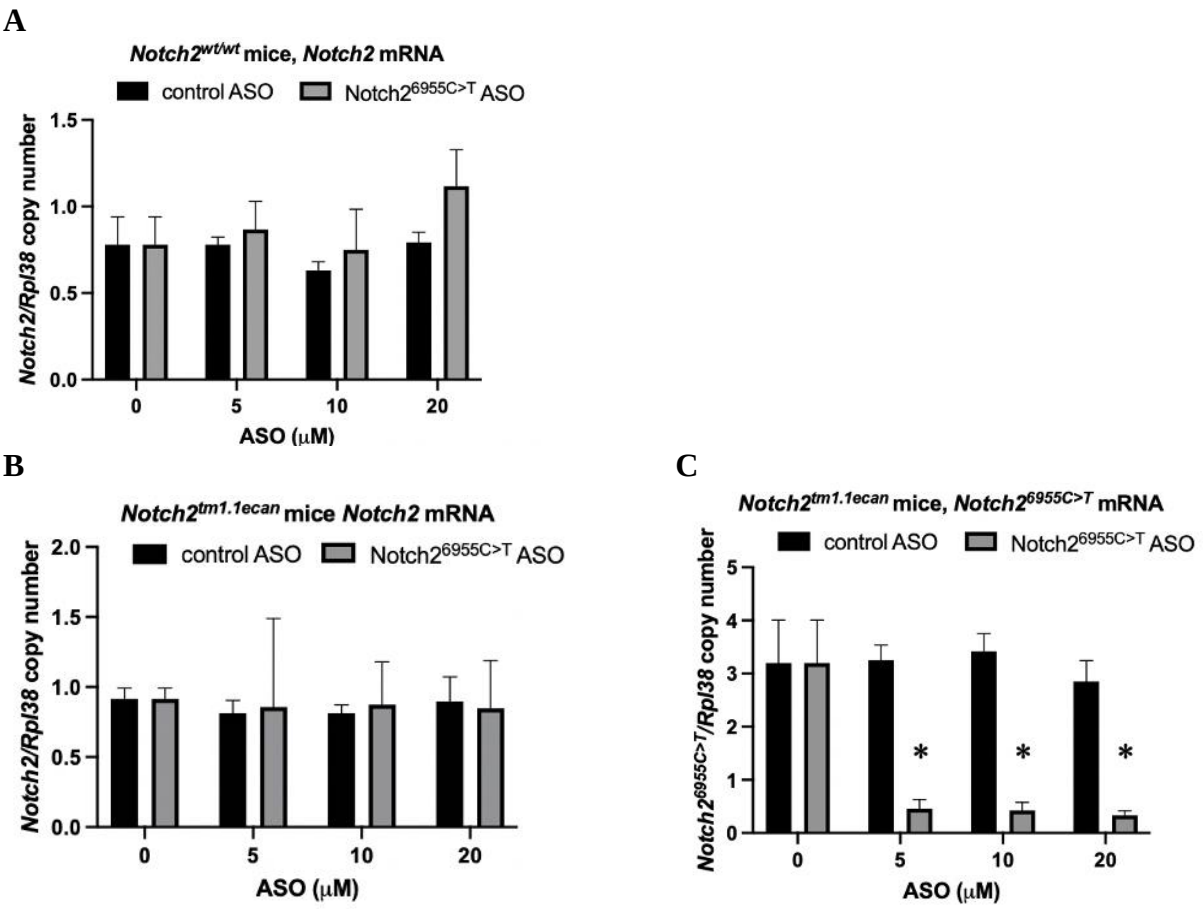

Figure 7. Effect of varying doses of mutant-specific Notch2$^{6955C>T}$ ASO in chondrocytes from *Notch2$^{wt/wt}$* mice and *Notch2$^{tm1.1Ecan}$* mice cultured on JAGGED1-coated plates for 72 hours. Panel A shows expression of *Notch2* copy number corrected for *Rpl38* expression in *Notch2$^{wt/wt}$* mice, Panel B shows *Notch2* copy number corrected for *Rpl38* expression in *Notch2$^{tm1.1Ecan}$* mice, and Panel C shows expression of *Notch2$^{6955C>T}$* copy number corrected for *Rpl38* expression. Values are means ± SD; n=3. * Significantly different between the same dose of control ASO and Notch2 ASO, $p < 0.05$ by unpaired t-test.

Effect of Notch2 ASOs on *Notch2* and *Notch2*$^{6955C>T}$ Expression In Vivo

Expression of *Notch2* mRNA from *Notch2*$^{wt/wt}$ chondrocytes decreased by 22% following subcutaneous injection of Notch2 ASO, although the effect did not reach statistical significance. Expression of *Notch2* mRNA from *Notch2*$^{tm1.1Ecan}$ chondrocytes showed a significant decrease following injection of Notch2 ASO (Figure 8A). Expression of *Notch2*$^{6955C>T}$ mRNA was not detected in cartilage from *Notch2*$^{wt/wt}$ mice, as expected. In *Notch2*$^{tm1.1Ecan}$ mice, expression of *Notch2*$^{6955C>T}$ transcript decreased by 36% when mice were injected with Notch2 ASO compared to control ASO, although the effect did not reach statistical significance (Figure 8B).

A **B**

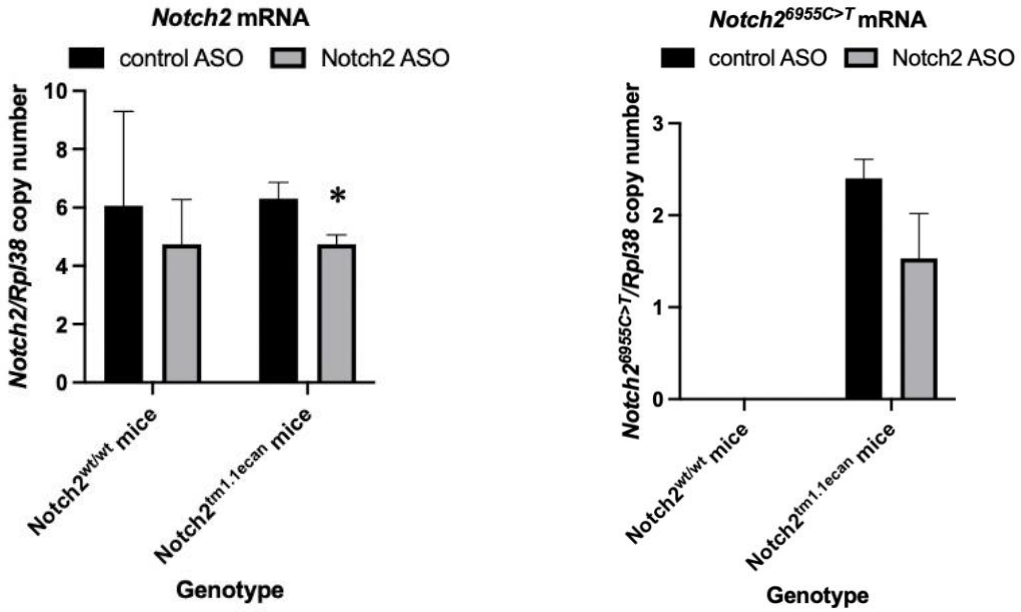

Figure 8. Effect of control and Notch2 ASOs on *Notch2* and *Notch2*$^{6955C>T}$ expression in *Notch2*$^{wt/wt}$ mice and *Notch2*$^{tm1.1Ecan}$ mice in vivo. Data are shown as copy number of *Notch2*, Panel A, and copy number of *Notch2*$^{6955C>T}$, Panel B, corrected for *Rpl38* expression. Values are means ± SD; n=3. * Significantly different between the same dose of control ASO and Notch2 ASO, $p < 0.05$ by unpaired t-test.

DISCUSSION

In this study, we report that wild type Notch2 ASO and mutant specific Notch2 ASO downregulate *Notch2* and *Notch2*$^{6955C>T}$ expression, respectively, in chondrocyte cultures from *Notch2*$^{tm1.1Ecan}$ mice. Notch2 ASO had the same effect on the expression of *Notch2* mRNA in *Notch2*$^{wt/wt}$ mice and *Notch2*$^{tm1.1Ecan}$ mice.

Previous work shows evidence of increased NOTCH2 signaling in cells of osteoblast, osteoclast, and osteocyte cell cultures from *Notch2*$^{tm1.1Ecan}$ mice, and suppression of NOTCH2 activation upon addition of Notch2 ASOs in these cultures (21). We raised the question as to whether Notch signaling was present in cartilage tissue. Expression of the Notch target genes *Hes1, Hey1, Hey2,* and *HeyL* was amplified in *Notch2*$^{tm1.1Ecan}$ mice compared to *Notch2*$^{wt/wt}$ mice, conforming to the gain-of-function NOTCH2 mutation expected in HCS. Notch2 ASO significantly decreased mRNA expression of *Hes1, Hey1, Hey2,* and *HeyL* in *Notch2*$^{tm1.1Ecan}$ mice, although levels of mRNA expression in ASO-treated *Notch2*$^{tm1.1Ecan}$ chondrocytes were not as low as the mRNA levels found in chondrocytes from *Notch2*$^{wt/wt}$ mice. This raises the concern that the Notch2 ASO is not completely effective in targeting the Notch signaling pathway.

Transcripts from the mutant gene *Notch2*$^{6955C>T}$, indicative of HCS, were present in cells from *Notch2*$^{tm1.1Ecan}$ mice, but not in *Notch2*$^{wt/wt}$ mice. Notch2$^{6955C>T}$ mutant ASOs downregulated *Notch2*$^{6955C>T}$ in *Notch2*$^{tm1.1Ecan}$ mice but did not downregulate *Notch2*, indicating that it is specific to the mutant transcript. This is consistent with the results of previous work, which found that *Notch2*$^{6955C>T}$ mRNA was present in osteocyte-enriched cultures only from *Notch2*$^{tm1.1Ecan}$ mice and found that Notch2 ASOs suppressed *Notch2*$^{6955C>T}$ mRNA levels in these cultures (21).

Previous work showed a 50% downregulation of *Notch2* mRNA in the spleen, kidney, femur, and liver after subcutaneous administration of Notch2 ASO (21). In the present work, Notch2 ASOs did not downregulate *Notch2* in cartilage when administered in vivo subcutaneously as efficiently as they did in vitro. This could be due to the limited vascularization of cartilage tissue, making it difficult for the ASO to access this tissue. In an inflammatory disease state, however, systemic administration of ASOs may be more effective, an effect that could be studied in the future.

Additional ways to apply ASOs in vivo are possible. Previous work revealed that mice harboring the HCS mutation are sensitized to osteoarthritis (26). Because of this, ASOs may be effective in vivo if they are applied in a localized fashion. This would directly deliver ASOs to cartilage tissue. Although a systemic application would be more practical, local administration of ASO could be more successful. Also, it may be worth studying the effects of an in vivo ASO applied for a longer duration than 72 hours.

CONCLUSION

In conclusion, gain-of-function NOTCH2 signaling, indicative of HCS, is present in chondrocytes from *Notch2*$^{tm1.1Ecan}$ mice. Notch2 ASOs downregulate *Notch2* mRNA in vitro in chondrocyte cultures from *Notch2*$^{wt/wt}$ mice and *Notch2*$^{tm1.1Ecan}$ mice, and Notch2$^{6955C>T}$ ASOs downregulate *Notch2*$^{6955C>T}$ mRNA in vitro in chondrocyte cultures from *Notch2*$^{tm1.1Ecan}$ mice.

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