HHS Public Access

Author manuscript

J Cell Physiol. Author manuscript; available in PMC 2019 March 27.

Published in final edited form as:

J Cell Physiol. 2018 January ; 234(1): 595–605. doi:10.1002/jcp.26802.

Postnatal deletion of *Alk5* gene in meniscal cartilage accelerates age-dependent meniscal degeneration in mice

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Abstract

Activation of transforming growth factor-β (TGF-β) signaling has been used to enhance healing of meniscal degeneration in several models. However, the exact role and molecular mechanism of TGF-β signaling in meniscus maintenance and degeneration are still not understood due to the absence of in vivo evidence. In this study, we found that the expression of activin receptor-like kinases 5 (ALK5) in the meniscus was decreased with the progression of age and/or osteoarthritis induced meniscal degeneration. *Col2a1* positive cells were found to be specifically distributed in the superficial and inner zones of the anterior horn, as well as the inner zone of the posterior horn in mice, indicating that *Col2a1-CreER*^{T2} mice can be a used for studying gene function in menisci. Furthermore, we deleted *Alk5* in *Col2a1* positive cells in meniscus by administering tamoxifen. Alterations in the menisci structure were evaluated histologically. The expression levels of genes and proteins associated with meniscus homeostasis and TGF-β signaling were analyzed by quantitative real-time PCR analysis (qRT-PCR) and immunohistochemistry (IHC). Our results revealed severe and progressive meniscal degeneration phenotype in 3- and 6-month-old *Alk5* cKO mice compared with Cre-negative control, including aberrantly increased hypertrophic meniscal cells, severe fibrillation, and structure disruption of meniscus. qRT-PCR and IHC results showed that disruption of anabolic and catabolic homeostasis of chondrocytes may contribute to the meniscal degeneration phenotype observed in *Alk5* cKO mice. Thus, TGF-β/ALK5 signaling plays a chondro-protective role in menisci homeostasis, in part, by inhibiting matrix degradation and maintaining extracellular matrix proteins levels in meniscal tissues.

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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

Keywords

activing receptor-like kinases 5 (ALK5); matrix degradation; meniscal degeneration; transforming growth factor-β (TGF-β) signaling

1 | INTRODUCTION

Meniscal degeneration is a common knee disease, which leads to knee joint pain and dysfunction. Furthermore, meniscal degeneration may cause degenerative joint changes such as osteophyte formation, articular cartilage degeneration, knee dysfunction, and symptomatic osteoarthritis (OA; Fox, Bedi,& Rodeo, 2012; Howell, Kumar, Patel, & Tom, 2014). The exact pathophysiology and mechanisms underlying meniscal degeneration are not well understood. Currently, there is no effective therapeutic intervention to decelerate the progress of meniscal degeneration.

The menisci of mammals are crescent-shaped fibrocartilaginous tissues located between the femoral condyles and the tibial plateau in the knee. The menisci are essential for joint stability, shock absorption, distribution of contact forces, joint lubrication, and proprioception (Fox et al., 2012; Fox, Wanivenhaus, Burge, Warren, & Rodeo, 2015; Howell et al., 2014). The meniscus is an inhomogeneous structure, and the local cellular and extracellular content of the menisci varies depending on the distance from the peripheral edge. The outer zone of the meniscus contains an abundance of type I collagen, which is similar to ligaments, tendons and other fibrous connective tissues. The cells in this region are akin to fibroblasts with a fusiform shape. In contrast, the superficial and inner zone of the meniscus contains an abundance of type II collagen and higher aggrecan content, which is more similar to hyaline articular cartilage, and fibrochondrocyte is the predominant cell type in this region (Beaufils, Becker, Kopf, Matthieu, & Pujol, 2017; McNulty, Moutos, Weinberg, & Guilak, 2007; Wilusz, Weinberg, Guilak, & McNulty, 2008). Meniscal cells are responsible for maintaining the balance between the anabolic and catabolic processes in menisci by synthesizing and degrading extracellular matrix (ECM) in response to environmental cues. Loss of the function of meniscal cells to maintain a balance between the anabolic and catabolic processes in meniscus leads to meniscal degradation (Howell et al., 2014; Meister, Indelicato, Spanier, Franklin, & Batts, 2004; Mesiha et al., 2007).

In recent years, a variety of molecules and signaling pathways, such as insulin-like growth factors 1 and 2 (Webber, Zitaglio, & Hough, 1988), fibroblast growth factor 2 (Adesida, Grady, Khan, & Hardingham, 2006), platelet-derived growth factor (Bhargava et al., 1999; Webber, Harris, & Hough, 1985), hepatocyte growth factor (Bhargava et al., 1999), and bone morphogenetic proteins (Bhargava et al., 1999) have been found to play critical roles in regulating the biological activities and maintenance of meniscal cells. However, the molecular mechanisms underlying the pathogenesis of meniscal degeneration are still not clearly understood.

Recently, the role of transforming growth factor-β (TGF-β) signaling in the regulation of meniscal cells has received increasing and specific attention. In canonical TGF-β-Smad signaling, the TGF-β ligands initiate signaling cascades by binding to the TGF-β type II

receptor (TGFβRII) first, and then TGFβRII and TGF-β type I receptor (also called activin receptor-like kinases 5 [ALK5]) assemble to form a heteromeric complex on the cell membranes and phosphorylate ALK5. The activated (phosphorylated) ALK5 subsequently recruits and phosphorylates Smad2 and Smad3, which then form a heteromeric complex with Smad4. The Smad complexes are subsequently translocated into the nucleus to regulate the transcriptions of the target genes in cooperation with other cofactors. In addition, TGF-β can also activate non-Smad signaling pathways such as mitogen-activating protein kinases, phosphatidylinositol 3 kinase/Akt and small GTPases in a context-dependent manner (Hata & Chen, 2016; Massague, 2012).

During aging and meniscal degeneration, the canonical TGF-β-Smad signaling molecule is reduced in the meniscal tissues (Katsuragawa et al., 2010; Muhammad et al., 2014). TGF-β1 has been studied as a therapeutical molecule for meniscal degeneration by increasing the proliferation and matrix synthesis of meniscal cells in several in vitro models (Collier & Ghosh, 1995; Cucchiarini, Schmidt, Frisch, Kohn, & Madry, 2015; de Mulder, Hannink, Giele, Verdonschot, & Buma, 2013; Gruber et al., 2008; Gunja, Uthamanthil, & Athanasiou, 2009; Pangborn & Athanasiou, 2005a, 2005b; Riera et al., 2011; Stewart et al., 2007). However, due to the lack of in vivo evidence, the accurate role and molecular mechanism of TGF-β signaling in menisci maintenance and degeneration remain largely unknown.

To determine the roles of TGF-β signaling in menisci homeostasis in vivo, we generated mice with inducible cartilage-specific deletion of *Alk5* by breeding $Alk5^{flox/flox}$ (Larsson et al., 2001) with $Col2a1\text{-}CreER^{T2}$ mice (Chen et al., 2007). By injection of tamoxifen into 2-week-old mice, we confirmed the deletion of *Alk5* gene in meniscal superficial and inner zones and found that it led to pathological changes in menisci at the age of 3 and 6 months. Our data demonstrate that TGF-β signaling plays an essential role in maintaining menisci homeostasis, and inhibition of TGF-β signaling in meniscal superficial and inner zones at the postnatal stage accelerate age-dependent meniscal degeneration in mice.

2 | MATERIALS AND METHODS

2.1 | Animals

$Alk5^{flox/flox}$ mice (Larsson et al., 2001) and $Col2\alpha1\text{-}CreER^{T2}$ transgenic mice (Chen et al., 2007) were kindly provided by Professor Yang Chai and Professor Di Chen, respectively. $ROSA^{mT/mG}$ reporter mice (Muzumdar, Tasic, Miyamichi, Li, & Luo, 2007) were purchased from Jackson Laboratory (Stock Number: 007676; USA). $Col2\alpha1\text{-}CreER^{T2}$ mice were crossed with $ROSA^{mT/mG}$ reporter mice to obtain $Col2\alpha1\text{-}CreER^{T2}$; $ROSA^{mT/mG}$ mice. The resulting Cre-negative and $Col2\alpha1\text{-}CreER^{T2}$; $ROSA^{mT/mG}$ mice were intraperitoneally injected with tamoxifen (1mg/10 g body weight, daily for 5 days) at the age of 2 weeks (from postnatal Day 14 to postnatal Day 18) and were killed at the age of 2 months. $Alk5^{flox/flox}$ mice were crossed with $Col2a1\text{-}CreER^{T2}$ mice to obtain $Alk5^{flox/flox}$; $Col2\alpha1\text{-}CreER^{T2}$ (hereafter referred to as $Alk5^{Col2ERT2}$). The resulting $Alk5^{Col2ERT2}$ and Cre-negative mice were intraperitoneally injected with tamoxifen (1mg/10 g body weight, daily for 5 days) at 2 weeks to specifically inactivate ALK5 signaling in a tamoxifen-inducible and chondrocyte-specific manner (hereafter referred to as *Alk5* cKO mice). The Cre-negative and *Alk5* cKO mice aged Day 21 (3 days after the last injection) were used for

immunohistochemistry (IHC), and mice aged 3 months and 6 months were used for histologic assessment. For the surgical OA model, wild-type mice at the age of 10 weeks were used to induced OA by destabilization of the medial meniscus (DMM) surgery as previously described (Glasson, Blanchet, & Morris, 2007). The mice were killed 4 and 8 weeks after surgery and the knee joints were used for IHC. For aging studies, wild-type mice aged 3, 6, 12, and 18 months were killed and the knee joints were sampled for IHC. All mice were maintained in a C57BL/6J background. The animal experiments were performed according to the protocols approved by the Laboratory Animal Welfare and Ethics Committee of the Third Military Medical University (Chongqing, China).

2.2 | Isolation and culture of knee menisci

Hind knee menisci were harvested from 21 days old (3 days after the last injection) Cre-negative and *Alk5* cKO mice under a microscope (at least ten were pooled as one sample). Freshly dissected meniscus samples were washed in sterile phosphate buffer saline (PBS) and rest for 48 hr in Dulbecco's Modified Eagle's Medium/F12 (1:1) supplemented with 1% penicillin/streptomycin in a CO_2 incubator at 37°C.

2.3 | Cryostat sections and fluorescence detection

For cryosection preparation, the knee joints were isolated from anesthetized mice perfused with cold 4% paraformaldehyde (PFA), fixed in 4% PFA at 4°C for 24 hr, decalcified in 14% EDTA (pH 7.4) for 3 days, incubated in 30% sucrose at 4°C for 24 hr and then embedded in optimal cutting temperature (OCT). Frozen sections were cut at 10 μm thickness with Leica cryostat until the anterior and posterior horns appeared as triangles between the femoral condyle and the tibial plateau.

For fluorescence detection, frozen sections were washed three times with PBS and imaged with a fluorescence microscope.

2.4 | Histologic assessment

The knee joints were fixed in 4% PFA overnight, decalcified in 20% formic acid for 7 days, and embedded in paraffin. Serial sagittal sections were cut at 5 μm thickness until the anterior and posterior horns appeared as triangles between the femoral condyle and the tibial plateau. The sections were then stained with Safranin O/Fast Green to assess meniscus destruction by three independent investigators using a histological scoring system. The sum scores for the anterior and posterior horns were calculated separately based on three criteria: (1) tissue surface structures, (2) cellularity, and (3) Safranin O staining distribution and intensity as previously described (Kwok et al., 2016).

2.5 | Immunohistochemistry

IHC was performed with the SP-9000 Histostain-Plus kits (ZSGB-BIO) according to the manufacturer's instructions as previously described (Wang et al., 2017). Briefly, sections were deparaffinized in xylene, rehydrated in a graded series of alcohol washes and washed twice in PBS for 5 min. Endogenous peroxidase activity was quenched with 3% H_2O_2 for 10 min followed by antigen retrieval using 0.1% trypsin for 15 min. The sections were then blocked with normal goat serum for 30 min and incubated with different antibodies at 4°C

overnight. On the second day, the sections were rinsed with PBS after incubation with horseradish peroxidase conjugated secondary antibodies for 30 min at 37°C. Finally, the sections were stained with a diaminobenzidine kit. Primary antibodies against the following proteins were used: ALK5 (1:100, Santa Cruz Biotechnology, Cat# sc-398, RRID: AB_632493), runt-related transcription factor 2 (RUNX2; 1:200, Santa Cruz Biotechnology, Cat# sc-390715, RRID: AB_2637033), Collagen II (Col II; 1:200, Millipore, Cat# MAB8887, RRID: AB_2260779), and matrix metalloproteinase 13 (MMP13; 1:200, Proteintech, Cat# 18165-1-AP, RRID: AB_2144858).

2.6 | Apoptosis assay

Apoptotic cells in the meniscus were detected by TdT mediated dUTP-X nick end labeling (TUNEL) assay using an in situ cell death detection kit (peroxidase) of Roche according to the manual.

2.7 | RNA isolation and quantitative real-time PCR analysis

Menisci tissues were flash-frozen for RNA extraction using TRIzol reagent (Invitrogen). Quantitative real-time PCR (qRT-PCR) were run in triplicate and independently repeated three times using Mx3000P PCR machine (Stratagene) and SYBR Premix Ex TaqTM kit. The primers for target genes are listed in Table 1. The results were calculated as the relative quantification compared to the control group, which was set at 1. Cyclophilin A was used as the internal control.

2.8 | Statistical analysis

Data were presented as the Mean ± 95% confidence intervals. Differences between two groups were assessed by the Student unpaired *t* test. Differences among multiple groups were assessed by one-way analysis of variance followed by Tukey's posthoc test. All data were analyzed using GraphPad Prism v.6.01 software. $P < 0.05$ was considered to be statistically significant.

3 | RESULTS

3.1 | Decreased expression of ALK5 is associated with age-related and OA induced meniscal degeneration

It has been shown that the expressions of TGF-β1 TGF-β3, and Smad2/3 were reduced with meniscal injury and degeneration (Katsuragawa et al., 2010; Muhammad et al., 2014). We questioned whether the expression of ALK5 is altered during meniscal injury and degeneration. Aging is a major risk factor for meniscal degeneration (Kwok et al., 2016). In this study, we first used IHC to detect the expression of ALK5 in meniscal tissues in young and aged mice. The results showed that ALK5 was abundantly expressed in the anterior and posterior horns in 3-month-old mice, which was gradually decreased in the meniscal tissues from aged mice (6–24 months; Figure 1a). Furthermore, we evaluated the ALK5 protein level in menisci of mice with post-traumatic OA induced by DMM surgery. The results showed that ALK5 was abundantly expressed in the meniscal anterior and posterior horns in the sham group. With OA progression, the expression of ALK5 in meniscal anterior and posterior horns was progressively decreased in mice of 4 and 8 weeks after DMM surgery

(Figure 1b). These findings suggest a potential involvement of dysregulated TGF-β/ALK5 signaling in age-related and OA induced meniscal degeneration.

3.2 | *Col2a1-CreER^{T2}* drives Cre recombination in meniscal cells

After observing the decreased ALK5 expression during meniscal degeneration, we further asked whether the dysregulation of TGF-β/ALK5 signaling is associated with meniscal injury and degeneration. We used mice with inducible cartilage-specific deletion of *Alk5* to elucidate the effect of TGF-β/ALK5 signaling in the pathophysiology of meniscal degeneration. To evaluate the specificity of the tamoxifen-induced *Col2a1-CreER^{T2}*-driven recombination in the meniscal tissues of postnatal mice, we first bred *Col2a1-CreER^{T2}* transgenic mice with *ROSA$^{mT/mG}$* reporter mice. Analysis of frozen sections from 2-month-old mice using fluorescence microscopy showed that *Col2a1-CreER^{T2}* targeting cells (GFP-labelled cells) were located in the superficial and inner zones of the anterior horns, as well as the inner zone of the posterior horn in *Col2a1-CreER^{T2}*; ROSA$^{mT/mG}$ mice (Figure 2a lower panel). Furthermore, we generated *Alk5^{Col2ERT2}* mice by crossing *Alk5$^{flox/flox}$* with *Col2a1-CreER^{T2}* mice. IHC analysis demonstrated that ALK5 protein levels were decreased in the superficial and inner zones of anterior horns, as well as the inner zone of the posterior horn in *Alk5* cKO mice compared with those in Cre-negative mice (Figure 2b). Taken together, these results demonstrated that *Col2a1-CreER^{T2}* mouse strain is a useful tool for studying gene function in the superficial and inner zones of menisci, and *Alk5* gene could be effectively deleted in these regions of menisci in a tamoxifen-dependent manner.

3.3 | Postnatal cartilage-specific deletion of Alk5 accelerates age-dependent meniscal degeneration

To investigate the role of TGF-β/ALK5 signaling in meniscal maintenance and degeneration at the postnatal stage, the above mentioned Cre-negative and *Alk5^{Col2ERT2}* mice were then subjected to a time-course experiment. Three-month-old *Alk5* cKO mice showed fibrillation in the superficial, inner and outer zones with faint or no Safranin O staining, and aberrant hypertrophic chondrocytes in the superficial and inner zones (Figure 3a), indicating the degeneration of menisci. The meniscal degeneration phenotype became more profound in 6-month-old *Alk5* cKO mice, which exhibited more severe fibrillation and structure disruption of meniscal tissues (Figure 3b). Accordingly, the meniscal histological grading system scores were significantly increased in 3- and 6-month-old *Alk5* cKO mice compared with aged matched Cre-negative mice (Figure 3a,b, right panels). Taken together, these results revealed that cartilage-specific deletion of *Alk5* accelerates age-dependent meniscal degeneration in mice, which demonstrates that postnatal chondrocytic TGF-β signaling plays an essential role in maintaining meniscal homeostasis and preventing meniscal degeneration.

3.4 | Postnatal cartilage-specific deletion of *Alk5* leads to increased expression levels of catabolic and hypertrophic marker genes and decreased expressions of anabolic marker genes in menisci

To explore the mechanisms underlying the accelerated meniscal degeneration in *Alk5* cKO mice, we extracted RNA from the knee menisci tissues of Cre-negative and *Alk5* cKO mice at 21-days-old (3 days after the last injection), and found that *Alk5* gene was effectively

deleted in the *Alk5* cKO menisci tissue (Figure 4a). Next, the expressions of genes associated with cartilage homeostasis were detected. The mRNA expressions of proteoglycan 4 (*Prg4*), *Aggrecan*, and *Col2a1* (anabolic marker genes) were decreased in the *Alk5*-deficient menisci tissue (Figure 4b–d). Meanwhile, the expressions of *Runx2, Mmp13,* a disintegrin and metalloproteinase with thrombospondin motif 5 (*Adamts5*) and *Col10a1* (catabolic and hypertrophic marker genes) were significantly increased in *Alk5*-deficient menisci tissues (Figure 4e–h). Consistently, the IHC results showed that the Col II protein level (Figure 5a) was significantly decreased whereas the RUNX2 (Figure 5b) and MMP13 (Figure 5c) protein levels were remarkably increased in *Alk5* cKO mice compared with the Cre-negative mice. Furthermore, we performed TUNEL assay to analyze the apoptosis of cells in meniscal cartilage. Our results showed that the percentage of positive cells in the anterior and posterior horns was significantly increased in *Alk5* cKO mice compared with the Cre-negative mice at 21-days-old (Figure 6). Together, these results indicated that deletion of *Alk5* in meniscal cartilage led to decreased synthesis and increased degradation of ECM and promoted meniscal fibrochondrocyte hypertrophic differentiation and meniscal cartilage cell apoptosis, which may partly disrupt menisci homeostasis and accelerate meniscal degeneration.

4 | DISCUSSION

In recent years, mouse models, especially genetic mouse models, have been widely used to investigate the cellular and molecular mechanisms of bone and joint diseases (Blaker, Clarke, & Little, 2017; Fang & Beier, 2014; Moon & Beier, 2015; Watanabe, Oue, & Ikegawa, 2011). Using the *Col2a1-CreER^{T2}* transgenic mouse model, a variety of interested genes have been deleted in *Col2a1*-expressing cells to dissect their role in bone and joint diseases in an inducible manner (Chen, Li, Xie, Wang, & Chen, 2014). In our study, using this transgenic mouse strain, we evaluated the role and underlying molecular mechanisms of TGF-β signaling in menisci homeostasis by specifically deleting the *Alk5* gene to inhibit TGF-β signaling in *Col2a1*-expressing meniscal cells at the postnatal stage.

To date, few studies have focused on the gene functions of genetically modulated genes in specific compartments of the menisci to explore their roles in menisci development, homeostasis, and disease. In the current study, for the first time we evaluated the specificity of the *Cre* recombination activity in meniscal tissues at the postnatal stage using *Col2a1-CreER^{T2}*; *ROSA$^{mT/mG}$*. The results showed that *Col2a1-CreER^{T2}* targeting cells were mainly located in the superficial and inner zones of the anterior horn, as well as the inner zones of the posterior horn meniscal cells, which was consistent with IHC staining of Col II in wild-type mouse menisci (Gamer, Xiang, & Rosen, 2017). Thus *Col2a1-CreER^{T2}* mice can be used as a valuable tool for identifying the role of interested genes in regulating menisci homeostasis. In this study, we only evaluated the *Col2a1-CreER^{T2}*-driven recombination at 2 months after birth; further assessment of the potential utility of *Col2a1-CreER^{T2}* mice will require a more detailed analysis of the spatio-temporal activity of the *Col2a1* promoter activity in these mice because of the dynamic expression of type II collagen during menisci development, homeostasis, and aging.

Our results demonstrated that the *Alk5* gene was effectively deleted in meniscal tissues, including the superficial and inner zones of the anterior horn, as well as the inner zone of the posterior horn. Importantly, we found that deletion of the *Alk5* gene in these meniscal cells at the postnatal stage accelerated age-dependent meniscal degeneration in mice, including increased hypertrophic chondrocytes in the superficial and inner zones, severe fibrillation, and structure disruption of meniscal tissues. It should be noted that the degeneration of the anterior horn was more severe than the posterior horn according to histological staining and meniscal histological grading system scores. The possible reason is that TGF-β signaling was more efficiently inhibited in the anterior horn than the posterior horn due to the higher *Col2α1* expression in the anterior horn as revealed by GFP fluorescence and ALK5 IHC results. To our knowledge, this is the first study showing the protective role of TGF-β signaling on meniscal tissue maintenance in vivo.

The ECM of menisci mainly contains type I and type II collagens (Col I and Col II) and Aggrecan, which could be degraded by specific collagenases such as MMP13, MMP9, and MMP3 and ADAMTS5 under pathological conditions, including meniscal degeneration (Fuller, Smith, Little, & Melrose, 2012; Kreinest et al., 2016; Ling, Lai, Wong, & Levenston, 2016; Upton, Chen, & Setton, 2006). Previous studies showed that TGF-β signaling pathway components exerted both proanabolic and anticatabolic effects on menisci during the repair and healing process of degenerative menisci (Collier & Ghosh, 1995; Cucchiarini et al., 2015; de Mulder et al., 2013; Gruber et al., 2008; Gunja et al., 2009; Pangborn & Athanasiou, 2005a, 2005b; Riera et al., 2011; Stewart et al., 2007). In this study, we found that the anabolic marker genes such as *Prg4, Aggrecan,* and *Col2α1* were decreased, whereas the catabolic and hypertrophic marker genes such as *Runx2, Mmp13, Adamts5,* and *Col10α1* were significantly increased in *Alk5*-deficient meniscal tissues. Consistently, the IHC results showed that Col II level was decreased, whereas the RUNX2 and MMP13 protein levels were increased in the meniscal tissues of *Alk5* cKO mice. The above changes in anabolism and catabolism related molecules in the meniscal tissues eventually tilt the balance away from matrix maintenance toward degradation, resulting in meniscal degeneration over time observed in *Alk5* cKO mice, together with the accelerated hypertrophic differentiation and increased cell apoptosis.

It has been shown that cartilage-specific deletion of *Alk5* (Wang et al., 2017) or *Tgfbr2* (Jin et al., 2011; Shen et al., 2013) in knee cartilage and intervertebral disc cartilage induces a progressive OA-like phenotype and intervertebral disc degeneration. Further studies showed that inhibition of TGF-β signaling in the knee cartilage and intervertebral disc cartilage up-regulates the expressions of downstream target genes, such as *Runx2, Mmp13,* and *Adamts5* in cartilage, leading to the development of knee OA and intervertebral disc degeneration (Jin et al., 2011; Shen et al., 2013; Wang et al., 2017). Our current study is the first to demonstrate that a similar set of molecules is also responsible for meniscal degradation. Further genetical deletion of *Runx2, Mmp13,* or *Adamts5* under the *Alk5* deficiency background could be helpful in dissecting the relationship between TGF-β signaling and the molecules in menisci homeostasis genetically. A previous study showed that broad-spectrum MMP inhibitors promote the integrative repair of meniscus in vitro (McNulty, Weinberg, & Guilak, 2009). Observing whether MMP13 inhibitors could rescue the meniscal

degeneration phenotypes observed in *Alk5* cKO mice will further justify the application of MMP13 inhibitor as a treatment for meniscal degeneration.

In summary, in this experiment, we found that cartilage-specific deletion of *Alk5* gene in meniscus leads to meniscus degeneration by dysregulating the ECM metabolism and enhancing the hypertrophic differentiation and apoptosis of meniscal chondrocytes. Our study provides experimental data suggesting that modulating TGF-β signaling could be a potential therapy for delaying meniscal degeneration.

ACKNOWLEDGMENTS

We thank Professor Yang Chai (University of Southern California, USA) and Professor Di Chen (Rush University Medical Center, USA) for providing *Alk5*$^{flox/flox}$ mice and *Col2α1-CreER*^{T2} mice, respectively.

FUNDING

Contract grant sponsor: Special Funds for Major State Basic Research Program of China (973 program); Contract grant number: 2014CB942904. Contract grant sponsor: International (Regional) Cooperation and Exchange; Contract grant number: 81220108020). Contract grant sponsor: National Natural Science Foundation of China; Contract grant number: 81472074).

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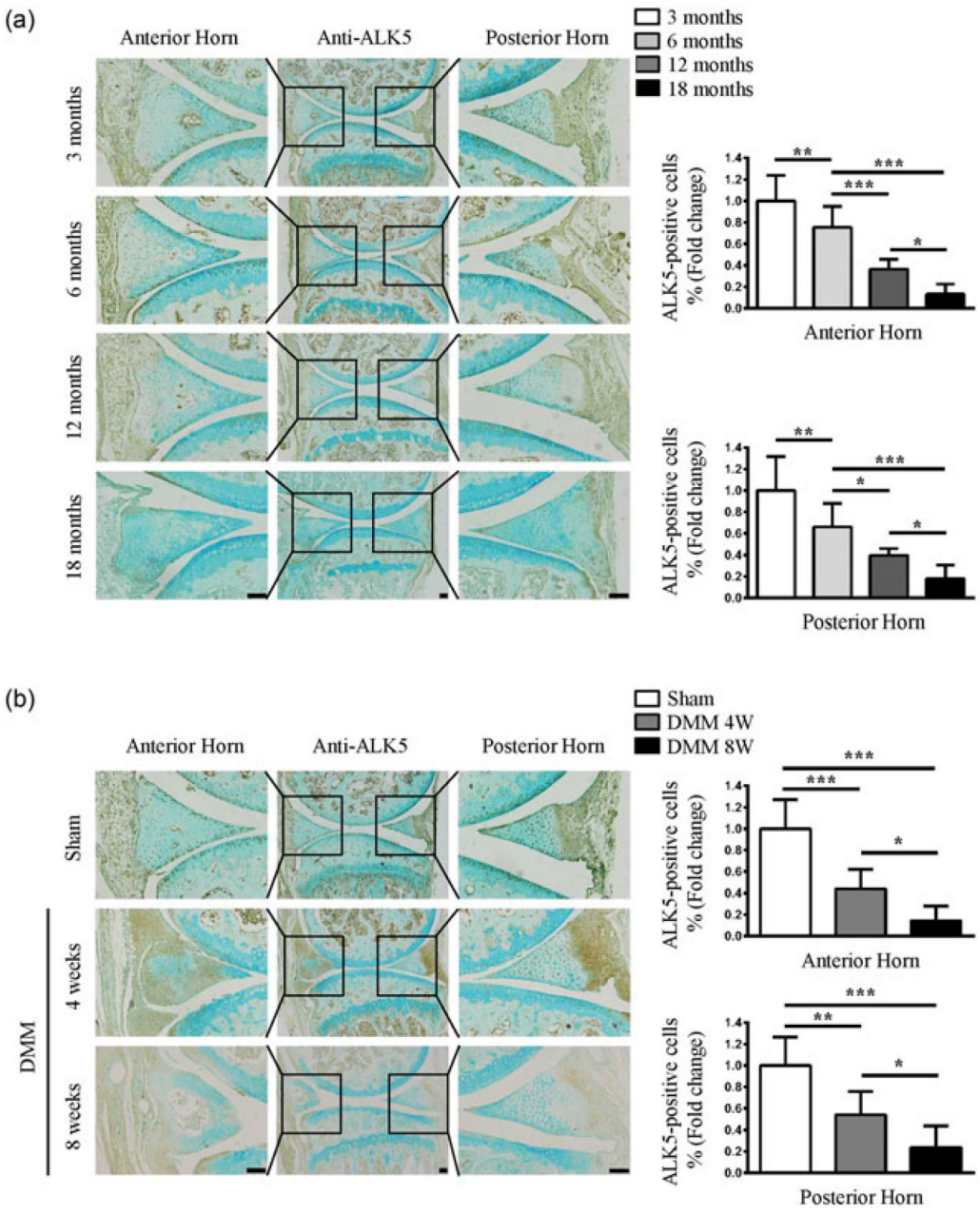

FIGURE 1.

Decreased expression of ALK5 in age-related and OA induced degenerative meniscus anterior and posterior horns. (a) Representative images of IHC staining for ALK5 showed that the number of positive cells in the anterior and posterior horns were strongly reduced with aging. Quantitative data are shown in the right panel (the percentage of positive cells in Cre-negative mice was defined as 1, $n = 3$ mice per group). (b) Representative images of IHC staining for ALK5 showed that the number of positive cells was gradually reduced in the anterior and posterior horns of mice at 4 and 8 weeks after DMM surgery. Quantitative data are shown in the right panel (the percentage of positive cells in Cre-negative mice was defined as 1, $n = 3$ mice per group). Scale bar: 100 μm. Data were expressed as the mean

± 95% confidence intervals. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. ALK5, activin receptor-like kinases 5; DMM, destabilization of the medial meniscus; IHC, immunohistochemistry; OA, osteoarthritis [Color figure can be viewed at wileyonlinelibrary.com]

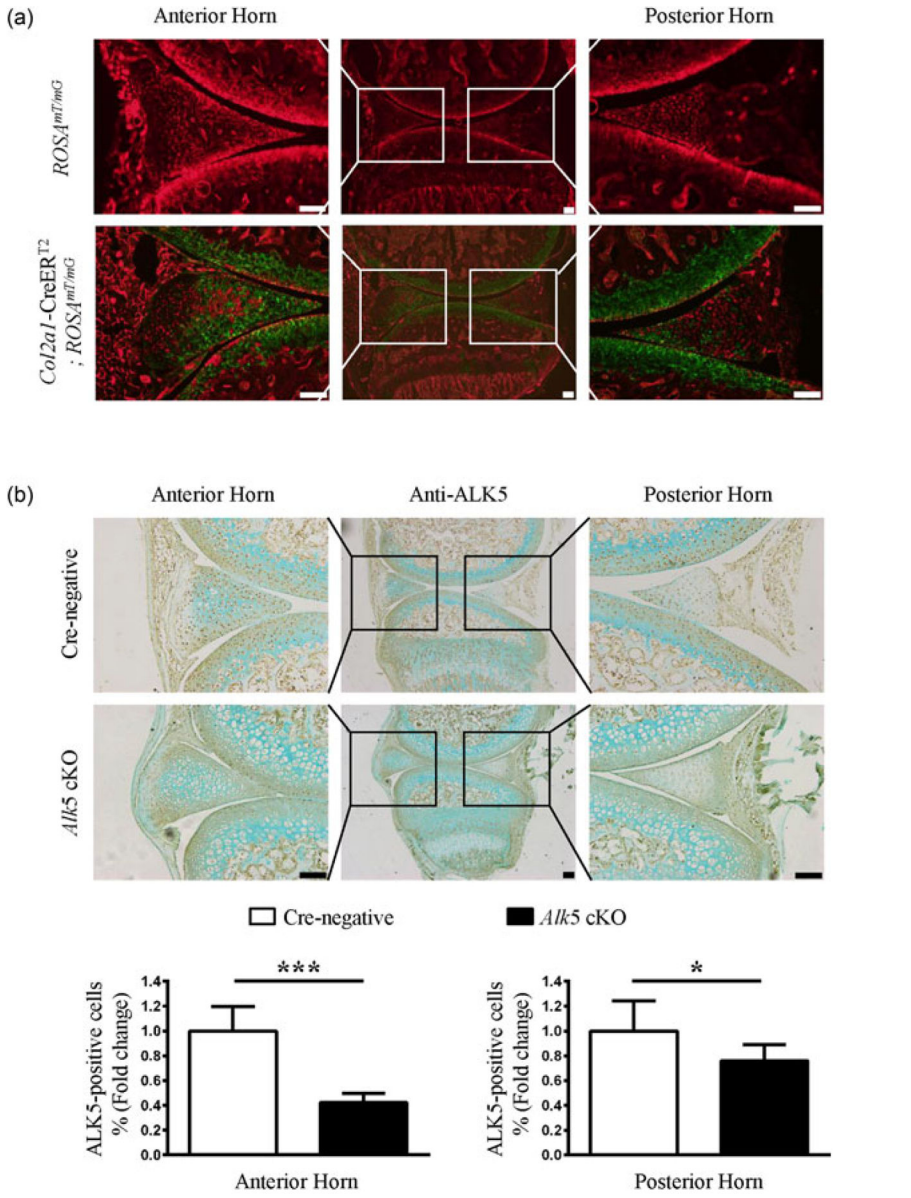

FIGURE 2.

Directed Cre recombination in meniscus from *Col2a1*-CreER^{T2} mice. (a) *Col2a1*-CreER^{T2}; *ROSA*$^{mT/mG}$ mice were generated by breeding *Col2a1*-CreER^{T2} transgenic mice with *ROSA*$^{mT/mG}$ reporter mice). Frozen histologic sections were harvested from 2-month-old Cre-negative and *Col2a1*-CreER^{T2}; *ROSA*$^{mT/mG}$ mice after they were injected with tamoxifen at the age of 2 weeks (1 mg/10 g body weight, i.p. injection, daily for 5 days) and analyzed by fluorescence microscopy. The representative images showed that *Col2a1*-CreER^{T2} targeting GFP-positive cells are located in the meniscus anterior and posterior horns. (b) The resulting *Alk5*^{Col2ERT2} and aged matched Cre-negative mice were intraperitoneally injected with tamoxifen from postnatal Day 14 to postnatal Day 18. The mice were sacrificed on postnatal Day 21, 3 days after the last injection. Representative images of IHC staining for ALK5 showed that the number of positive cells in anterior and

posterior horns is strongly reduced in 21-day-old *Alk5* cKO mice compared with Cre-negative mice. Quantitative data are shown in the lower panel (the percentage of positive cells in Cre-negative mice was defined as 1, $n = 3$ mice per group). Scale bar: 100 μm. Data were expressed as the mean ± 95% confidence intervals, $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. ALK5, activing receptor-like kinases 5; GFP, green fluorescent protein; IHC, immunohistochemistry; i.p., intraperitoneal [Color figure can be viewed at wileyonlinelibrary.com]

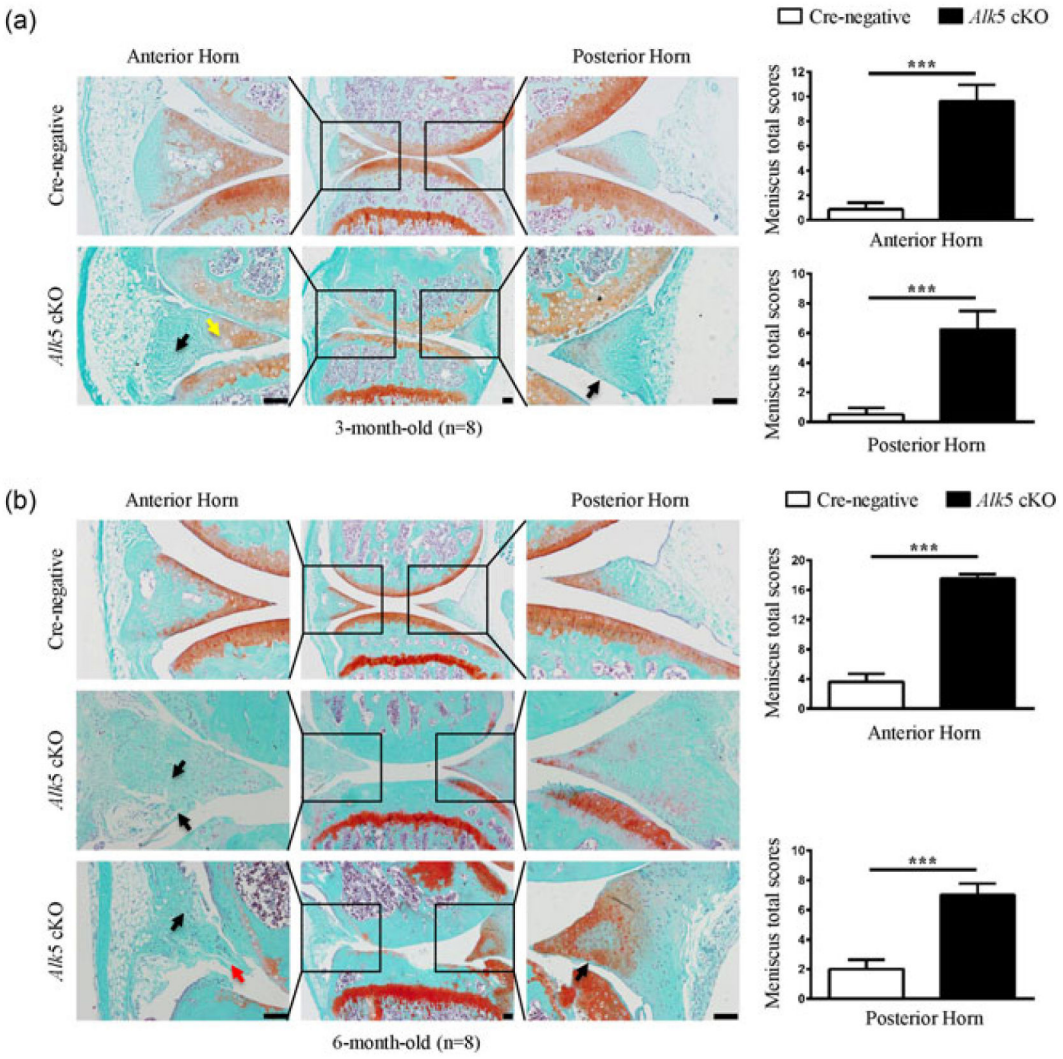

FIGURE 3.

Histological analysis of structural damage in the meniscus of *Alk5* cKO mice. (a) Knee joint samples were dissected from 3-month-old mice, and Safranin O/Fast green staining was performed. Representative images showed fibrillation in the superficial zone, inner and outer regions with faint or no Safranin O staining (black arrow) and aberrantly increased hypertrophic chondrocytes (yellow arrow) in menisci of *Alk5* cKO mice. Quantitative data are shown in the right panel ($n = 8$ mice per group). (b) Knee joint samples were dissected from 6-month-old mice, and Safranin O/Fast green staining was performed. Representative images showed more severe fibrillation (black arrow) and disruption of meniscal tissue (red arrow) in meniscus of *Alk5* cKO mice. Quantitative data are shown in the right panel ($n = 8$ mice per group). Scale bar: 100 μm. Data were expressed as the mean ± 95% confidence intervals. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. *Alk5,* activing receptor-like kinases 5 [Color figure can be viewed at wileyonlinelibrary.com]

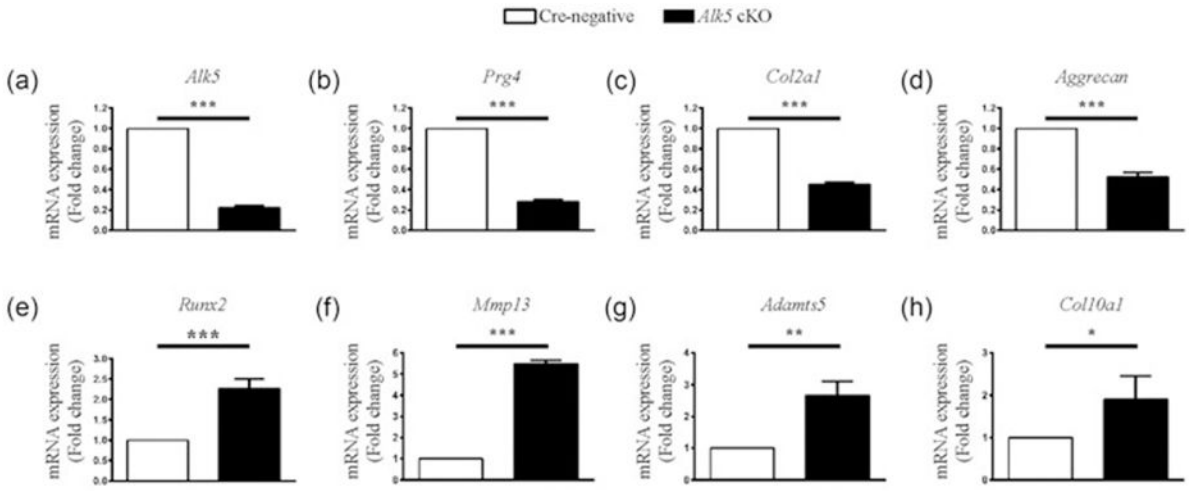

FIGURE 4.

Loss of TGF-β/ALK5 signaling in meniscal cells decreased mRNA level of anabolic marker genes and increased mRNA level of catabolic and hypertrophic marker genes. qRT-PCR analysis showed effectively deletion of *Alk5* gene (a), a significant decrease in the gene expressions of *Prg4* (b), *Col2a1* (c) and *Aggrecan* (d), and a significant increase in the gene expressions of *Runx2* (e), *Mmp13* (f), *Adamts5* (g), and *Col10a* (h) in 21-day-old *Alk5* cKO mice meniscl cells compared with Cre-negative mice meniscus cells. The analysis was repeated for three times. Data were expressed as the mean ± 95% confidence intervals. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. ALK5, activing receptor-like kinases 5; Mmp13, matrix metalloproteinase 13; Prg4, proteoglycan 4; qRT-PCR, quantitative real-time PCR analysis; Runx2, runt-related transcription factor 2; TGF-β, transforming growth factor-β

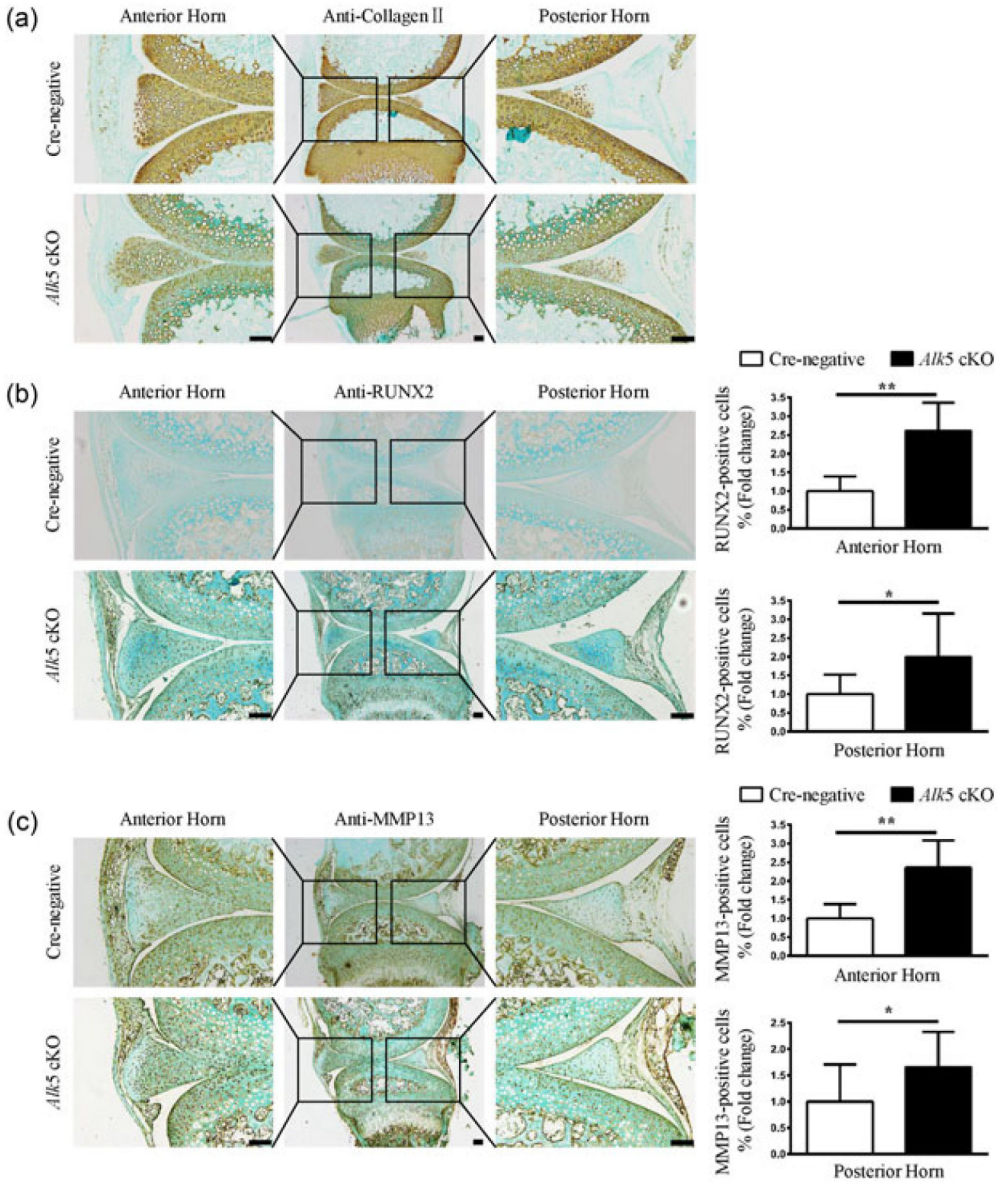

FIGURE 5.

Loss of TGF-β/ALK5 signaling in meniscal cells decreased protein levels of anabolic marker genes and increased protein levels of catabolic and hypertrophic marker genes. (a) Representative images of IHC staining for Col II showed that the expression of Col II in anterior and posterior horns were significantly reduced in 21-day-old *Alk5* cKO mice compared with Cre-negative mice ($n = 3$ mice per group). (b,c) Representative images of IHC staining for RUNX2 (b) and MMP13 (c) showed that the number of positive cells in anterior and posterior horns were significantly increased in 21-day-old *Alk5* cKO mice compared with Cre-negative mice. Quantitative data were shown in the right panel (the percentage of positive cells in Cre-negative mice was defined as 1, $n = 3$ mice per group). Scale bar: 100 μm. Data were expressed as the mean ± 95% confidence intervals. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. ALK5, activing receptor-like kinases 5; Col II, Collagen II; IHC, immunohistochemistry; MMP13, matrix metalloproteinase 13; RUNX2, runt-related

transcription factor 2; TGF-β, transforming growth factor-β [Color figure can be viewed at wileyonlinelibrary.com]

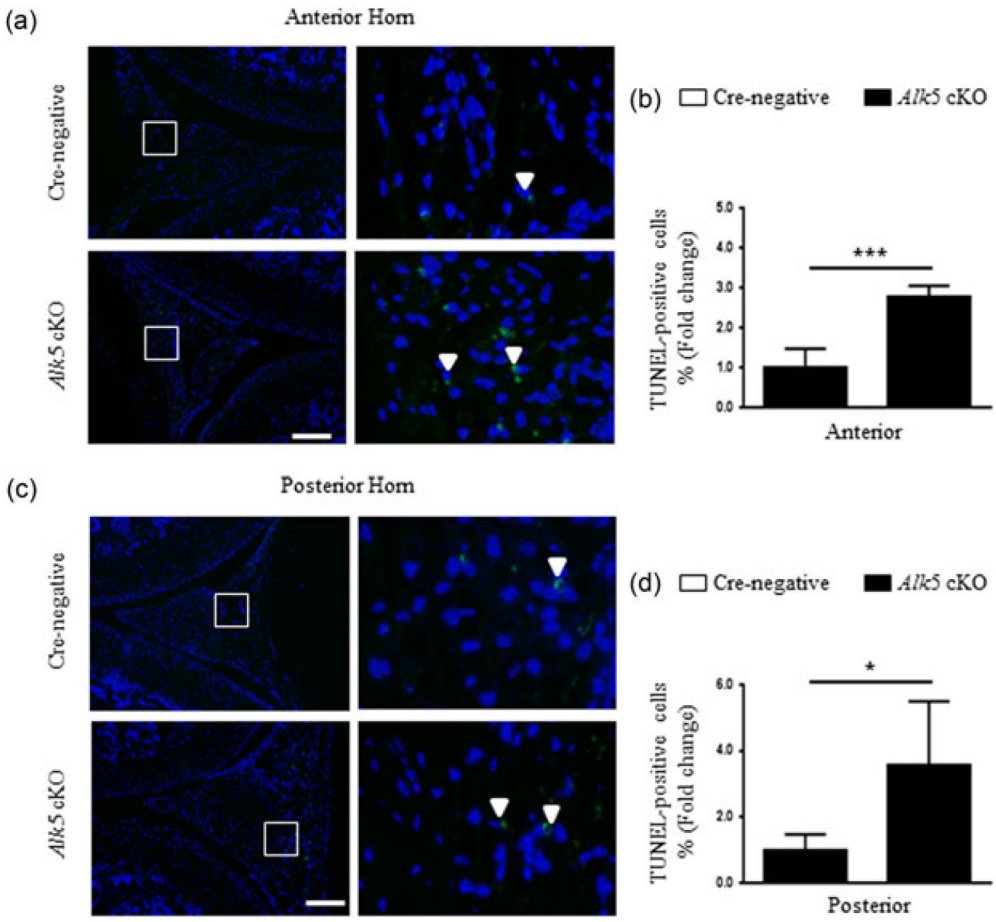

FIGURE 6.

Loss of TGF-β/ALK5 signaling in meniscal increased cell apoptosis. Representative images of TUNEL staining for cell apoptosis showed that the apoptotic cells in anterior (a) and posterior horns (c) were significantly increased in 21-day-old *Alk5* cKO mice compared with Cre-negative mice (high magnification of the region occupied by white squares in the right panel, the positive cells are shown in white arrows). Quantitative data are shown in (b,d) (the percentage of positive cells in Cre-negative mice was defined as 1, $n = 3$ mice per group). Scale bar: 100 μm. Data were expressed as the mean ± 95% confidence intervals. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. ALK5, activing receptor-like kinases 5; TGF-β, transforming growth factor-β; TUNEL, TdT mediated dUTP-X nick end labeling [Color figure can be viewed at wileyonlinelibrary.com]

TABLE 1

Primers and sequences for real-time PCR analysis

	Forward primer	Reverse primer
Alk5	5′-CATCAGGGTCTGGATCAGGTT-3′	5′-GTAACACAATGGTCCTGGCAA-3′
Runx2	5′-AGAGTCAGATTACAGATCCCAGG-3′	5′-TGGCTCTTCTTACTGAGAGAGG-3′
Mmp13	5′-CTTCTTCTTGTTGAGCTGGACTC-3′	5′-CTGTGGAGGTCACTGTAGACT-3′
Adamts5	5′-GGAGCGAGGCCATTTACAAC-3′	5′-CGTAGACAAGGTAGCCCACTTT-3′
Aggrecan	5′-CCTGCTACTTCATCGACCCC-3′	5′-AGATGCTGTTGACTCGAACCT-3′
Col2	5′-CTGGTGGAGCAGCAAGAGCAA-3′	5′-CAGTGGACAGTAGACGGAGGAAAG-3′
Col10	5′-ACCCCAAGGACCTAAAGGAA-3′	5′-CCCCAGGATACCCTGTTTTT-3′
Prg4	5′-GAAAATACTTCCCGTCTGCTTGT-3′	5′-ACTCCATGTAGTGCTGACAGTTA-3′
Cyclophilin A	5′-CGAGCTCTGAGCACTGGAGA-3′	5′-TGGCGTGTAAAGTCACCACC-3′

Note. Alk5, activing receptor-like kinases 5; *Mmp13,* matrix metalloproteinase 13; *Prg4,* Proteoglycan 4.