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Dwarfism in Homozygous *Agc1^{CreERT}* Mice is Associated with Decreased Expression of Aggrecan

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SUMMARY

Aggrecan (*Acan*), a large proteoglycan is abundantly expressed in cartilage tissue. Disruption of *Acan* gene causes dwarfism and perinatal lethality of homozygous mice. Due to sustained expression of *Acan* in the growth plate and articular cartilage, *Agc^{Cre}* model has been developed for the regulated ablation of target gene in chondrocytes. In this model, the IRES-CreERT-Neo-pgk transgene is knocked-in the 3' UTR of the *Acan* gene. We consistently noticed variable weight and size among the *Agc^{Cre}* littermates, prompting us to examine the cause of this phenotype. Wild-type, Cre-heterozygous (*Agc^{+Cre}*), and Cre-homozygous (*Agc^{Cre/Cre}*) littermates were indistinguishable at birth. However, by 1-month, *Agc^{Cre/Cre}* mice showed a significant reduction in body weight (18–27%) and body length (19–22%). Low body weight and dwarfism was sustained through adulthood and occurred in both genders. Compared with wild-type and *Agc^{+Cre}* littermates, long bones and vertebrae were shorter in *Agc^{Cre/Cre}* mice. Histological analysis of *Agc^{Cre/Cre}* mice revealed a significant reduction in the length of the growth plate and the thickness of articular cartilage. The amount of proteoglycan deposited in the cartilage of *Agc^{Cre/Cre}* mice was nearly half of the WT littermates. Analysis of gene expression indicates impaired differentiation of chondrocyte in hyaline cartilage of *Agc^{Cre/Cre}* mice. Notably, both *Acan* mRNA and protein was reduced by 50% in *Agc^{Cre/Cre}* mice. A strong correlation was noted between the level of *Acan* mRNA and the body length. Importantly, *Agc^{+Cre}* mice showed no overt skeletal phenotype. Thus to avoid misinterpretation of data, only the *Agc^{+Cre}* mice should be used for conditional deletion of a target gene in the cartilage tissue.

Keywords

Cre model; Cartilage; Proteoglycan; Growth plate; Chondrocytes

INTRODUCTION

Formation of a transient cartilaginous structure is required for the development of endochondral bone. Condensation of mesenchyme during embryogenesis initiates progressive differentiation of chondroblast to terminally mature hypertrophic chondrocyte (Long and Ornitz, 2013). Developing chondrocytes deposit collagen and proteoglycan rich

extracellular matrix (ECM), undergo hypertrophy and apoptosis. The cartilage matrix is then resorbed and replaced by mineralized matrix synthesized by osteoblasts (Javed et al., 2010). Proteoglycan constitutes ~7% of the cartilage ECM volume and plays an essential role in the matrix organization, storage of growth factors, chemokine, cytokine, and in establishing a morphogen gradient during tissue development (Knudson C, 1993; Melrose et al., 2016). Proteoglycans also regulate diffusion of nutrients and metabolites through cartilage ECM (Rojas et al., 2014). Mutations in genes involved in the synthesis, assembly, and modification of ECM are associated with chondrodysplasia and other skeletal disorder (Kozhemyakina et al., 2015).

Aggrecan is the most abundant proteoglycan component of ECM in the growth plate and articular cartilage (Gibson and Briggs, 2016). Acan protein consists of a 250 kDa core with approximately 100 chondroitin sulfate glycosaminoglycan and 30 keratan sulfate chains (Gibson and Briggs, 2016). Presence of sulfate ions in proteoglycan imparts a high negative charge that is critically important for both structure and function of cartilage tissue. The negative ions in proteoglycan attract huge amount of water molecules and thereby provide osmotic properties to the cartilage tissue against high mechanical compression in the skeletal joint (Horkay et al., 2012). The Acan aggregates facilitate the organization and assembly of collagen and remodeling of ECM in the cartilage tissue (Kiani et al. 2002). Healthy and proper functioning of articular cartilage require a high amount of sulfonated aggrecan to form aggregates. The size of Acan aggregates is decreased in both aging and osteoarthritis (Stern et al., 2007; Bayliss et al., 2000).

Aggrecan interacts with cell surface receptors and plays important roles in the cell-ECM cross talk, storage and release of signaling factors, cell division and migration (Mouw et al., 2014). For example, hyaluronan in the Acan aggregates interacts with CD44 and syndecan receptor expressed on the surface of chondrocytes to regulate cartilage homeostasis (Knudson et al. 2000; Bishop et al., 2007). Acan also binds with a number of ECM proteins such as tenascin, fibulin, and fibrillin to regulate the functional stability of matrix (Miura et al. 1999). The missense mutation in human *Acan* gene, that disrupts Acan-tenascin interaction, causes spondyloepimetaphyseal dysplasia characterized by short stature and premature osteoarthritis (Gleghorn et al., 2005; Thompson et al. 2009). In mice, the homozygous frameshift mutation in the *Acan* gene leads to perinatal lethality and a cartilage matrix deficiency phenotype, characterized by cleft palate and dwarfism (Watanabe et al., 1994). Heterozygous mice survive but are dwarf and develop herniation and degeneration of vertebral discs with age (Watanabe et al., 1997). Thus, the structural and functional integrity of cartilage matrix largely depends on quantity and quality of Acan protein.

LoxP-Cre system is routinely used for cell-type specific ablation of a target gene *in vivo*. Traditional transgenic mice created by random insertion are fraught with an aberrant and poor expression of Cre due to differences in site of integration and copy-number (Eleftheriou and Yang; 2011). To circumvent these problems, new Cre-lines are being developed by homologous recombination whereby Cre expression is under the control of an endogenous gene promoter. In the tamoxifen inducible $Agc^{tm}(IRES-CreERT-Neo-pgk)$ model, defined herein as (Agc^{Cre}), a 3.8 kb transgene is knocked-in the 3' UTR of the endogenous *Acan* gene (Henry et al., 2009). The IRES-CreERT-Neo-pgk cassette contains sequences for internal

ribosomal entry site, Cre recombinase fused with the estrogen receptor, SV40 polyadenylation signal and neomycin resistant gene controlled by pgk promoter (Fig. S1). In the Agc^{Cre} model, endogenous aggrecan promoter transcribes a bicistronic mRNA containing coding sequences of aggrecan and Cre gene. Presence of IRES ensures synthesis of two separate proteins from the bicistronic mRNA. Tamoxifen treatment results in nuclear translocation of the Cre-ERT fusion protein and subsequent excision of a floxed gene. Several studies have utilized the Agc^{Cre} model to investigate the role of target genes in developing cartilage tissue (Shen et al., 2017; Liao et al., 2017; He et al., 2017; Hu et al., 2017; Sinha et al., 2017; Jing et al. 2016; Liu et al., 2015; Maihiot et al., 2015; Zhou et al. 2014; Ono et al., 2014; Jing et al. 2014; 2013; Henry et al., 2012). Prior to breeding with our floxed mice, we noted variable weight and size among the Agc^{Cre} littermates. To understand if this Cre line displays a skeletal phenotype, we analyzed Agc^{Cre} mice from birth to adulthood.

RESULTS AND DISCUSSION

Presence of Cre-transgene in Both Alleles of *Acan* Gene Affect Postnatal Skeletal Growth

At birth, wild-type ($Agc^{+/+}$), Cre-heterozygous (Agc^{Cre}) and Cre-homozygous ($Agc^{Cre/Cre}$) littermates were similar in body weight and length (Fig. 1 and data not shown). However, by 1-month of age male $Agc^{Cre/Cre}$ mice showed 18% reduction in their body weight when compared with $Agc^{+/+}$ and $Agc^{+/Cre}$ littermates (Fig. 1a). The decrease in body weight continues through early post-natal life and by 3-months of age, the weight of $Agc^{Cre/Cre}$ mice was 27% less than the $Agc^{+/+}$ and $Agc^{+/Cre}$ littermates (Fig. 1a). Impaired growth in post-natal body weight was also noted in female $Agc^{Cre/Cre}$ mice (Fig. 1b). Accordingly, the 3-months old $Agc^{Cre/Cre}$ mice appeared dwarf when compared to $Agc^{+/+}$ and $Agc^{+/Cre}$ littermates (Fig. 1c, d). Total body length of male $Agc^{Cre/Cre}$ mice was reduced by 19% and 22% at 1 and 3-months respectively (Fig. 1e). A similar reduction in body length was evident in female $Agc^{Cre/Cre}$ mice (Fig. 1f). However, body length of the $Agc^{+/Cre}$ mice was comparable to wild-type littermates. To quantify changes in the skeletal growth, both appendicular and axial skeleton were analyzed (Fig. 2). A significant reduction in the overall length of forelimb (28%) and hindlimb (41%) was detected in 1-month old $Agc^{Cre/Cre}$ mice (Fig. 2a, b). The reduction in limbs length persisted through 3-months and was observed in both genders (Fig. 2 c, d, and data not shown). In addition, $Agc^{Cre/Cre}$ mice exhibited a 43% reduction in length of vertebrae (Fig. 2e). Consequently, the tail of $Agc^{Cre/Cre}$ mice is shorter when compared to $Agc^{+/+}$ and $Agc^{Cre/+}$ littermates (Fig. 1b). Consistent with the body weight and size, the lengths of limbs and vertebra of $Agc^{+/Cre}$ mice were comparable to the $Agc^{+/+}$ littermates (Fig. 2). Together these data indicate that post-natal skeletal growth is affected by dosage of the Cre transgene.

Growth Plate and Articular Cartilage Length is Shortened in $Agc^{Cre/Cre}$ Mice

To better understand the dwarfism in the $Agc^{Cre/Cre}$ mice, we performed histological analysis through knee joints of 1-month old littermates. Central sections stained with H&E revealed that growth plate in the distal femur and proximal tibia were smaller in the $Agc^{Cre/Cre}$ mice (Fig. 3a). Measurement from 3 independent $Agc^{Cre/Cre}$ littermates showed a 25% reduction in the average length of the growth plate (Fig. 3c). Importantly, the zone of

proliferative chondrocyte was decreased by 27% in the $Agc^{Cre/Cre}$ mice (Fig. 3b, c). Furthermore, level of the proteoglycan was reduced by 47% in the growth plate cartilage of $Agc^{Cre/Cre}$ mice (Fig. 4a,b). Abundant cartilage remnant present in the metaphyseal region of the wild-type and the $Agc^{+/Cre}$ mice were barely noticed in the $Agc^{Cre/Cre}$ mice (data not shown). Since *Acan* continues to express in the adult articular cartilage, we evaluated knee joints of 3-months old littermates. The thickness of the articular cartilage was decreased by 45% in the $Agc^{Cre/Cre}$ mice (Fig. 4c, d). The articular cartilage of $Agc^{+/Cre}$ mice was comparable to wild-type littermates (Fig. 3a and data not shown). Thus, growth plate and articular cartilage synthesis are affected by dosage of Cre transgene in the $Agc^{Cre/Cre}$ mice.

Aggrecan Expression is Markedly Decreased in Mice Carrying Cre-transgene in Both Alleles of *Acan* Gene

For a molecular understanding of endochondral defects in the $Agc^{Cre/Cre}$ mice, RNA and protein were isolated from the epiphyseal region of femur and tibia including knee-joint of 1 and 3-months old littermates. Expression of chondrocyte differentiation marker genes was determined by real time PCR. Importantly, *Sox9*, *Pthrp*, *Col2a*, and *Col10a* expression was significantly decreased in the $Agc^{Cre/Cre}$ mice (Fig. 5a). *Runx2* expression was decreased in the $Agc^{Cre/Cre}$ mice, however, the change was not significant (Fig. 5a). Decreased expression of chondrocyte markers was also noted in 3-months old $Agc^{Cre/Cre}$ littermate (data not shown). Consistent with lack of any skeletal phenotype, expression of chondrocyte marker genes in the $Agc^{+/Cre}$ mice was comparable to the wild-type littermates (Fig. 5a).

Transcription from the *Acan* locus is sustained in the adult growth plate and articular cartilage. Therefore, we evaluated *Acan* expression in the $Agc^{Cre/Cre}$ littermates at 1-month and 3-months of age. Interestingly, *Acan* mRNA was decreased by 45–50% in $Agc^{Cre/Cre}$ mice at both time points (Fig. 5b). Western blot analysis further confirmed a 50% decrease in the levels of Acan protein in $Agc^{Cre/Cre}$ mice (Fig. 5c). The $Agc^{+/Cre}$ mice, on the other hand, exhibited 18–20% decrease in *Acan* mRNA but levels of Acan protein was comparable to the wild-type littermates (Fig. 5b). Together, these results demonstrate that bi-allelic presence of Cre transgene in the $Agc^{Cre/Cre}$ mice impairs expression of *Acan* mRNA and causes dwarfism.

The phenotype of $Agc^{Cre/Cre}$ mice is similar to the *Acan* heterozygous loss of function mice which show a reduced amount of proteoglycan and also exhibit dwarfism (Watanabe et al. 1997). Therefore, regression analyses were performed to evaluate the correlation between aggrecan expression and dwarfism. Data from 3-months old littermates revealed a strong correlation ($R=0.9422$) between the level of *Acan* expression and the total body length (Fig. S1b). Interestingly, mutations of *Acan* gene in humans are associated with dwarfism and skeletal dysplasia with an early onset of osteoarthritis (Tompson et al. 2009; Gleghorn et al. 2005). Consistent with the human phenotype, the thickness of the articular cartilage was reduced in the $Agc^{Cre/Cre}$ mice (Fig. 4). Moreover, visual observation suggests a gaiting abnormality in 3-month old $Agc^{Cre/Cre}$ mice.

The IRES-CreERT-Neo-pgk cassette used to generate Agc^{Cre} line do not excise or replace any part of the endogenous aggrecan gene, so it is not clear why *Acan* expression is reduced in $Agc^{Cre/Cre}$ mice. The most plausible reason for decreased expression is the presence of

the pgk-Neo transgene. The constitutively active pgk promoter that transcribes neomycin gene is placed in an opposite orientation to the endogenous Acan gene promoter (Fig. S1a). Presence of pgk-Neo cassette either in the intron or the 3'UTR is known to cause a position dependent long-range disruption of endogenous gene expression (Pham et al. 1996, Seidl et al. 1999, Strathdee et al.; 2008, Lou et al. 2009; Pan et al. 2016).

Reduced Acan expression may also be due to the requirement of 3'UTR in regulating the stability of mRNA. The aggrecan mRNA consists of 823bp of 3'UTR. Only the initial 421bp are conserved between mouse, rat, and human. As described earlier, the IRES-CreERT-Neo-pgk cassette contains SV40 poly(A) sequences after CreERT transgene (Fig. S1a). Placement of SV40 poly(A) sequences in the 3'UTR of Acan gene likely disrupt read-through by RNA polymerase. Thus, the bicistronic mRNA produced in the Agc^{Cre/Cre} mice will lack 760bp of 3'UTR. Missing portion of the 3'UTR contains perfect consensus sequences for several RNA binding proteins including eukaryotic initiation factor that is essential for pre-mRNA processing, RNA transport and to prevent nonsense-mediated mRNA decay (Matoulkova et al., 2012; Fatscher et al., 2011).

The development and use of new Cre-lines for spatio-temporal deletion of a target gene are rapidly growing. Several investigators have reported the use of Agc^{Cre} model in their studies. Two articles have clearly indicated the use of Agc^{+/-Cre} mice (He et al. 2017; Jing J et al. 2013). One article reported the use of Agc^{Cre/Cre} mice for their analysis (Maihiot et al, 2015). However, most of the articles do not state if Agc^{+/-Cre} or Agc^{Cre/Cre} mice were used for phenotypic analysis (Shen et al., 2017; Liao et al., 2017; Hu et al., 2017; Sinha et al., 2017; Jing et al., 2015, 2016; Liu et al., 2015; Zhou et al. 2014; Ono et al., 2014; Jing et al. 2014; Henry et al., 2012). Therefore, caution must be exercised when interpreting data using this model as Agc^{Cre/Cre} mice themselves have a skeletal phenotype. We propose the Agc^{+/-Cre} mice, which are comparable to the wild-type counterparts, should be used for regulated ablation of target in the cartilage.

MATERIALS AND METHODS

Genotyping and Postnatal Growth Assessment of Aggrecan Cre Mice

All animal experiments were performed with the approval from the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham and conformed to relevant federal and state guidelines and regulations. Agc1^{CreERT2} mice were obtained from Dr. deCrombrughe laboratory at MD Anderson, TX. This model was generated by inserting a 3.8kb IRES-CreERT2- Neo-pgk cassette at the 3'UTR of *Acan* gene by homologous recombination (Henry et al., 2009). We have backcrossed the original Agc^{Cre/+} line with C57BL/6 mice for up to ten generations. All analysis was conducted on Agc^{Cre} littermates maintained on C57BL/6 background. For genotyping of littermates, genomic DNA was extracted from the tail snippets using DirectPCR[®] DNA extraction buffer (Viagen Biotech Inc. CA). Genotyping of Agc1^{CreERT2} mice was performed according to the Jackson laboratory protocol. Sequences of three primers used to specifically amplify *Acan* and *Cre* genes are: 5'GTTATATTCCGGAGCCCACA3', 5'AAAAGCGAC AAGACACCA3' and 5'CTCCAGACTGCCTTGGGAAA3'. The results were interpreted according to the size of the PCR product; Agc^{Cre/Cre} (200 bp), Agc^{+/-Cre} (200 bp and 299 bp) and Agc^{+/+} (299 bp).

Body weight of littermates was recorded weekly to quantify post-natal growth. Whole body and the tail length of littermates was measured at 1 and 3-months of age. Alizarin red and alcian blue stained skeletons were used to determine limb and vertebrae length of 1 and 3-month old littermates.

Skeletal Staining and Histology

For skeletal staining, littermates of all genotypes were processed at 1 and 3-months of age. Mice were deskinning, eviscerated, and muscles partially removed for alizarin red/alcian blue double staining. Cartilage and bone tissue staining was performed essentially as described previously (Adhami et al., 2015). Stained skeletons were cleared of soft tissues by gentle shaking in 1% KOH for 2-weeks for 1-month old and for 4-weeks for 3-month old animals. The subsequent clearing was carried with 1% KOH and 20% glycerol solution for 1-week and then with 1% KOH and 50% glycerol solution for 2 weeks. Stained skeletal elements stored in 100% glycerol were imaged simultaneously in a single frame using Nikon Cool PIX S10 digital camera.

For histology, left hindlimbs from 1-month and 3-months old littermates were fixed overnight in 10% formalin solution (Fisher Scientific, Pittsburgh, PA) at room temperature. Bones were decalcified in 10% EDTA (pH 7.5) for 2–3 weeks and processed for paraffin embedding as previously published (Adhami et al., 2014). Mid femur through mid-tibia was sectioned at 7µm, deparaffinized, and processed for hematoxylin/eosin or alcian blue staining. Images were captured at 4×, 10× and 40× magnifications using Nikon Eclipse 80i microscope.

RNA Isolation and Real-Time PCR

The epiphyseal region of the femur and tibia including knee joints of 1 and 3-months old littermates were collected for isolation of total RNA and protein. Tissues were flash frozen and homogenized in ice cold PBS containing 0.1% RNAase inhibitor (Roche Applied Sciences, Indianapolis, IN). Total RNA was extracted using TRIzol[®] reagent (Invitrogen Inc., Carlsbad, CA) and 2 µg of total RNA was used to prepare cDNA using cDNA synthesis kit (BioRad Laboratories Inc. Hercules, CA). Quantitative real-time PCR was performed using iTag SYBR Green Universal Supermix (BioRad Laboratories Inc. Hercules, CA) essentially as described previously (Chen et al., 2014). Gene expression was normalized to levels of β -actin used as internal control. Relative gene expression was quantified by a CT method. Primer pairs used for RT-PCR are summarized in Table 1.

Antibody and Western Blot Analysis

Flash frozen knee joints were homogenized to obtain tissue lysates as described previously (Chen et al., 2014). An equal volume of tissue lysates was separated on 10% SDS-PAGE and proteins transferred to Immobilon-Fl PVDF membrane (Millipore Corporation, Billerica, MA) using BioRad Mini Trans-Blot Cell. The transfer was carried out at 4° C for 1-hour using 100v. Blots were probed overnight with 1:500 dilution of aggrecan polyclonal antibody (Thermo Fisher Scientific, Rockford, IL), and washed in phosphate-buffered saline Tween-20. Blots were then incubated with anti-rabbit IRDye700 secondary antibody for 1-hour at 22°C and after washing scanned with Odyssey Imaging system (LI-COR, Lincoln,

NB). Blots were stripped and reprobed with goat polyclonal Lamin-B antibody (Santa Cruz, San Diego, CA). The intensity of Acan protein was quantified using Odyssey Imaging system and normalized with Lamin-B used as loading control and shown in the graph.

Statistical Analysis

Data were collected from at least three mice of each genotype (n=3) from independent litters and is presented as average with a standard error of the mean. Student t-test for two independent mean was used to determine the level of statistical significance. Asterisk indicate *P*-value * (0.05 – >0.01), ** (0.01 – >0.001), *** (<0.001). Pearson's correlation coefficient was determined between the level of Acan mRNA and whole body length of 3-month old littermates.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Mice carrying Cre transgene in both alleles of *Acan* gene exhibit dwarfism. Body weight of male (a) and female (b) littermates was recorded weekly from 3 to 12 weeks of age. NB indicates weight of newborn pups. The weekly average weight of 7 male and 7 female mice of each genotype (n=7) with standard error is presented in the growth curve. Representative images of 3-months old male (c) and female (d) littermates. Body length from snout to the tail of 1 and 3-months old male (e) and female (f) littermates was measured. The bar graph shows average length with standard error for all three genotype. The average length of 1-month old littermates is based on *Agc*^{+/+} (n=7), *Agc*^{+/Cre} (n=15), and *Agc*^{Cre/Cre} (n=11) mice. The average length of 3-months old littermates is based on at least 7 mice of each genotype (n=7). Asterisk represents ****P*<0.001.

Figure 2.

Postnatal limb growth is retarded in the $Agc^{Cre/Cre}$ mice. Skeleton from 1-month (a) and 3-months (c) old littermates were stained with alizarin red and alcian blue. Representative image of forelimb (upper panel) and hindlimb (lower panel) from the $Agc^{+/+}$, $Agc^{+/Cre}$, and $Agc^{Cre/Cre}$ male littermates are shown. The total length of forelimb from scapula to phalanges (upper panel) and hindlimb from the femur to phalanges (lower panel) were measured using a digital scale. Average limb length from at least three mice ($n=3$) of each genotype at 1-month (b) and 3-month (d) old littermates is presented in the bar graph. Representative image of caudal vertebrae (e) of 3-months old mice. An average length of caudal vertebrae (C2–C5) from three mice ($n=3$) is presented with standard error in the bar graph. Asterisk indicate $*P<0.05$, $**P<0.01$, $***P<0.001$.

Figure 3.

Dwarfism in Agc^{Cre/Cre} mice is associated with shorter growth plate and proliferative zone of chondrocytes. Hindlimbs from 1-month old male littermates were processed for H&E staining. Central image through knee joints was captured at 4× (a) and 10× (b) magnification using Nikon Eclipse 80i microscope. Boxed region in the growth plate of the tibia is shown at 10× magnification. The double headed arrow indicates the length of the growth plate. NIS element software was used to measure the length of growth plate and the proliferative zone. Average length calculated from individual sections of 3 independent littermates with standard error is shown in the bar graph. Scale bar; 500μm in 4× and 100μm in 10×. Asterisk indicate ** $P < 0.01$, *** $P < 0.001$.

Figure 4.

Decreased thickness of articular cartilage and amount of proteoglycan in *Agc*^{Cre/Cre} mice. Hindlimbs from 3-months old *Agc*^{+/+} and *Agc*^{Cre/Cre} littermates were processed for histology and alcian blue staining. Images of the tibia were captured at 4× (a) and the boxed region is shown at 40× magnification (lower panel). The intensity of the alcian blue signal (b) was quantified by NIS element software and presented in the graph. Representative images of alcian blue stained articular cartilage in the tibia (c) captured at 40× magnification. Articular cartilage length is indicated by the double headed arrow. Graph shows the average thickness of the articular cartilage calculated from three mice (n=3) of indicated genotype. Scale bar; 500μm in 4× and 100μm in 40×. Asterisk indicate ***P*<0.01, ****P*<0.001.

Figure 5.

Expression of chondrocyte marker genes is significantly decreased in the Agc^{Cre/Cre} mice. Quantitative RT-PCR was performed with RNA isolated from the knee joint of 1 and 3-months old male littermates. Expression of *Sox9*, *Pthrp*, *Col2a*, *Col10a*, and *Runx2* (a) was determined in triplicate reaction and normalized with β -actin. Average expression values from three independent mice (n=3) of each genotype are presented in the graph. Expression of *Acan* (b) in 1 and 3-months old Agc^{+/+}, Agc^{+/Cre} and Agc^{Cre/Cre} littermates. Western blot analysis of Acan protein (c) in the knee joints of 1-month old littermates. Lamin-B is shown as loading control. Average of normalized Acan protein signal from two blots are presented in the graph. Asterisk indicate * P <0.05, ** P <0.01, *** P <0.001.

Table 1

Sequences of primers used for quantitative real time PCR.

Gene	Forward (5' to 3')	Reverse (5' to 3')
<i>Sox9</i>	AGGAAGCTGGCAGACCAGTA	TGTTAATCGGGGTGGTCTTTC
<i>Col2a1</i>	TCCTCTGCGATGACATTATCT	GATCACCTCTGGGTCCTTGTT
<i>Pthrp</i>	GAACATCAGCTACTGCATGACAAG	TCTGATTTCGGCTGTGTGGATC
<i>Acan</i>	CCCTCGGGCAGAAGAAAGAT'	CGCTTCTGTAGCCTGTGCTTG
<i>Runx2</i>	GCCCTCCCTGAACTCTGCAC	TCATGCCTGGCTCTTCTTACT
<i>Col10a1</i>	GCAGCATTACGACCCAAGAT	CCTGAAGCCTGATCCAGGTA
<i>β-actin</i>	CGAGCGGTTCCGATGC	TGGATGCCACAGGATCCAT