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Characterization of Cre recombinase mouse lines enabling cell type-specific targeting of postnatal intervertebral discs

Yixin Zheng^{1,2,*}, Xuejie Fu^{1,2,*}, Qingbai Liu^{1,3,*}, Shengqi Guan^{4,*}, Cunchang Liu², Chunmei Xiu², Tingting Gong², Hongting Jin⁵, Saijilafu^{1,2}, Zunyi Zhang⁶, Di Chen⁷, Jianquan Chen^{1,2}

¹Department of Orthopaedics, The First Affiliated Hospital of Soochow University, Suzhou, Jiangsu, China

²Orthopedic Institute, Medical College, Soochow University, Suzhou, Jiangsu, China

³Department of Orthopedics, Lianshui County People's Hospital, Huaian, Jiangsu, China

⁴Laboratory Animal Center, Soochow University, Suzhou, Jiangsu, China

⁵Institute of Orthopaedics and Traumatology, The First Affiliated Hospital of Zhejiang Chinese Medical University, Hangzhou, Zhejiang, China

⁶Key Laboratory of Mammalian Organogenesis and Regeneration, College of Life and Environmental Science, Institute of Life Sciences, Hangzhou Normal University, Hangzhou, Zhejiang, China

⁷Department of Orthopedic Surgery, Rush University Medical Center, Chicago, Illinois

Abstract

Cre/loxP technology is an important tool for studying cell type-specific gene functions. Cre recombinase mouse lines, including *Agc1-CreER^{T2}*, *Col2a1-Cre*, *Col2a1-CreER^{T2}*, *Shh-Cre*, *Shh-CreER^{T2}*, and *Osx-Cre*, have been proven to be valuable tools to elucidate the biology of long bones, yet the information for their activity in postnatal intervertebral disc (IVD) tissues was very limited. In this study, we used *R26-mTmG* fluorescent reporter to systematically analyze cell specificity and targeting efficiency of these six mouse lines in IVD tissues at postnatal growing and adult stages. We found that *Agc1-CreER^{T2}* is effective to direct recombination in all components of IVDs, including annulus fibrosus (AF), nucleus pulposus (NP), and cartilaginous endplate (CEP), upon tamoxifen induction at either 2 weeks or 2 months of ages. Moreover, *Col2a1-Cre* targets most of the cells in IVDs, except for some cells in the outer AF (OAF) and NP.

Correspondence Jianquan Chen, Orthopedic Institute, Soochow University, 708 Renmin Rd, Suzhou, Jiangsu 215007, People's Republic of China. chenjianquan@suda.edu.cn.

*These authors contributed equally to this study.

AUTHOR CONTRIBUTIONS

YZ, XF, QL, and JC conceived and designed the experiments. YZ, SG, XF, CL, QL, CX, and TG performed the experiments. YZ, CL, QL, JC, HJ, SL, ZZ, and DC analyzed and interpreted the data. YZ, CL, QL, and JC wrote the draft. JC, HJ, SL, ZZ, and DC critically revised the manuscript. SG, HJ, ZZ, and DC provided study materials. All authors read and approved the final manuscript. YZ, XF, and JC take responsibility for the integrity of the data and the accuracy of the data analysis.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

In contrast, the activity of *Col2a1-CreERT²* is mainly limited to the IAF of IVD tissues at either stage of tamoxifen injection. Similarly, *Shh-Cre* directs recombination specifically in all NP cells, whereas *Shh-CreERT²* is active only in a few NP cells when tamoxifen is administered at either stage. Finally, *Osx-Cre* targets cells in the CEP, but not in the NP or AF of IVDs tissues at these two stages. Thus, our data demonstrated that all these Cre lines can direct recombination in IVD tissues at postnatal stages with different cell type specificity and/or targeting efficiency, and can, therefore, serve as valuable tools to dissect cell type-specific gene functions in IVD development and homeostasis.

Keywords

annulus fibrosus; Cre recombinase; intervertebral disc; nucleus pulposus

1 | INTRODUCTION

Low back pain is a common musculoskeletal problem, with its lifetime incidence reaching as high as 84% (Risbud & Shapiro, 2014). As the leading cause of musculoskeletal disabilities, low back pain can bring devastating consequences to the patients, and add enormous socioeconomic burdens to the societies (Bedore et al., 2013; Wang et al., 2012). Although it is well accepted that low back pain is primarily caused by degeneration of intervertebral disc (IVD), no disease-modifying therapeutic approach is currently available, largely due to our limited understanding of the molecular mechanisms underlying IVD homeostasis and degeneration.

The mouse has emerged as a valuable animal model to study IVD biology, since IVDs in mouse and human have the similar structure, and exhibit similar age-related histological changes (Zhang et al., 2018). As a specialized structure located between two adjacent vertebral bodies, IVD has three major components in mice, including the nucleus pulposus (NP), the annulus fibrosus (AF), and the cartilaginous endplate (CEP) (Zheng et al., 2018). Each of these components contains distinct cell types that are believed to express specific matrix components, as well as signal molecules and growth factors to delicately regulate IVD development and homeostasis. Disruption of these regulations may cause IVD degeneration (Bedore, Quesnel, Quinonez, Seguin, & Leask, 2016). However, efforts to identify the contribution of the specific cell type to the disease development are hindered by the limited tools that can target specific cell type populations within the IVDs.

Cre/LoxP technology is an important genetic tool that enables gene deletion or overexpression in a cell type-specific and/or temporal-controlled manner, and has made significant contributions to the understanding of roles of various genes under both physiological and pathological conditions in mice (Henry et al., 2009; Lim, Burclaff, He, Mills, & Long, 2017). In this system, expression of Cre recombinase is driven by a cell type-specific promoter/enhancer, therefore allowing for cell type-specific targeting. Moreover, the temporal control can be achieved by the approach of tamoxifen-inducible Cre recombinase, commonly known as the CreER^{T2} system that is a fusion protein of Cre with a mutated ligand-binding domain of estrogen receptor (Leone et al., 2003). In this modified

Cre system, CreER^{T2} fusion protein is translocated to the nucleus and subsequently directs recombination only upon tamoxifen induction (Hayashi & McMahon, 2002; Leone et al., 2003). In recent decades, a number of Cre and CreER^{T2} mouse lines have been generated for targeting osteoblast or chondrocyte lineage cells in long bones (Chen et al., 2007; Chen, Li, Xie, Wang, & Chen, 2014b; Harfe et al., 2004; Henry et al., 2009; Long, Zhang, Karp, Yang, & McMahon, 2001; Rodda & McMahon, 2006), and have been proven to be valuable tools to study the biology of bone and cartilage (Chen & Long, 2018; Long, 2011; Long & Ornitz, 2013). Although several studies indicated that at least some of these Cre lines also targeted the disc cells (Chen et al., 2007; Choi & Harfe, 2011; Henry et al., 2009), these tools have not been widely used to study IVD development and homeostasis, partly due to the lack of a detailed and systematic analysis of their targeting specificity and efficiency in IVDs tissues at postnatal stages.

In this study, we aimed to systematically analyze recombination directed by *Agc1-CreER^{T2}*, *Col2a1-Cre*, *Col2a1-CreER^{T2}*, *Shh-Cre*, *Shh-CreER^{T2}*, and *Osx-Cre* mice, in IVDs at postnatal growing and adult stages, by employing *R26-mTmG*, a robust and sensitive dual fluorescent Cre reporter mouse. We found that all these Cre lines can mediate recombination in IVD cells at these two postnatal stages with different cell type specificity and/or targeting efficiency. Thus, our study has provided critical insights into the utilization of these genetic tools, either alone or in combination, to dissect the cellular and molecular mechanisms underlying IVD development and homeostasis.

2 | MATERIALS AND METHODS

2.1 | Mouse lines and breeding

Mouse lines used in this study, including *Col2a1-Cre* (Long et al., 2001), *Col2a1-CreER^{T2}* (Chen et al., 2007, Chen et al., 2014b), *Osx-Cre* (Rodda & McMahon, 2006), *Agc1-CreER^{T2}* (Henry et al., 2009), *Shh-Cre* (Harfe et al., 2004), *Shh-CreER^{T2}* (Harfe et al., 2004), and *R26-mTmG* mice (Muzumdar, Tasic, Miyamichi, Li, & Luo, 2007), were described previously. *Col2a1-Cre*, *Col2a1-CreER^{T2}*, and *Agc1-CreER^{T2}* mice were provided by Dr. Di Chen (Rush University, Chicago). *Osx-Cre* and *Shh-CreER^{T2}* mice were gifts from Dr. Ting Chen (Beijing Institute of Biological Sciences, Beijing, China) and Dr. Xiaochun Bai (Southern Medical University, Guangzhou, China), respectively. *Shh-Cre* and *R26-mTmG* mice were purchased from Jackson Laboratories. To map Cre target cells in IVD tissues at postnatal stages, the above Cre mouse lines were separately crossed with *R26-mTmG* mice to get double heterozygous mice. For conventional Cre mouse lines (*Col2a1-Cre*, *Shh-Cre*, and *Osx-Cre*), the double heterozygous were killed at 2 weeks and 2 months of ages. For tamoxifen-inducible Cre mouse lines (*Agc1-CreER^{T2}*, *Col2a1-CreER^{T2}*, and *Shh-CreER^{T2}*), the double heterozygous mice were subjected to tamoxifen or vehicle (corn oil) administration before analysis. All mice were housed in a specific pathogen-free facility with controlled temperature and humidity at a 12-hr day/night cycle. All mice had free access to standard chow diet and sterilized water, and were monitored daily for health status. No adverse events were observed throughout the experiments. All animal protocols in this study were reviewed and approved by the Animal Studies

Committee at Soochow University and all experimental methods and procedures were carried out in accordance with the approved guidelines.

2.2 | Tamoxifen administration

Tamoxifen (T5684; Sigma, St. Louis, MI) was dissolved in corn oil at a concentration of 10 mg/ml. Two-week-old or two-month-old double heterozygous mice (*Col2a1-CreER^{T2}*; *R26-mTmG*, or *Agc1-CreER^{T2}*; *R26-mTmG*, or *Shh-CreER^{T2}*; *R26-mTmG* mice) were randomly divided into two groups (three mice per group for each genotype). Group I and II mice were injected intraperitoneally with tamoxifen (100 µg per gram of body weight) and corn oil once daily for 5 consecutive days, respectively. All these mice were harvested one day after the last injection.

2.3 | Sample preparation

Cryojane sections were prepared as previously reported (Chen & Long, 2013; Chen et al., 2014a). Briefly, IVD samples were isolated and fixed in freshly made 4% paraformaldehyde overnight at 4°C. After three washes in PBS (5 min each), the fixed IVDs were decalcified in 14% EDTA for 3 days at 4°C on a rocker. After decalcification, IVDs were cryoprotected in 15% sucrose in PBS for 2 hr and 30% sucrose in PBS overnight at 4°C. Samples were then snap-frozen in OCT embedding medium. Frozen tissue blocks were sectioned at a thickness of 8 µm using a Leica CM3050S cryostat and the CryoJane Tape Transfer System (Leica, Buffalo Grove, IL). IVD sections were mounted onto adhesive coated slides (Leica), and stored at -20°C until they were analyzed.

2.4 | Immunofluorescence staining

Immunofluorescence staining was performed to enhance GFP signals as described previously (Chen et al., 2014a). Briefly, frozen sections were incubated with 5% goat serum in PBS to prevent nonspecific binding and then incubated with a chicken polyclonal GFP antibody (1:2,500; Abcam, Cambridge, MA) in a humidified chamber overnight at 4°C. After three washes in PBST, sections were incubated with Alexa Fluor® 488-conjugated goat anti-chicken antibody (1:250; Life Technologies, Grand Island, NY) for 1 hr. Sections were mounted with VECTASHIELD Mounting Medium and imaged with a fluorescence microscope. The primary and secondary experimental outcomes were assessing IVD components that exhibited GFP signals, and the percentage of GFP-positive cells in these structures, respectively.

2.5 | Statistical analysis

All quantitative results were shown as mean ± standard deviation. Student's *t* test was used to determine the statistical difference between two groups. Any difference with a *p* value < 0.05 was considered as statistically significant. For each test, the experimental unit was a single mouse. The quantitative results were calculated from three independent mice for each group.

3 | RESULTS

3.1 | *Agc1-CreER^{T2}* is active in all cell types of postnatal IVDs

Agc1-CreER^{T2} (also known as *aggrecan-CreER^{T2}*) is a knock-in mouse line expressing a tamoxifen-inducible Cre recombinase (*CreER^{T2}*) from the endogenous aggrecan locus (Henry et al., 2009). Initial characterization of this mouse line using R26R reporter detected inducible expression of Cre recombinase in IVDs of 6-month-old mice (Henry et al., 2009). To further determine the cell type specificity and targeting efficiency of *Agc1-CreER^{T2}* in IVD tissues at postnatal growing and adult stages, we generated 2-week-old or 2-month-old *Agc1-CreER^{T2}; R26-mTmG* mice and treated these mice with either corn oil or tamoxifen for 5 consecutive days ($n=3$ independent mice for each group). We harvested IVDs 1 day after the last treatment. GFP staining of CryoJane sections revealed abundant GFP-positive cells in IVDs in the mice administered with tamoxifen (Figure 1a,b). In contrast, IVDs from corn oil-injected mice exhibited little to no GFP signal (Supporting Information Figure S1). A closer examination of magnified images from tamoxifen-treated mice revealed that all the cells in the NP and all the chondrocytes within the growth plate of the vertebral body expressed GFP when tamoxifen was injected at either 2 weeks or 2 months of age (Figure 1c–f). Similarly, $47.1 \pm 4\%$ and $57.3 \pm 4.3\%$ of GFP-positive cells were detected in the CEP in 2-week-old and 2-month-old mice, respectively (Figure 1c,d). Moreover, GFP-labeled cells were observed in both OAF and IAF of *Agc1-CreER^{T2}; R26-mTmG* mice, but their numbers decreased when tamoxifen was administered in older mice (Figure 1g,h). Nearly 100% of IAF and OAF cells were GFP-positive when tamoxifen was injected at 2 weeks of age, whereas these numbers dropped to $56 \pm 4\%$ ($n=3$) and $35 \pm 5\%$ ($n=3$) respectively, when tamoxifen was injected at 2 months of age (Figure 1g,h). Taken together, our data confirmed the activity of *Agc1-CreER^{T2}* and determined its cell type specificity and targeting efficiency in IVD tissues at postnatal stages.

3.2 | *Col2a1-Cre* mediates recombination in the majority of cells in IVD tissues at postnatal stages

Col2a1-Cre is a transgenic mouse line in which the expression of Cre recombinase is under the control of a type II collagen promoter (*Col2a1*; Long et al., 2001). *Col2a1-Cre* was shown to target chondrocytes and osteoblastic lineage cells (including osteocytes, osteoblasts, and their precursors/progenitors) in long bones (Nagao, Cheong, & Olsen, 2016). To characterize its activity in IVDs, we generated *Col2a1-Cre; R26-mTmG* compound heterozygous mice, and harvested IVDs at 2 weeks and 2 months of ages. GFP staining showed that IVD sections from *Col2a1-Cre; R26-mTmG* mice contained many GFP-positive cells (Figure 2a,b). Examination of higher magnification images revealed that the pattern and level of GFP expression were similar between 2-week-old and 2-month-old mice. First, all the cells in the CEP and all the chondrocytes within the GP were GFP-positive ($n=3$) (Figure 2c,d). Secondly, about 73% of cells in the IAF expressed GFP at both ages (Figure 2e), whereas only 27.7% and 10.6% cells in the OAF were GFP-positive at 2 weeks and 2 months of ages, respectively (Figure 2e). Finally, about 75% of NP cells, are GFP-positive at either age (Figure 2g,h). Collectively, our data demonstrated that *Col2a1-Cre* targets most of the cells in postnatal IVDs, except for some cells in the OAF and NP.

3.3 | *Col2a1-CreER^{T2}* is leaky, and mainly targets cells in the IAF of IVD tissues at postnatal stages

Col2a1-CreER^{T2} is a tamoxifen-inducible version of *Col2a1-Cre* in which the Cre recombinase was fused to a mutated ligand-binding domain of estrogen receptor (ER^{T2}) as a fusion protein (CreER^{T2}) (Chen et al., 2007). In *Col2a1-CreER^{T2}* mice, the expression of CreER^{T2} is regulated by *Col2a1* promoter, whereas its nuclear translocation and subsequent Cre-mediated recombination is dependent on the tamoxifen induction. To determine the cell type specificity and targeting efficiency of *Col2a1-CreER^{T2}* in IVD tissues at postnatal stages, we administered either corn oil or tamoxifen into 2-week-old or 2-month-old *Col2a1-CreER^{T2}*; *R26-mTmG* mice, and harvested IVD tissues 1 day after the last injection. Visualization of Cryo-jane sections revealed that the numbers of GFP-labeled cells in IVD tissues from *Col2a1-CreER^{T2}*; *R26-mTmG* mice decreased with ages when tamoxifen was administered (Figure 3a,b). Specifically, GFP expression was present in nearly all chondrocytes within the GP and all cells in the IAF of *Col2a1-CreER^{T2}*; *R26-mTmG* mice when tamoxifen was injected at 2 weeks of age (Figure 3c,e). However, only $54 \pm 4\%$ ($n = 3$) of growth plate chondrocytes and $30 \pm 3\%$ ($n = 3$) of cells in the IAF were GFP-positive when tamoxifen was injected at 2 months of age (Figure 3d,f). Interestingly, about 99% OAF cells were GFP-negative in both 2-week-old and 2-month-old mice (Figure 3c,d). Moreover, only $6.7 \pm 3\%$ and $5.2 \pm 0.7\%$ of cells in the CEP exhibited GFP expression in mice at 2 weeks and 2 months of ages, respectively (Figure 3e,f). Surprisingly, cells in the NP did not show any GFP signal regardless of ages when tamoxifen was injected (Figure 3g,h). To determine the leakage expression of Cre recombinase in *Col2a1-CreER^{T2}* mice, we also examined IVD sections from corn oil-treated *Col2a1-CreER^{T2}*; *R26-mTmG* mice. As shown in Supporting Information Figure S1, about 5.1% cells in the IAF and 4.2% cells in the CEP exhibited GFP expression, indicating the leakiness for Cre expression in these mice. Taken together, our data demonstrated that *Col2a1-CreER^{T2}* mice exhibit the leakage expression of Cre recombinase, and mainly target cells in the IAF of IVD tissues at postnatal stages.

3.4 | *Shh-Cre* mediates recombination in all NP cells of IVD tissues at postnatal stages

Shh-Cre (also known as *Shh::GFPCre*) is a knock-in mouse line in which a GFP::Cre fusion gene is targeted into the *Shh* locus so that the expression of Cre recombinase is turned on in cells that normally express *Shh* (Harfe et al., 2004). Previous fate-mapping studies showed *Shh-Cre* specifically targeted NP cells in the IVDs of mouse embryos and the aged mice (Choi, Cohn, & Harfe, 2008), which is consistent with the fact that *Shh* is expressed in notochord where nucleus pulposus tissues are derived. To further characterize cell type specificity and targeting efficiency of *Shh-Cre* in IVD tissues at postnatal stages, we generated *Shh-Cre*; *R26-mTmG* compound heterozygous mice, and harvested IVDs at 2 weeks and 2 months of ages. A thorough examination of IVD sections from three independent mice for each stage demonstrated that 100% NP cells were labeled by GFP (Figure 4). Interestingly, we also noticed a few GFP-positive cells in the GP and CEP (Figure 4c,d), which probably represent notochordal remnants as previously suggested (Choi et al., 2008). In contrast, all other IVD tissues did not show any GFP signal (Figure 4). Together, our data indicated that *Shh-Cre* drives recombination specifically in the NP cells of IVD tissues at postnatal stages.

3.5 | *Shh-CreER^{T2}* specifically targets a few cells located at the NP in IVD tissues at postnatal stages

Shh-CreER^{T2} is a tamoxifen-inducible Cre mouse line in which the gene encoding the CreER^{T2} fusion protein has been inserted into the *Shh* locus so that expression of Cre recombinase is controlled by endogenous *Shh* locus, and its nuclear translocation and subsequent Cre-mediated recombination, are induced by tamoxifen (Harfe et al., 2004). *Shh-CreER^{T2}* has been shown to specifically target the NP cells when induced at early embryonic stages (E8.5-E11.5; Choi & Harfe, 2011). To determine the activity of *Shh-CreER^{T2}* in IVD tissues at postnatal stages, we administered either corn oil or tamoxifen into 2-week-old or 2-month-old *Shh-CreER^{T2}; R26-mTmG* mice, and harvested IVD tissues 1 day after the last injection. GFP staining of Cryojane sections showed that only a few cells are GFP-positive in IVDs from the tamoxifen-injected mice (Figure 5a,b). Specifically, an average of 1.6% GFP-positive cells ($1.6 \pm 0.2\%$, $n = 3$) were detected in each IVD section of *Shh-CreER^{T2}; R26-mTmG* mice that were injected with tamoxifen at 2 weeks of age (Figure 5c,e,g), whereas only 0.5% GFP-positive cells ($0.5 \pm 0.3\%$, $n = 3$) appeared in each IVD section when tamoxifen was given at 2 months of age (Figure 5d,f,g). Interestingly, all GFP-labeled cells are within the NP tissues that are immediately underneath the CEP. To determine the leakage expression of Cre recombinase in *Shh-CreER^{T2}* mice, we also examined IVD sections from three corn oil-treated *Shh-CreER^{T2}; R26-mTmG* mice and no GFP-positive cells were observed (data not shown), indicating no leakiness for Cre expression in these mice. Together, our data indicated that *Shh-CreER^{T2}* specifically targets NP cells with low targeting efficiency in IVD tissues at postnatal stages.

3.6 | *Osx-Cre* targets cells in the CEP of IVDs tissues at postnatal stages

Osx-Cre (also known as *Osx1-GFP::Cre* and *Sp7-Cre*) is a BAC transgenic mouse line in which expression of a GFP::Cre fusion protein is under the control of the Osterix gene regulatory elements (Rodda & McMahon, 2006). *Osx-Cre* was shown to target multiple cell types in long bones in mice at the postnatal stage, including osteoblasts, osteocytes, and prehypertrophic chondrocytes, as well as stromal cells, adipocytes, and perivascular cells in the bone marrow (Chen et al., 2014a; Liu et al., 2013; Mizoguchi et al., 2014). To characterize the potential targeting of IVDs by *Osx-Cre* in postnatal mice, we generated *Osx-Cre; R26-mTmG* mice compound heterozygous mice, and harvested IVDs at 2 weeks and 2 months of ages. GFP staining detected some GFP-positive cells in IVD sections in *Osx-Cre; R26-mTmG* mice (Figure 6a,b). Examination of higher magnification images revealed that IVDs from both 2-week-old and 2-month-old mice exhibited a similar pattern and level of GFP expression (Figure 6C–H). First, some prehypertrophic and hypertrophic chondrocytes within the growth plate of vertebral bodies were GFP-positive in both 2-week-old and 2-month-old mice (Figure 6e,f), consistent with the previous reports of its similar activity in long bones (Chen et al., 2014a; Liu et al., 2013). Secondly, $28.9 \pm 6\%$ and $47.8 \pm 2.5\%$ cells in the CEP were GFP-positive at 2 weeks and 2 months of ages, respectively (Figure 6e,f). Finally, the NP and AF tissues did not exhibit any GFP signal at either age (Figure 6c,d,g,h). Collectively, our data indicated that *Osx-Cre* targets cells in the CEP of IVD tissues as well as some prehypertrophic and hypertrophic chondrocytes within the GP at postnatal stages.

4 | DISCUSSION

Low back pain is mainly caused by IVD degeneration, a degenerative disease initiated in the postnatal life. The Cre/LoxP technology has significantly advanced our understanding of gene functions in development and disease of various tissues. In recent years, several Cre mouse lines have been generated to target specific cell types in long bones. However, the information for their activity in IVD tissues at the postnatal stages was very limited, which prevented the utilization of these genetic tools to study IVD homeostasis and degeneration. In this study, we systematically assessed recombination specificity and efficiency of *Agc1-CreER^{T2}*, *Col2a1-Cre*, *Col2-CreER^{T2}*, *Shh-Cre*, *Shh-CreER^{T2}*, and *Osx-Cre* mice in the IVDs of both 2-week-old and 2-month-old mice, and provided the first detailed description of their activity in postnatal IVDs. Our data will facilitate the utilization of these Cre lines to study IVD biology.

Our data clearly showed that *Col2a1-Cre* targets the majority of cells in the NP. In contrast, we did not detect any GFP expression in NP cells of *Col2a1-CreER^{T2}*; *R26-mTmG* mice when induced by tamoxifen at either 2 weeks or 2 months of age. This finding is consistent with the previous report using *Col2a1-CreER^{T2}*; *R26-lacZ* mice (Jin et al., 2011). The reason behind the discrepancy between *Col2a1-Cre* and *Col2a1-CreER^{T2}* is still not clear. One possibility is that *Col2a1* gene only turns on at an early embryonic stage but not the postnatal stage in NP cells. Alternatively, it is possible that *Col2a1* is normally expressed in postnatal NP cells, but its expression is lost due to the positional effect of a transgene, a common phenomenon that the expression level or pattern of a transgene is altered by its integration site in the chromosome.

Our data showed that *Osx-Cre*-targeted cells include cells in the CEP and GP, but not in the NP or AF tissues in both 2-week-old and 2-month-old mice. Therefore, *Osx-Cre* could be used for targeting CEP cells without affecting NP and AF cells. Moreover, our direct visualization of IVDs from postnatal *Osx-Cre* mice detected GFP-positive cells specifically in the CEP and GP, likely reflecting the endogenous expression of *Osx* in these cells (data not shown). Many studies used extracellular matrix(ECM)-related genes (such as *Sox9*, *Col2a1*, and *Agc1*) as markers to assess phenotypes of NP cells. However, these genes are also expressed by chondrocytes, therefore not specific markers for either NP cells or chondrocytes. Since *Osx* is expressed in prehypertrophic and early hypertrophic chondrocytes, but not in NP or AF cells, *Osx* may be used as a marker to distinguish between NP cells and chondrocytes, when used in combination with these ECM-related genes.

Our data showed that *Col2a1-CreER^{T2}* mainly targets cells in the IAF of IVD tissues at postnatal stages. In contrast, none of these six Cre mouse lines enables specific targeting of cells in the OAF of postnatal IVDs. Interestingly, Bedore et al., 2016 recently characterized Cre activity of *Col1a2-CreER*, a transgenic mouse line in which expression of a CreER fusion protein is driven by the regulatory elements of the type I collagen gene (*Col1a2*), in IVD tissues (Ponticos et al., 2004). They showed that *Col1a2-CreER* mediated recombination specifically in the OAF, but not in the IAF or NP, when induced by tamoxifen at 3 weeks of ages and analyzed 100 days later (Bedore et al., 2016). Thus, *Col2a1-CreER^{T2}*

and *Col1a2-CreER* may be useful for delineating IAF- and OAF-specific gene functions in IVD development and homeostasis, respectively.

Our studies also added some important insights into the embryonic origin of the NP tissues and the heterogeneity of their resident cells. On one hand, our detailed analysis of IVDs from *Shh-Cre; R26-mTmG* mice revealed that the postnatal NP is entirely derived from *Shh*-expressing cells, supporting previous observations that all NP cells were descendants of *Shh*-positive notochord cells. Our results were also in line with a previous study that traced notochord-derived cells using *Noto-Cre*, a knock-in mouse generated by targeting Cre into the Noto (a notochord-specific homeobox transcription factor) locus (McCann, Tamplin, Rossant, & Seguin, 2012). Like *Shh-Cre*, *Noto-Cre* was found to specifically target all cells within the NP of postnatal IVDs (McCann et al., 2012). On the other hand, our fate-mapping of *Col2a1-Cre*-labeled cells indicated that the NP cells are not homogenous, and can be divided into two populations based on whether they are targeted by *Col2a1-Cre*. Earlier studies suggested that there were two distinct cell populations residing in the NP, including larger notochordal cells and smaller chondrocyte-like cells (Zhang et al., 2018). It will be interesting to determine whether *Col2a1-Cre* specifically targets chondrocyte-like NP cells. In addition, future studies are warranted to determine when notochordal cells or their descendants become *Col2a1*-positive cells.

In conclusion, our study revealed that all these Cre lines direct recombination in IVD tissues at the postnatal growing and adult stages, but with different cell type specificity and/or targeting efficiency. Thus, these tools can be utilized to dissect cell type-specific gene functions in IVD homeostasis and degeneration.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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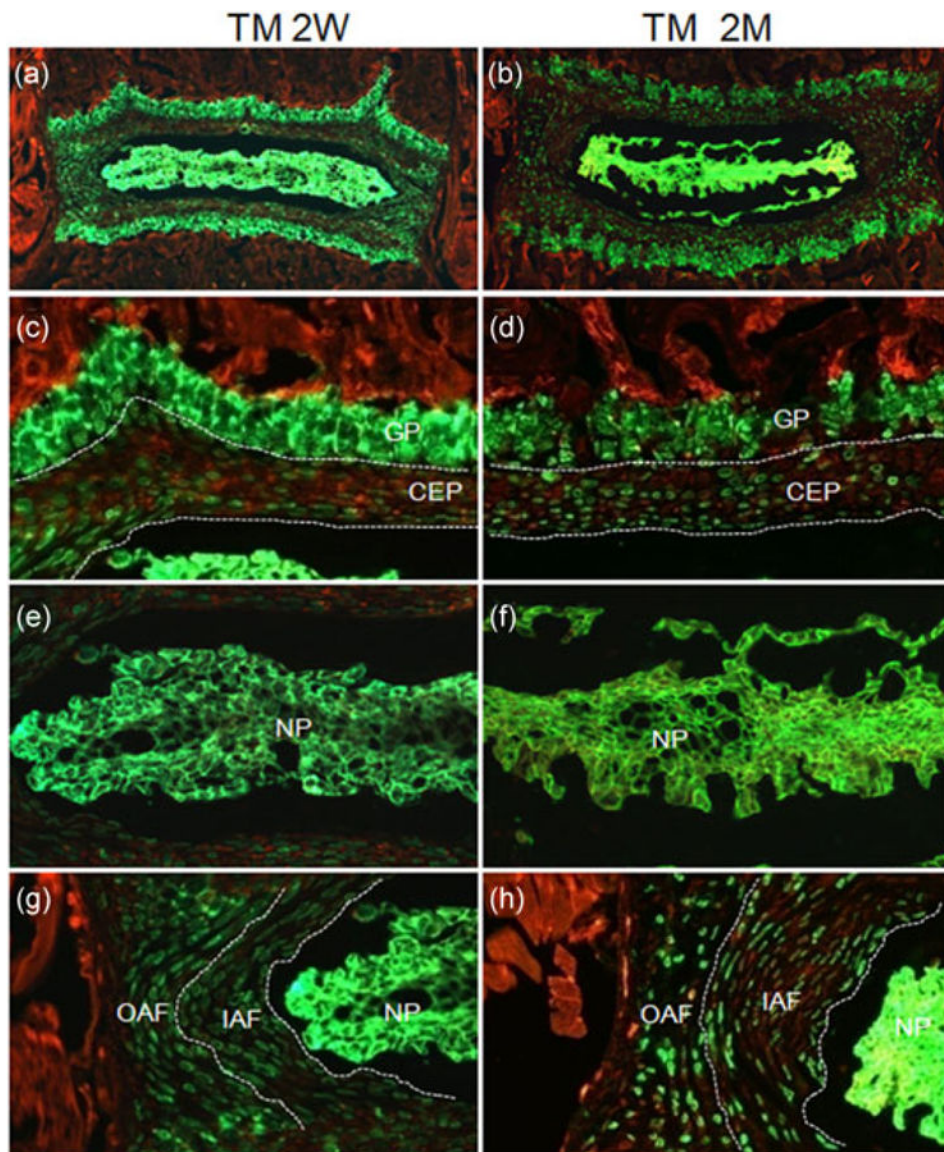


FIGURE 1. *Agc1-CreERT2* is active in all cell types of IVD tissues at postnatal stages. *Agc1-CreERT2*; *R26-mTmG* mice were treated with tamoxifen at 2 weeks (TM 2W) or 2 months (TM 2M) of ages. (a,b) Fluorescence images of EGFP/ mTomato on intervertebral disc sections from TM 2W (a) and TM 2M (b) mice. (c–h) Higher magnification EGFP/mTomato images for CEP/GP (c,d), NP (e,f), and AF (g, h) from TM 2W (c,e,g) and TM 2M (d,f,h) mice. Red: membrane-targeted tdTomato; Green: membrane-targeted EGFP. CEP: cartilaginous endplate; GP: growth plate cartilage; IAF: inner annulus fibrosus; IVD: intervertebral disc; NP: nucleus pulposus; OAF: outer annulus fibrosus. Images shown were representative results from three independent mice analyzed [Color figure can be viewed at wileyonlinelibrary.com]

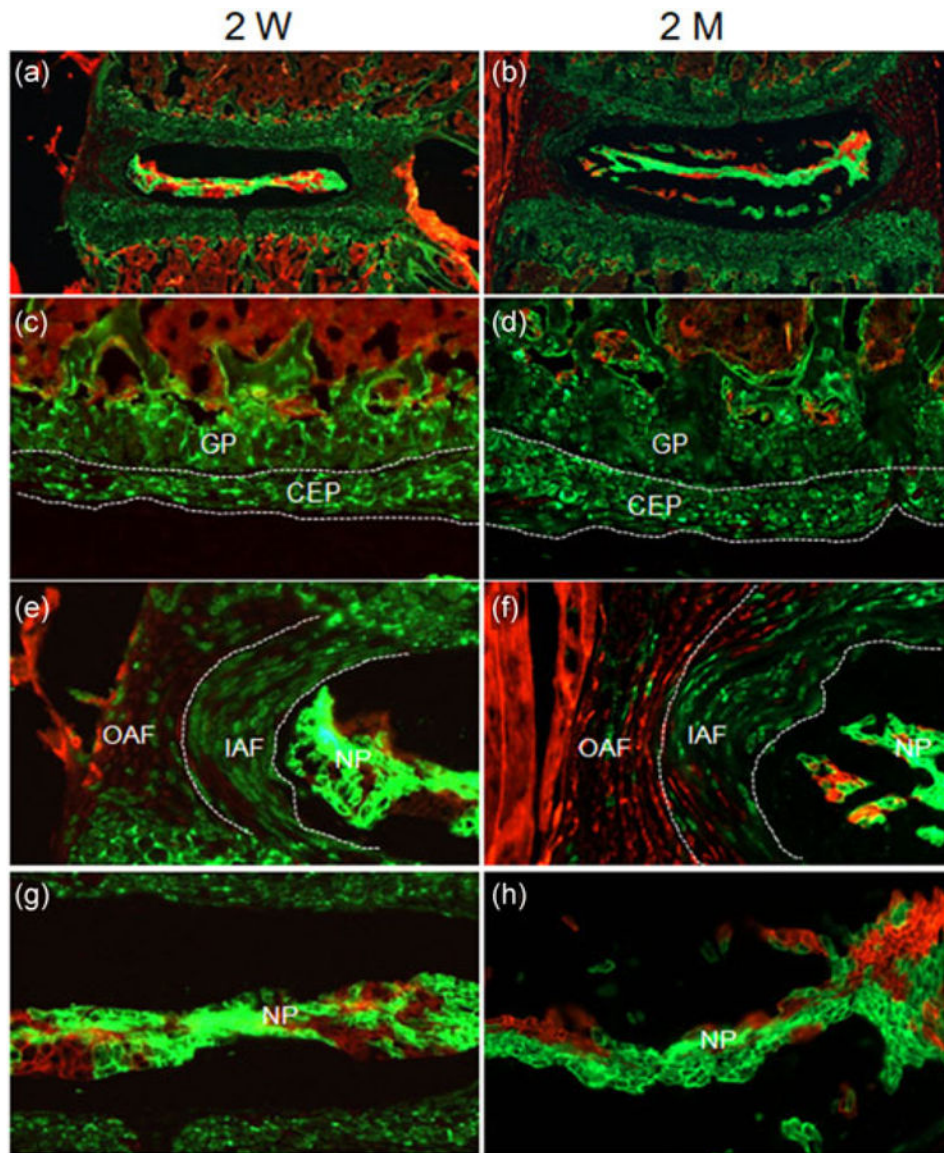


FIGURE 2.

Col2a1-Cre mediates recombination in the majority of cells in IVD tissues at postnatal stages. (a,b) Fluorescence images of EGFP/mTomato on intervertebral disc sections from 2-week-old (a) and 2-month-old (b) *Col2a1-Cre; R26-mTomG* mice. (c–h) Higher magnification EGFP/mTomato images for CEP/GP (c,d), AF (e,f), and NP (g,h) from 2-week-old (c,e,g) and 2-month-old (d,f,h) *Col2a1-Cre; R26-mTomG* mice. Red: membrane-targeted tdTomato; Green: membrane-targeted EGFP. CEP: cartilaginous endplate; GP; growth plate cartilage; IAF: inner annulus fibrosus; IVD: intervertebral disc; NP: nucleus pulposus; OAF: outer annulus fibrosus. Images shown were representative results from three independent mice analyzed [Color figure can be viewed at wileyonlinelibrary.com]

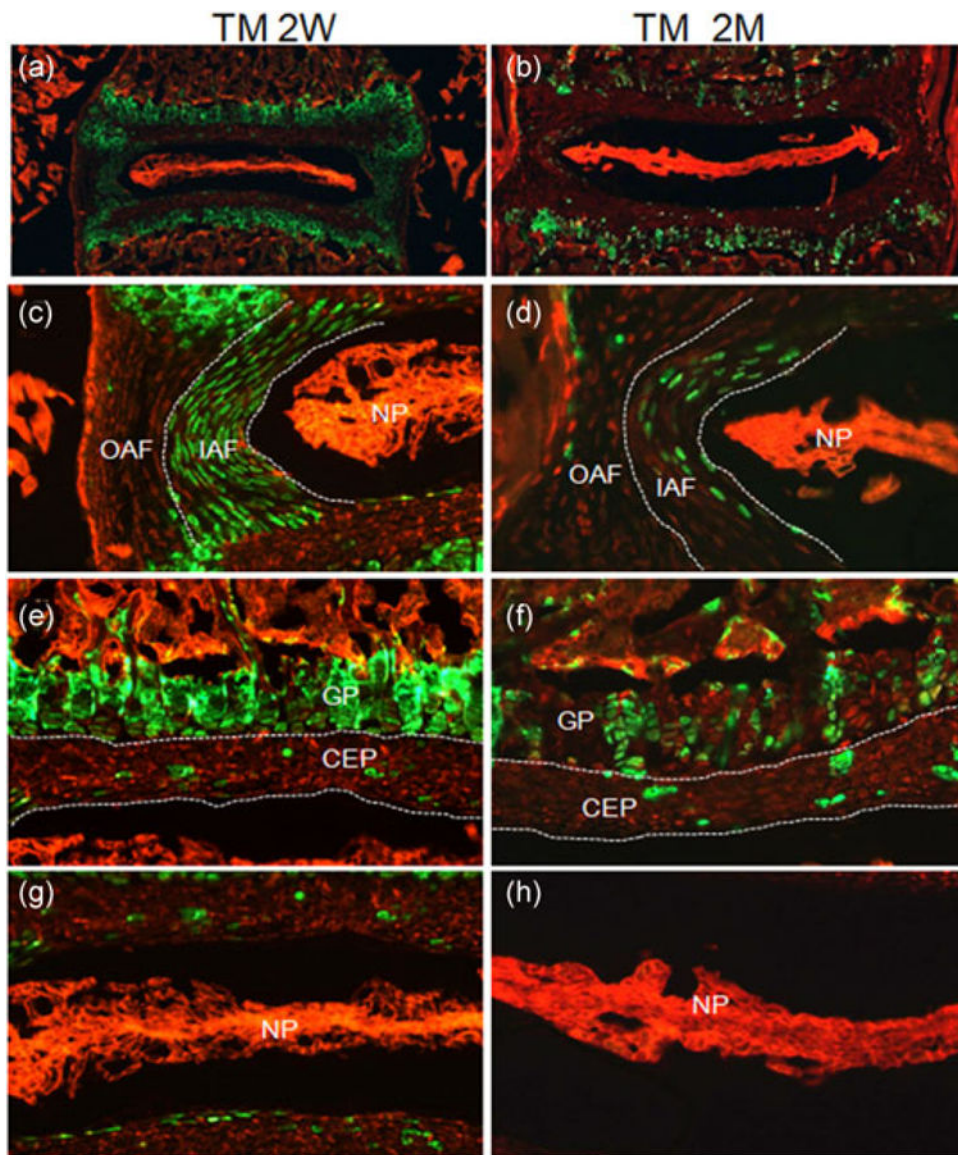


FIGURE 3.

Col2a1-CreER^{T2} mainly targets cells in the IAF of IVD tissues at postnatal stages. *Col2a1-CreER^{T2}* mice were treated with tamoxifen at 2 weeks (TM 2W) or 2 months (TM 2M) of ages. (a,b) Fluorescence images of EGFP/ mTomato on intervertebral disc sections from TM 2W (a) and TM 2M (b) *Col2a1-CreER^{T2}; R26-mTmG* mice. (c–h) Higher magnification EGFP/mTomato images for AF (c,d), CEP/GP (e,f), and NP (g,h) from TM 2W (c,e,g) and TM 2M (d,f,h) *Col2a1-CreER^{T2}; R26-mTmG* mice. Red: membrane-targeted tdTomato; Green: membrane-targeted EGFP. CEP: cartilaginous endplate; GP: growth plate cartilage; IAF: inner annulus fibrosus; IVD: intervertebral disc; NP: nucleus pulposus; OAF: outer annulus fibrosus. Images shown were representative results from three independent mice analyzed [Color figure can be viewed at wileyonlinelibrary.com]

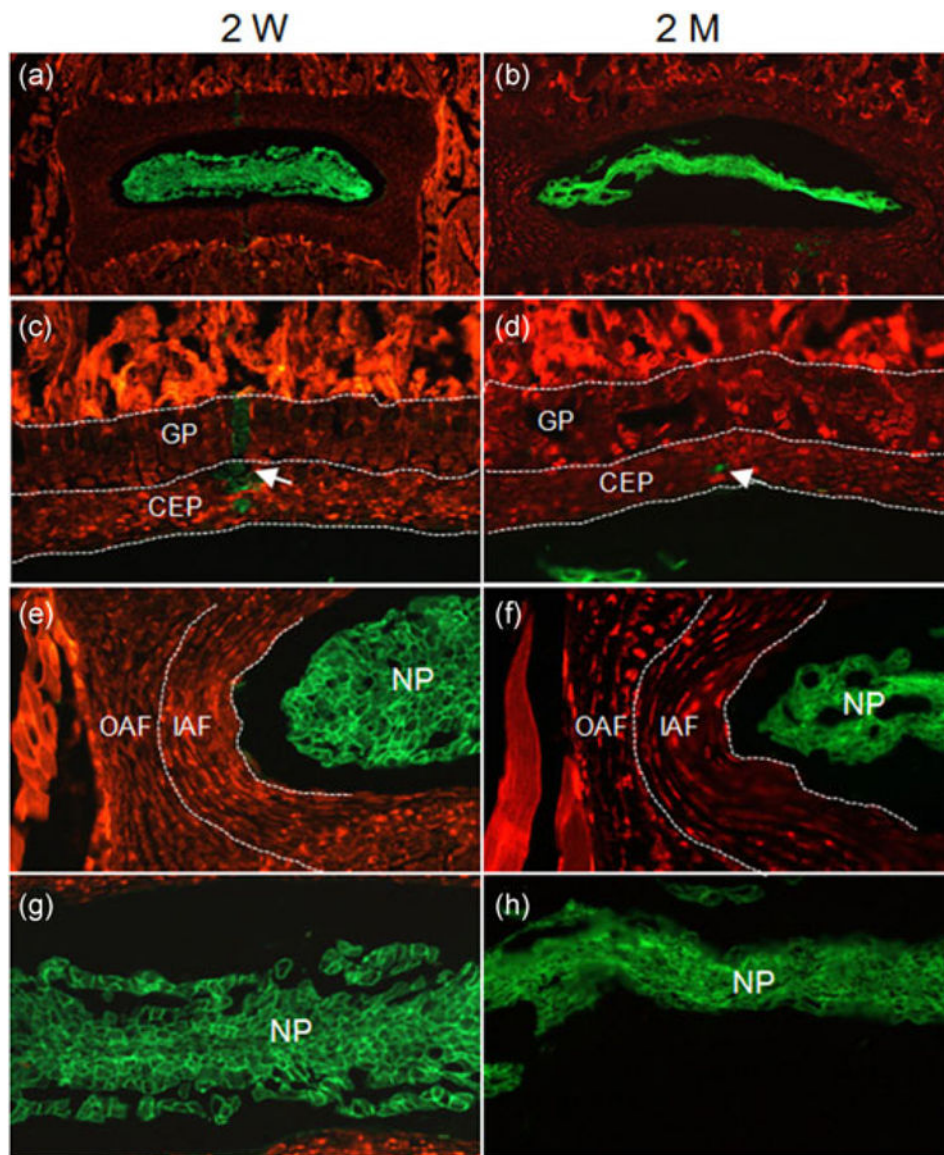


FIGURE 4. *Shh-Cre* mediates recombination in all NP cells of IVD tissues at postnatal stages. (a,b) Fluorescence images of EGFP/mTomato on intervertebral disc sections from 2-week-old (a) and 2-month-old (b) *Shh-Cre; R26-mTomG* mice. (c–h) Higher magnification EGFP/mTomato images for CEP/GP (c,d), AF (e,f) and NP (g,h) from 2-week-old (c,e,g) and 2-month-old (d,f,h) *Shh-Cre; R26-mTomG* mice. Red: membrane-targeted tdTomato; Green: membrane-targeted EGFP. CEP: cartilaginous endplate; GP: growth plate cartilage; IAF: inner annulus fibrosus; IVD: intervertebral disc; NP: nucleus pulposus; OAF: outer annulus fibrosus. Arrows point to cells that likely represents notochordal remnants in the CEP/CP. Images shown were representative results from three independent mice analyzed [Color figure can be viewed at wileyonlinelibrary.com]

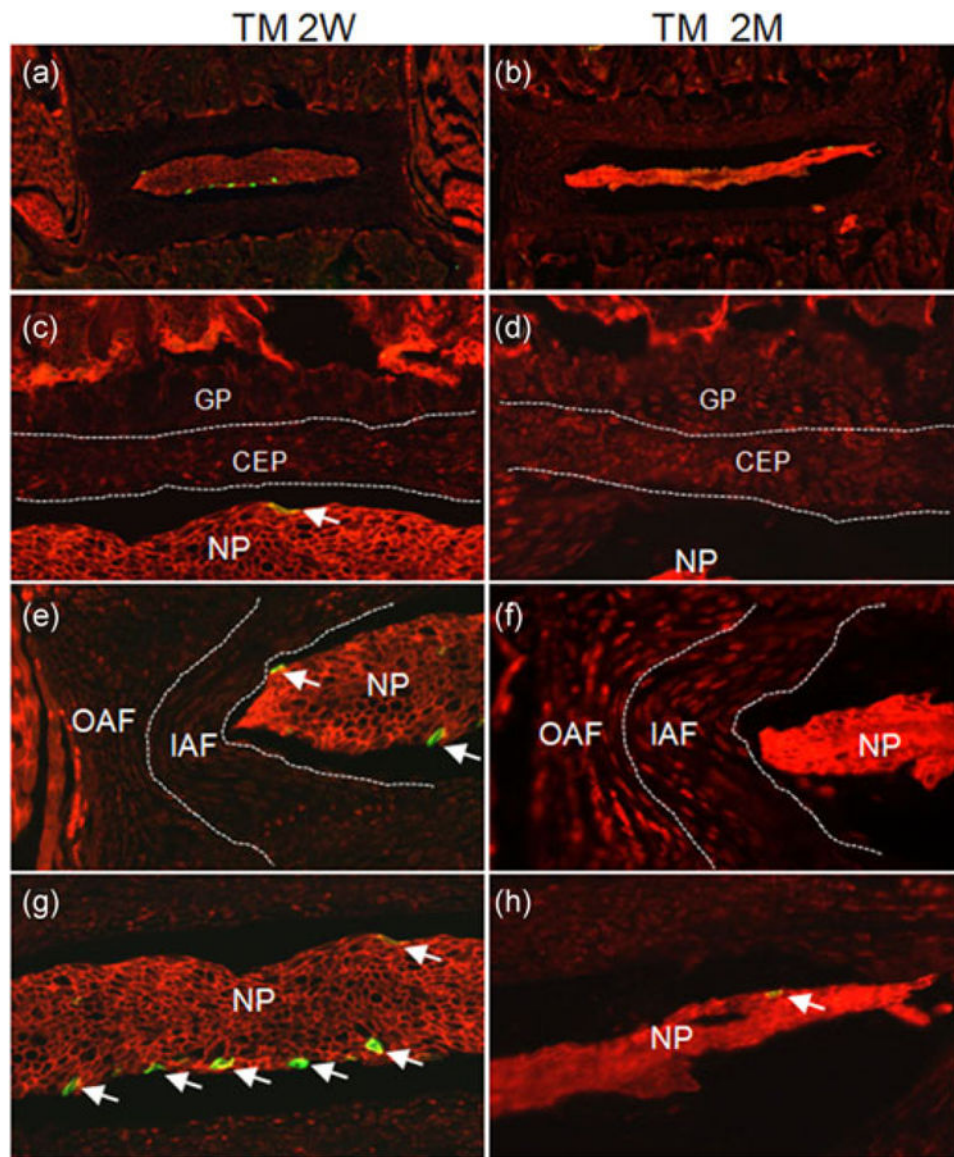


FIGURE 5. *Shh-CreER^{T2}* specifically targets a few cells located at the NP in IVD tissues at postnatal stages. *Shh-CreER^{T2}* mice were treated with tamoxifen at 2 weeks (TM 2W) or 2 months (TM 2M) of ages. (a,b) Fluorescence images of EGFP/mTomato on intervertebral disc sections from TM 2W (a) and TM 2M (b) *Shh-CreER^{T2}; R26-mTomG* mice. (c-h) Higher magnification EGFP/mTomato images for CEP/GP (c,d), AF (e,f), and NP (g,h) from TM 2W (c,e,g) and TM 2M (d,f,h) *Shh-CreER^{T2}; R26-mTomG* mice. Red: membrane-targeted tdTomato; Green: membrane-targeted EGFP. CEP, cartilaginous endplate; GP, growth plate cartilage; IAF, inner annulus fibrosus; IVD: intervertebral disc; NP, nucleus pulposus; OAF, outer annulus fibrosus. Arrows point to GFP-positive NP cells that locate at the border between the NP and CEP. Images shown were representative results from three independent mice analyzed [Color figure can be viewed at wileyonlinelibrary.com]

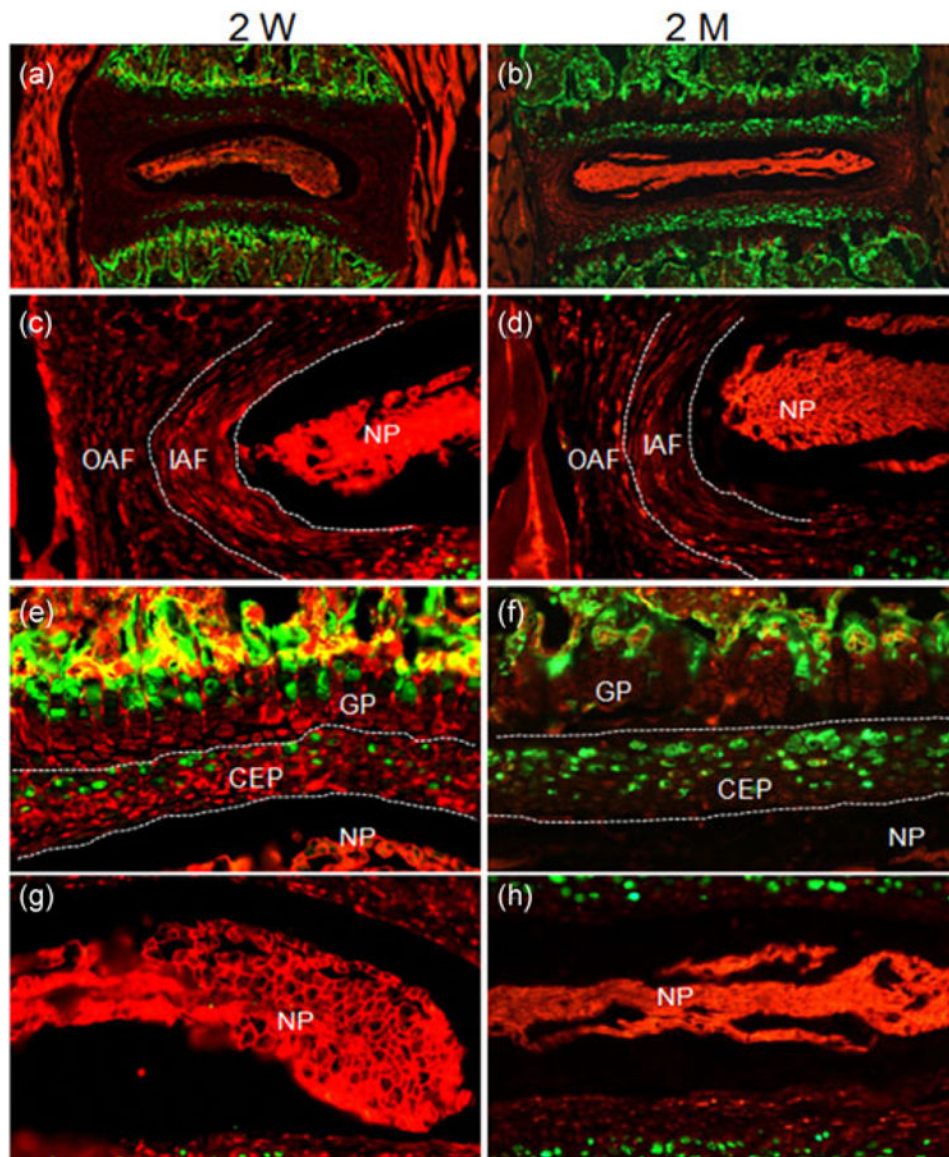


FIGURE 6.

Osx-Cre targets cells in the CEP of IVD tissues at postnatal stages. (a, b) Fluorescence images of EGFP/mTomato on intervertebral disc sections from 2-week-old (a) and 2-month-old (b) *Osx-Cre; R26-mTmG* mice. (c–h) Higher magnification EGFP/mTomato images for AF (c,d), CEP/GP (e,f), and NP (g,h) from 2-week-old (c,e,g) and 2-month-old (d,f,h) *Osx-Cre; R26-mTmG* mice. Red: membrane-targeted tdTomato; Green: membrane-targeted EGFP. CEP: cartilaginous endplate; GP: growth plate cartilage; NP: nucleus pulposus; IAF: inner annulus fibrosus; OAF: outer annulus fibrosus. Images shown were representative results from three independent mice analyzed [Color figure can be viewed at wileyonlinelibrary.com]