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## Runx2 is required for postnatal intervertebral disc tissue growth and development

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### Abstract

Runx2 plays an essential role in embryonic disc tissue development in mice. However, the role of runt-related transcription factor 2 (Runx2) in postnatal disc tissue growth and development has not been defined. In the present studies, we generated *Runx2* conditional knockout (KO) mice (*Runx2<sup>Agc1ER</sup>*), in which *Runx2* was deleted in Aggrecan-expressing cells in disc tissue at postnatal 2-weeks of age. We then analyzed changes in disc tissue growth and development using histology and immunohistochemical methods in 3-month-old mice. We found that large vacuolated notochordal cells were accumulated in the nucleus pulposus (NP) in *Runx2* KO mice. The growth plate cartilage tissue in the disc was thicker in *Runx2* KO mice. We also found a significant upregulation of Indian hedgehog (Ihh) expression in the cells in NP cells and in annulus fibrosus cells of *Runx2* KO mice. These results demonstrated that Runx2 may play an important role in postnatal disc tissue development through interacting with Ihh signaling.

### Keywords

growth plate (GP) cartilage; Indian hedgehog (Ihh); intervertebral disc (IVD); notochordal cells; runt-related transcription factor 2 (Runx2)

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#### AUTHOR CONTRIBUTIONS

L. Liao, H. Jiang, Y. Fan, R.S. Lu, and C. Wei carried out experiments. L. Liao and D. Chen prepared the manuscript. T. Takarada and S. He helped revising the manuscript. D. Chen contributed to the experimental design, data interpretation and finalized the manuscript.

#### CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

## 1 | INTRODUCTION

Intervertebral disc (IVD) is a specialized connective tissue which consists of three distinct interdependent tissues: the central viscous nucleus pulposus (NP), the outer fibrillary annulus fibrosus (AF) and the cartilage growth plate (GP) and endplate (EP), which anchor the disks to the adjacent vertebral body. The NP is composed of a proteoglycan and water gel held together loosely by an irregular network of type II collagen and elastin fibers. It contains two distinct cell types: large clusters of notochordal cells (M. McCann & Seguin, 2016) and smaller, more disperse chondrocyte-like cells (Roberts, 2000). There are large vacuoles inside the notochordal cells and these vacuoles increase the cell size of notochordal cells and occupy about 80% of the cell volume (Hunter, Bianchi, Cheng, & Muldrew, 2007). The presence of these large intracellular vacuoles makes notochordal cells directly contribute to the mechanical properties of the notochord (Hunter et al., 2007). Actually, these notochordal cell vacuoles are lysosome related organelles that function in axis and spine morphogenesis (Ellis, Bagwell, & Bagnat, 2013). In most vertebrates, including humans and mice, there is a progressive loss of large vacuolated notochord cells immediately after birth and the NP becomes populated by small chondrocyte-like NP cells (Hunter, Matyas, & Duncan, 2003; Roberts, 2000). Interestingly, the loss of large vacuolated notochordal cells is also associated with the onset of disc degeneration, suggesting that these cells are required for the maintenance of the NP (Aguiar, Johnson, & Oegema, 1999; Boos et al., 2002; Hunter et al., 2003).

Understanding signals which control the normal IVD growth and differentiation may provide potential therapies for the treatment of disc degenerative disease. The runt-related transcription factor 2 (Runx2) is an essential transcription factor regulating endochondral ossification through control of chondrocyte proliferation and differentiation (Chen et al., 2014; Komori 2008; Zaidi et al., 2002). Over the past two decades, major progress has been made in the understanding the critical role of Runx2 in regulating osteoblast differentiation. In addition, Runx2 has also been reported to regulate the chondrocyte differentiation and hypertrophy (Kamekura et al., 2006; Liao et al., 2017; Takarada et al., 2013). In mice, Runx2 expression has been detected in prehypertrophic and hypertrophic chondrocytes (Kamekura et al., 2006; Sato et al., 2008) and osteoblast lineage cells (Ducy, Zhang, Geoffroy, Ridall, & Karsenty, 1997). Although the role of Runx2 in long bone has been well documented, less is known about its role in disc tissue.

In 1997, Runx2 was found to be essential for embryonic skeletal development and vertebral bone formation in mice (Ducy et al., 1997). During embryonic development, Runx2 expression was detected to be restricted to cells of the ossification centers of vertebrae before they became mineralized (Ducy et al., 1997). Subsequently, another study demonstrated that, during mouse embryogenesis, *Runx2*, but not *Runx1* or *Runx3*, was expressed in the IVD and Runx2 expression was significantly increased in the mouse model of IVD degeneration and in patients with IVD degeneration (Sato et al., 2008). These studies suggest that Runx2 is essential for the disc development during embryogenesis, and may play an important role in disc degeneration after birth. However, the effect of Runx2 on postnatal disc tissue development has not been fully defined.

*Agc1-CreER* transgenic mice are a mouse model which could be used to target cartilage cells at postnatal and adult stages (Henry et al., 2009). In the present studies, we generated *Runx2<sup>Agc1ER</sup>* conditional KO mice in which *Runx2* is deleted in Aggrecan-expressing cells. We analyzed the disc phenotype of *Runx2* knockout (KO) mice and found that the transition of notochordal cell to chondrocyte-like cells in the NP was inhibited in *Runx2<sup>Agc1ER</sup>* KO mice. Furthermore, Indian hedgehog (*Ihh*) expression was found to be significantly increased in NP and AF areas of *Runx2* KO mice. Our findings demonstrate that *Runx2* expression is required for postnatal disc tissue growth and development.

## 2 | MATERIALS

### 2.1 | Animals

*Runx2<sup>Agc1ER</sup>* mice were generated by breeding *Runx2<sup>lox/lox</sup>* mice (Liao et al., 2017; Takarada et al., 2013) with *Agc1-CreER* mice (Henry et al., 2009). *Agc1-CreER* transgenic mice were obtained from Jackson laboratories (Bar Harbor, ME). *Runx2<sup>Agc1ER</sup>* conditional KO mice were administered with tamoxifen (1 mg/10 g body weight, intraperitoneal injection [i.p.] injection, daily for 5 days) at age of 2-week-old. Mice were killed at age of 3-month-old for microcomputed tomography ( $\mu$ CT) and histologic analyses. Cre-negative littermates were used as controls,  $n = 5$  per group. Animal protocol of this study has been approved by the IACUC of the Rush University Medical Center and all experimental methods and procedures were carried out in accordance with the approved guidelines.

### 2.2 | Cre-recombination efficiency

To examine whether *Agc1-CreER* could efficiently target disc cells in postnatal mice, *Agc1-CreER* transgenic mice (Henry et al., 2009) were bred with *ROSA<sup>mT/mG</sup>* reporter mice to generate *Agc1-CreER; ROSA<sup>mT/mG</sup>* mice. *Agc1-CreER; ROSA<sup>mT/mG</sup>* mice express red fluorescence in all cell types before Cre-recombination and express green fluorescence following recombination after tamoxifen induction. *Agc1-CreER; ROSA<sup>mT/mG</sup>* mice are administered tamoxifen at 2-week-old by i.p. injection (1 mg/10 g body weight, daily for five consecutive days) and killed 7 weeks after tamoxifen induction.

### 2.3 | Histology analysis

We dissected lumbar spine from *Runx2<sup>Agc1ER</sup>* mice and Cre<sup>-</sup> control mice. Samples were fixed in 10% formalin, decalcified, and processed as previously described (B. Wang et al., 2014; Liao et al., 2017; M. Wang et al., 2012; Shen et al., 2013; T. Wang et al., 2018). And then, 3- $\mu$ m mid-sagittal sections at three different levels (15- $\mu$ m apart) were cut from the medial compartment of the L3-L5 intervertebral bodies. These sections were stained with Alcian blue/H&E Orange G. Three slides per mouse, six mice per group were analyzed in the experiment.

### 2.4 | Microcomputed tomography

Before histologic processing, we evaluated formalin-fixed mouse spines by  $\mu$ CT-35 cone-beam scanner (Scanco Medical, Switzerland) with a 55 kVp source and a 145  $\mu$ Amp current. We scanned the mouse spines at a resolution of 10.5  $\mu$ m. The scanned images from each

group were evaluated at the same thresholds to allow 3-dimensional structural rendering of each sample.

## 2.5 | Immunohistochemistry

Three  $\mu\text{m}$  thick paraffin sections were baked at  $60^{\circ}\text{C}$  overnight. And then deparaffinized, rehydrated and heated at  $95^{\circ}\text{C}$  with Antigen Unmasking solution (Vector Laboratories, Burlingame, CA) for 5–10 min. Slices were then treated with 3% hydrogen peroxide for 10 min at room temperature for 10 min, incubated with 0.5% Triton X-100 (Sigma-Aldrich, St. Louis, MO) for 1 hr, and then blocked with Avidin/Biotin Blocking Kit (Invitrogen, CA, 004303).

Slides were then blocked with the blocking serum at 10% normal goat serum (Vector Laboratories) in 1% bovine serum albumin for 30 min at room temperature and incubated with primary antibodies against Runx2 (1:200 dilution) (Mouse IgG; MBL, Woburn, MA) or Ihh (1:500 dilution; Rabbit IgG; Abcam, Cambridge, MA) at  $4^{\circ}\text{C}$  overnight. The next day, secondary biotinylated goat anti-mouse antibody (Vector Laboratories) was added to slices for 30 min, followed by the incubation with VECTASTAIN Elite ABC HRP Kit (Vector Laboratories) for 30 min. Afterwards, positive staining was detected by ImmPACT DAB Peroxidase (HRP) Substrate (Vector Laboratories). Slides were then counterstained with CAT Hematoxylin (Biocare Medical, Pacheco, CA), dehydrated with graded ethanol and cleared with three changes of Xylene and cover-slipped finally.

## 2.6 | Real-time PCR analysis

Twelve-week-old *Runx2<sup>Agc1ER</sup>* mice and their  $\text{Cre}^{-}$  control mice were killed. Disc tissues were collected from freshly killed mice to obtain total RNA. Total mRNA was extracted with Trizol (Invitrogen Life Technologies). 1  $\mu\text{g}$  total RNA was used to synthesize complementary DNA (cDNA) using an iScript cDNA Synthesis kit (Quanta Biosciences, MD). Subsequently, real-time PCR amplification was performed using specific primers of target genes and a SYBR Green real-time PCR kit (Quanta Biosciences). The primer names and sequences were listed in Table 1. Data were collected from cartilage of three independent mice ( $n = 3$ ).

## 2.7 | Statistical analysis

Data are presented as the mean  $\pm$  standard deviation. For experiments comparing two groups of data, unpaired Student's *t* test was performed. A value of  $p < 0.05$  was considered to be significant.

# 3 | RESULTS

## 3.1 | Agc1-CreER mice target disc tissue with high recombination efficiency

To evaluate *Agc1-Cre* expression and recombination efficiency in disc tissue, we bred *Agc1-CreER* transgenic mice (Henry et al., 2009) with *ROSA<sup>mT/mG</sup>* reporter mice (Chelberg, Banks, Geiger, & Oegema, 1995; Dahia, Mahoney, Durrani, & Wylie, 2009; Liao et al., 2018) to generate *Agc1-CreER; ROSA<sup>mT/mG</sup>* mice. Mice express red fluorescence in all cell types before Cre recombination and green fluorescence after breeding with *Agc1-CreER*

mice and administered with tamoxifen. The red fluorescent image of disc in *Agc1-CreER*; *ROSA<sup>mT/mG</sup>* mice indicates the absence of *Agc1-CreER*-expressing cells (Figure 1, left panel) and the green-labeled cells marked *Agc1-CreER* targeting cells (Figure 1, right panel).

### 3.2 | Deletion of *Runx2* in Aggrecan-expressing disc cells led to defects in disc tissue

$\mu$ CT scanning displayed that there was no obvious phenotype in spine and disc tissues in *Runx2* KO mice compared with the Cre<sup>-</sup> control mice (Figure 2), suggesting that there is no changes in bone mineralization in spine and disc tissue in *Runx2* KO mice. However, the histologic results showed obvious changes in disc tissue morphology. Most NP cells are chondrocyte-like cells which are characterized by their smaller size and high ratio of proteoglycan matrix to cells in the NP in 3-month-old Cre<sup>-</sup> control mice (Figures 3a,b). In contrast, large numbers of large vacuolated notochordal cells were observed in the NP in 3-month-old *Runx2<sup>Agc1ER</sup>* KO mice. Furthermore, less proteoglycan matrix, demonstrated by Alcian blue staining, was found in the NP of *Runx2* KO mice than that of Cre<sup>-</sup> control mice (Figure 3b). In addition, results of Alcian blue staining also demonstrated that the GP cartilage tissue was expanded in *Runx2* KO mice. In some mice these expanded GP tissue was protruded to the vertebral bone (Figure 3a,b).

### 3.3 | Deletion of *Runx2* in Aggrecan-expressing disc cells increased *Ihh* expression

*Runx2* is an essential transcription factor regulating endochondral ossification through control of chondrocyte proliferation and differentiation. Results of immunohistochemical (IHC) analysis showed that *Runx2* expression was detected in the nucleus of chondrocyte in the Cre<sup>-</sup> control mice, whereas almost no *Runx2* positive cells was detected in the disc tissue in *Runx2* KO mice (Figure 4a). In contrast, *Ihh* protein levels were greatly increased in NP and AF cells of *Runx2* KO mice (Figures 4b,c). We then further analyzed expression of other genes related to *Ihh* signaling, such as *Ptch1* and *Smo*, and found that expression of both *Ptch1* and *Smo* was significantly upregulated in the disc tissue of *Runx2* KO mice (Figure 4d,e).

### 3.4 | Deletion of *Runx2* in disc cells altered chondrocyte maker gene expression

Total RNA was extracted from disc tissue from 3-month-old Cre<sup>-</sup> or *Runx2<sup>Agc1ER</sup>* KO mice followed by the real-time PCR analysis. Expression of *Runx2*, *Osx*, *Sox9*, *Col10a1*, *Mmp9*, *Mmp13*, *Adamts4*, and *Adamts5* was significantly reduced, respectively (Figures 5a–h). These observations are consistent with previous reports (Komori, 2008; Liao et al., 2017; M. Wang et al., 2012; Pratap et al., 2005). To further confirm changes in expression of *Col10a1* and *Mmp13*, we performed IHC assays and found that Col-X and MMP13 protein levels were significantly reduced in the disc tissue of *Runx2* KO mice (Figure 5i,j). Abnormal regulation of the genes encoding for matrix degradation enzymes may play an important role in the development and progression of disc degeneration.

## 4 | DISCUSSION

In this study, we found that *Agc1-CreER* mice could efficiently target disc cells, including GP cartilage cells, AF and NP cells (Figure 1). Using *Agc1-CreER* mice, we generated

*Runx2<sup>Agc1ER</sup>* KO mice and analyzed disc phenotype of these mice. As a result, we have demonstrated an essential role of Runx2 in disc tissue development and maintaining normal disc structures at postnatal and adult stages. Our results suggest that Runx2 expression in Aggrecan-expressing cells at the postnatal stage is required for promoting the transition from notochordal cells to chondrocyte-like cells in the NP.

The NP is an aggrecan-rich gel-like tissue. In the neonate, the NP is highly cellular with relatively little proteoglycan production. In contrast, in the adult, the proportion of cells to matrix is low. As the disc matures, the composition of the NP changed. The numbers of large vacuolated cells (notochordal origin) are decreased and replaced by smaller chondrocyte-like cells. These cellular events are also accompanied with changes in the extracellular microenvironment (Risbud & Shapiro, 2011). In adult human, beginning as early as the second decade of life, notochordal cells are no longer detected within the NP, which is replaced by smaller or disperse chondrocyte-like cells (Chelberg et al., 1995). Notochordal cells survive at postnatal age, but at greatly reduced levels, at least until 48 weeks of age in mice (Dahia et al., 2009; Fujita et al., 2005). Currently, identification of these two cell types is restricted to cell morphology analysis and use of limited numbers of notochord-specified makers (Fujita et al., 2005; Gilson, Dreger, & Urban, 2010; Minogue, Richardson, Zeef, Freemont, & Hoyland, 2010). And there have been two conflicting hypotheses about the origin of the chondrocyte-like cells in the NP. Originally, it was suggested that these cells were of mesenchymal origin, a consequence of the migration of cells to the NP from the surrounding EP (Vujovic et al., 2006). Alternatively, it has been suggested that notochordal cells are progenitors for all NP cells and undergo terminal differentiation to give rise to chondrocyte-like cells (Boos et al., 2002; Liebscher, Haefeli, Wuertz, Nerlich, & Boos, 2011; Pazzaglia, Salisbury, & Byers, 1989). In these two scenarios, notochordal cells play distinct roles, either as organizer or as tissue-specific progenitors, respectively. Due to the technical limitations, no definitive conclusion has been arrived. In 2012, there is evidence that notochordal cells are the precursor cells of the NP, and undergo differentiation to generate chondrocyte-like cells during postnatal development (M.R. McCann, Tamplin, Rossant, & Seguin, 2012). This transition might be induced by a variety of factors present in the extracellular environment of the IVD. In addition, rabbit notochordal cells have been shown to be able to differentiate toward chondrocyte-like cells when maintained in culture (Kim et al., 2009). However, notochordal cells in the NP have the morphology very different from that of chondrocyte-like NP cells. Notochordal cells are markedly greater in diameter and contain large vacuoles (Guehring, Urban, Cui, & Tirlapur, 2008; Hunter et al., 2003; Mollenhauer, 2006). In this study we found that most of cells within the NP of *Runx2* KO mice are notochordal cells compared with those of corresponding Cre<sup>-</sup> control mice with most of chondrocyte-like cells (Figure 3b). This observation suggests that Runx2 promotes the transition of notochordal cells into chondrocyte-like cells within the NP. *Runx2* deficiency leads to the accumulation of notochordal cells in the NP. These findings suggest that Runx2 may play an important role in maintaining NP tissue normal morphology, homeostasis and normal function.

Ihh is essential for chondrocyte and osteoblast proliferation and differentiation during prenatal endochondral bone formation (Maeda et al., 2007). Hedgehog signaling is required for the formation of the notochord sheath and NP tissue patterning within the IVD (Choi &

Harfe, 2011). It has been reported that deletion of *Smo* in *Shh*-expressing cells results in abnormal development of the IVD. Disc tissue with *Smo* removal has a smaller NP than controls and concentric lamellae were absent in the AF (Choi & Harfe, 2011). In postnatal control animals, the NP was located inside the AF throughout the vertebral column. In contrast, *Smo* KO animals contained small NP with the majority of notochord cells dispersed throughout the vertebral column (Choi & Harfe, 2011). In contrast, the postnatal *Ihh* specific deletion in *Col2a1*-expressing cells (P14 mice) showed loss of GP cartilage and AF tissue with enlarged NP tissue (Maeda et al., 2007). These findings clearly demonstrated the critical role of hedgehog signaling in disc tissue development, especially in the NP formation. In the current study we found that *Ihh* expression was significantly increased in both NP and AF tissues and *Ihh* signaling was activated in the disc tissue. These findings suggest that *Runx2* may regulate notochordal cell transition through an *Ihh*-dependent mechanism. Our observations also suggest that *Runx2* may play an important role in disc tissue growth and the development of degenerative disc disease. In this study, we found significant upregulation of *Ihh* expression in disc tissue in *Runx2* KO mice, suggesting that *Runx2* may suppress *Ihh* expression under normal conditions during postnatal disc tissue development. The detail molecular mechanism by which *Runx2* regulates *Ihh* expression in disc tissue and the significance of this regulation in NP cell function needs to be further explored.

Our current findings suggest that *Runx2* regulates multiple genes, including *Osx*, *Col10a1*, *Mmp9*, *Mmp13*, *Adamts4* and *Adamts5* in the disc tissue. However, we know much less about upstream regulation of *Runx2* in the disc tissue. In previous studies, we found that *Runx2* is regulated by  $\beta$ -catenin signaling in the disc tissue (M. Wang et al., 2012). We also found that microRNA miR-204/miR-211 regulate *Runx2* expression in mesenchymal progenitor cells (Huang, Zhao, Xing, & Chen, 2010). To fully understand the role of *Runx2* in disc tissue homeostasis, more detail mechanism studies regarding *Runx2* regulation need to be further investigated.

In summary, we found that *Runx2* plays an important role in regulation of *Ihh* expression and notochordal cell transition into chondrocyte-like cells in the NP and is required for disc tissue development.

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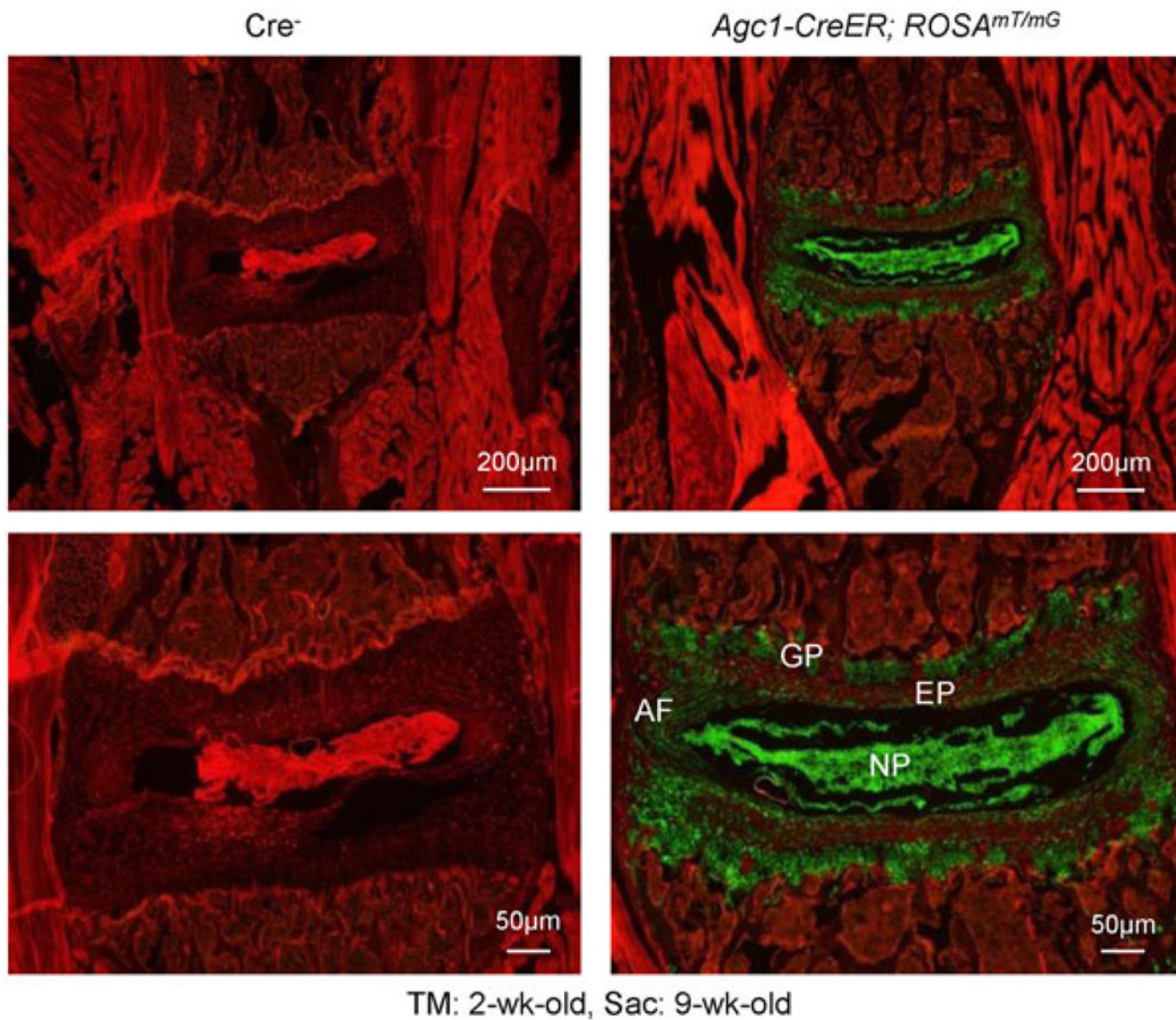
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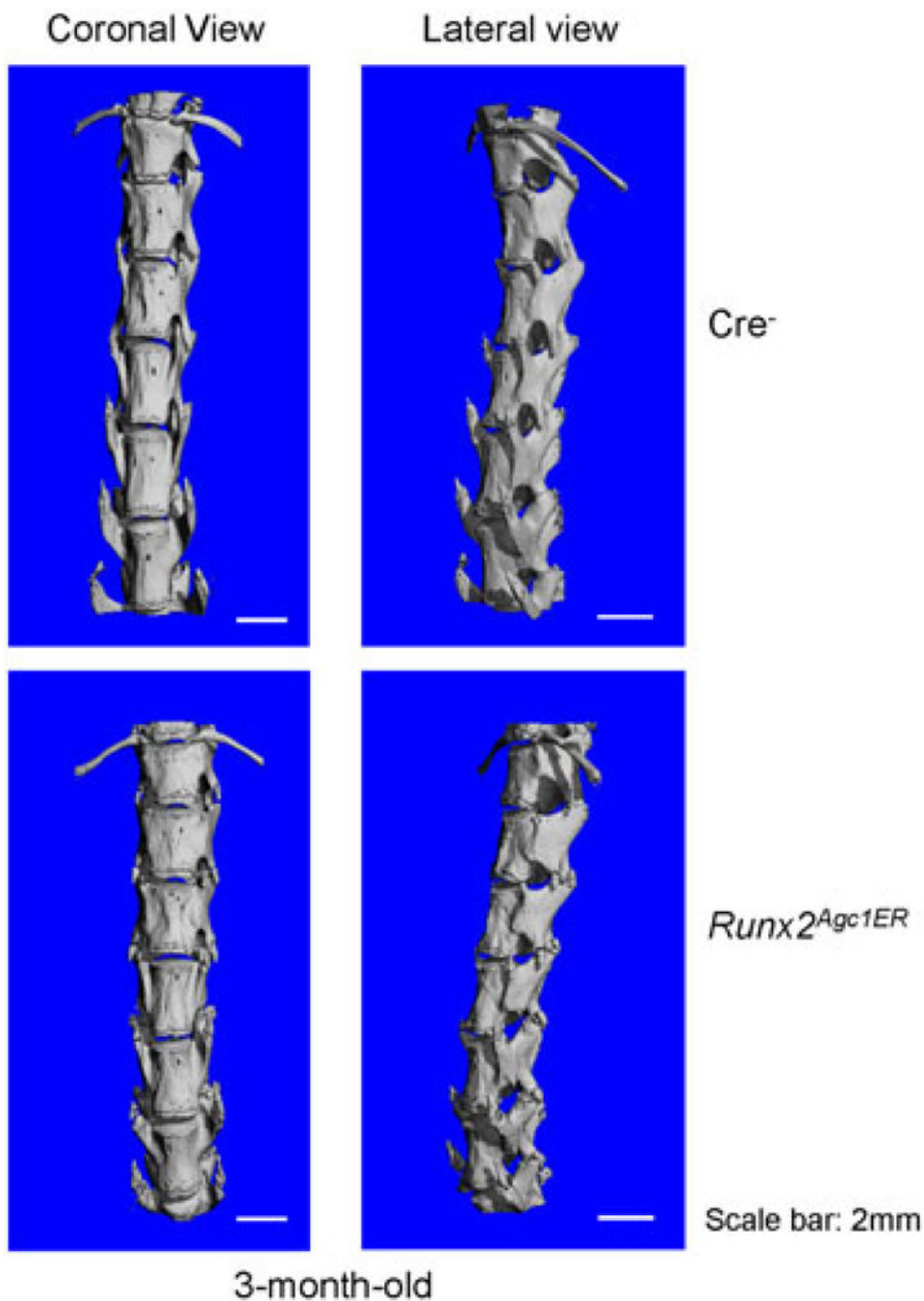
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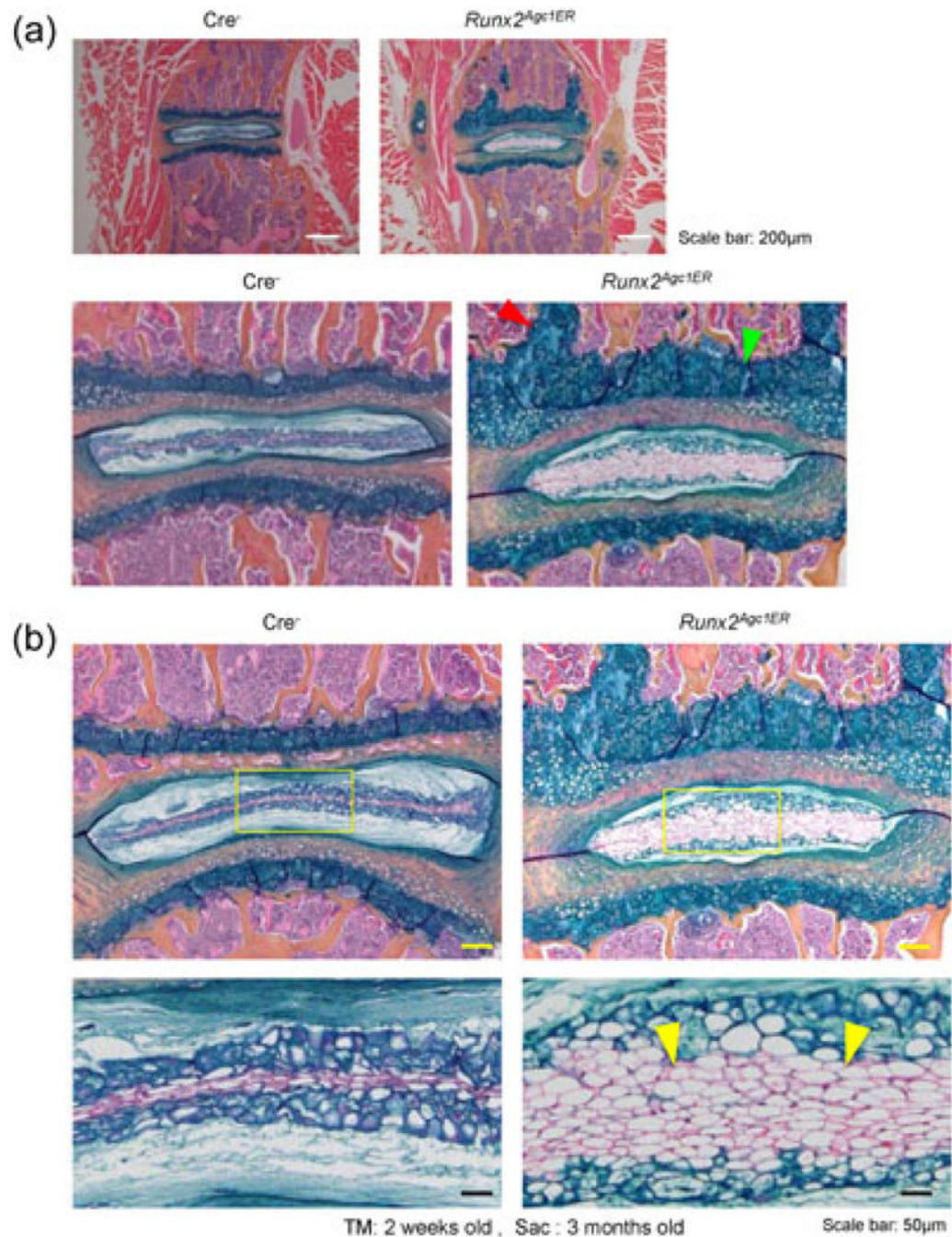
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**FIGURE 1.**

*Agc1-CreER* mice efficiently target disc cells. To determine the Cre-mediated targeting specificity and recombination efficiency, we have bred *Agc1-CreER* mice with *ROSA<sup>mT/mG</sup>* reporter mice. Tamoxifen was administered into 2-week-old mice and immunofluorescence analysis was performed in disc tissue of 9-week-old mice. Results of fluorescence microscope analysis showed that most disc tissues were efficiently targeted by *Agc1-CreER* mice, including growth plate (GP) cartilage cells, AF and NP cells (right panel). AF: annulus fibrosus; NP: nucleus pulposus [Color figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]



**FIGURE 2.** Micro-CT analysis of *Runx2<sup>Agc1ER</sup>* KO mice. Two-week-old Cre<sup>-</sup> control and *Runx2<sup>Agc1ER</sup>* KO mice were treated with tamoxifen and 3-month-old Cre<sup>-</sup> control and *Runx2<sup>Agc1ER</sup>* KO mice were analyzed by micro-CT ( $\mu$ CT). The results showed that no significant changes in the length of the spine. However, early sign of osteophyte formation was observed in *Runx2<sup>Agc1ER</sup>* KO mice (marked with red arrowheads). KO: knockout; Runx2: runt-related transcription factor 2 [Color figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]



**FIGURE 3.**

Defects in disc tissues of *Runx2<sup>Agc1ER</sup>* KO mice. (a) Results of histologic analysis showed that the growth plate thickness was significantly increased in *Runx2<sup>Agc1ER</sup>* KO mice, suggesting that Runx2 inhibits growth plate cartilage growth in normal mice (green arrowhead: increased growth plate cartilage thickness; red arrowhead: the expanded growth plate tissue was protruded to the vertebral body). (b) Increased numbers of notochordal cells in NP were observed in *Runx2<sup>Agc1ER</sup>* KO mice (yellow arrowheads: notochordal cells). KO:

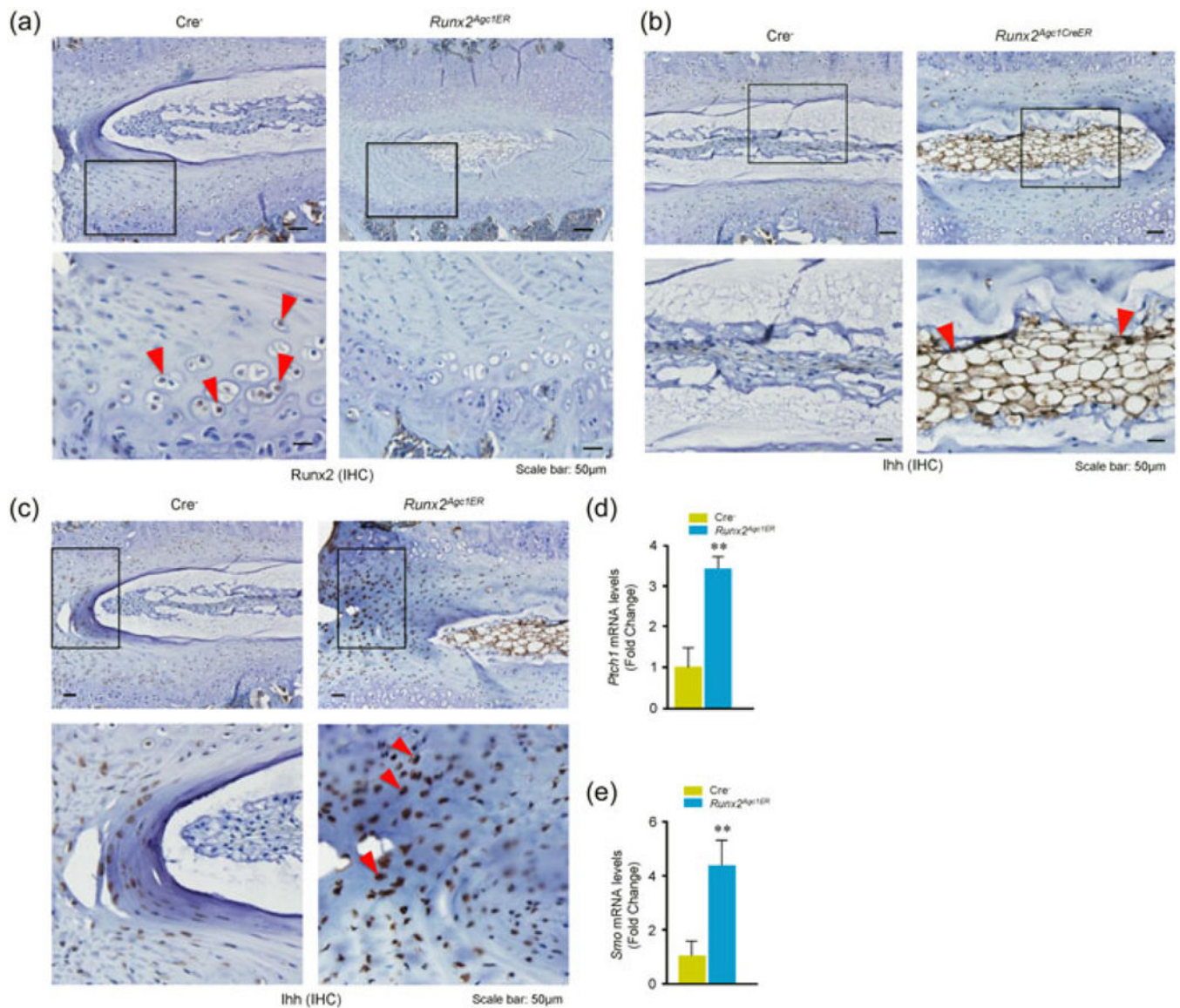
knockout; NP: nucleus pulposus; Runx2: runt-related transcription factor 2 [Color figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

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**FIGURE 4.**

Changes in Ihh signaling in disc tissues of *Runx2<sup>Agc1ER</sup>* KO mice. Two-week-old Cre<sup>-</sup> control and *Runx2<sup>Agc1ER</sup>* KO mice were treated with tamoxifen and immunohistochemical (IHC) assay was performed using disc tissues of 3-month-old Cre<sup>-</sup> and *Runx2* KO mice. (a) Results showed that Runx2 expression (red arrowheads indicate the Runx2-positive cells in Cre<sup>-</sup> mice) was significantly reduced in the annulus fibrosus (AF) cells of *Runx2<sup>Agc1ER</sup>* KO mice (right panels). (b) Expression of Ihh was significantly increased in the annulus fibrosus (AF) cells and nucleus pulposus (NP) cells in disc tissue of 3-month-old *Runx2<sup>Agc1ER</sup>* KO mice. Red arrowheads marked Ihh-positive cells. (c) Increased Ihh expression was also detected in AF cells (red arrowheads marked Ihh-positive cells) of disc tissues of *Runx2<sup>Agc1ER</sup>* KO mice. (d and e) Changes in mRNA expression of Ihh signaling related genes were analyzed by real-time PCR using the RNA extracted from disc tissues of 3-month-old Cre<sup>-</sup> and *Runx2* KO mice. The results showed that expression of *Ptch1* and *Smo*

was significantly increased in disc tissues of *Runx2* KO mice. KO: knockout; Runx2: runt-related transcription factor 2 [Color figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

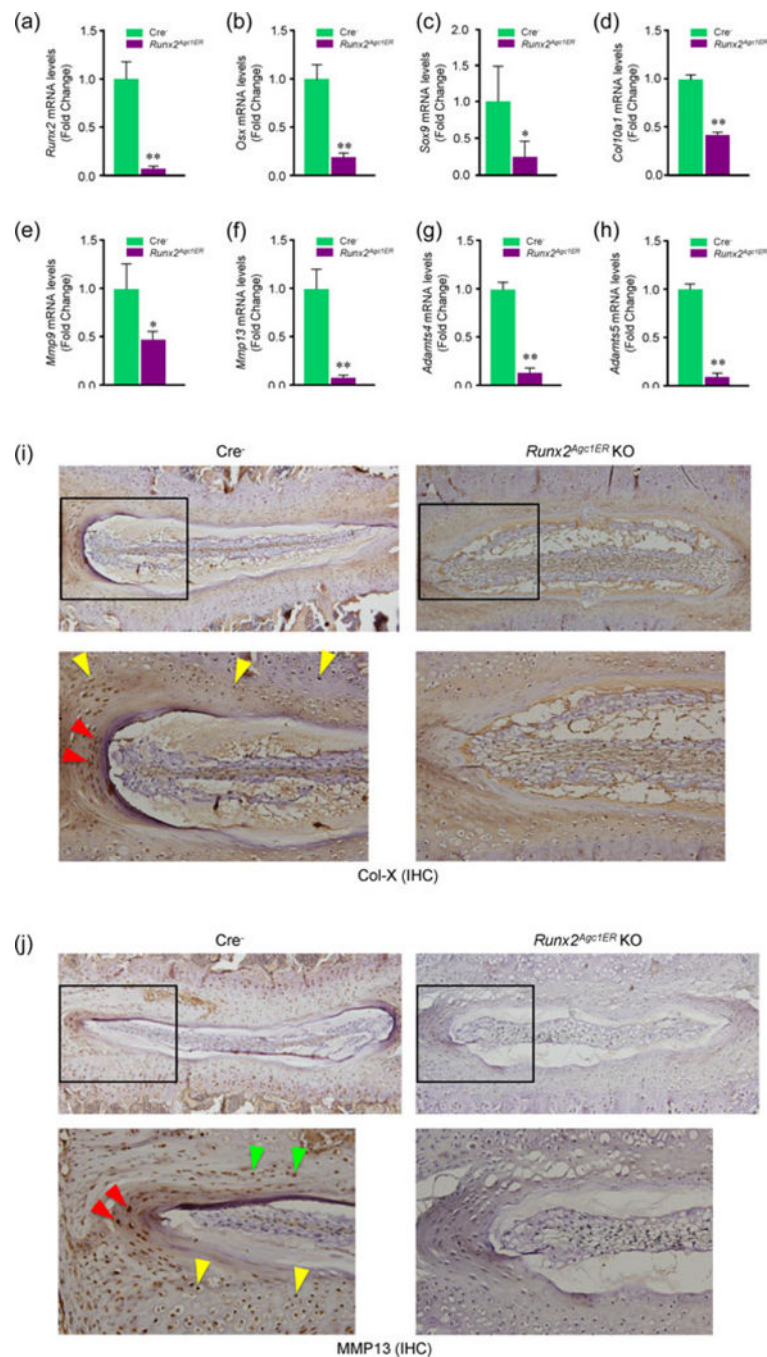
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**FIGURE 5.** Deletion of *Runx2* in Aggrecan-expressing disc tissue alters the expression of genes encoding for matrix proteins in disc tissue. (a–h) Two-week-old Cre<sup>-</sup> control and *Runx2<sup>Agc1ER</sup>* KO mice were treated with tamoxifen and total RNAs were extracted from disc tissue of 3-month-old Cre<sup>-</sup> and *Runx2* KO mice and real-time PCR assay was performed. Expression of *Runx2*, *Osx*, *Sox9*, *Col10a1*, *Mmp9*, *Mmp13*, *Adamts4*, and *Adamts5* was significantly decreased in *Runx2<sup>Agc1ER</sup>* KO mice. Statistical analysis was performed using unpaired Student's *t* test (\**p* < 0.05, \*\**p* < 0.01, *n* = 3 mice per group). (i

and j) Expression of Col-X and MMP13 protein was also analyzed by immunohistochemistry (IHC). The results showed that expression of both Col-X and MMP13 proteins was significantly reduced in disc tissues of *Runx2* KO mice. KO: knockout; Runx2: runt-related transcription factor 2 [Color figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

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**TABLE 1**

The names of sequences of primers used in this project

| Genes          | Primer sequence (forward primers) | Primer sequence (reverse primers) |
|----------------|-----------------------------------|-----------------------------------|
| <i>Rumx2</i>   | GACTGTGGTTACCGTCATGGC             | ACTTGGTTTTTCATAAACACAGCGGA        |
| <i>Col10a1</i> | TTCTGCTGCTAATGTTCTTGACC           | GGGATGAAAGTATGTGTCTTGGG           |
| <i>Mmp9</i>    | GCAGAGGCATACTTGTACCCG             | TGATGTTATGATGGTCCCACCTTG          |
| <i>Mmp13</i>   | CTTCTTCTTGTGTGAGCTGGACTC          | CTGTGGAGGTCACCTGTAGACT            |
| <i>Actin</i>   | GGCTGTATTCCCCTCCATCG              | CCAGTTGGTAACAATGCCATGT            |

Note. Rumx2: runt-related transcription factor.