Mohawk promotes the maintenance and regeneration of the outer annulus fibrosus of intervertebral discs

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The main pathogenesis of intervertebral disc (IVD) herniation involves disruption of the annulus fibrosus (AF) caused by ageing or excessive mechanical stress and the resulting prolapse of the nucleus pulposus. Owing to the avascular nature of the IVD and lack of understanding the mechanisms that maintain the IVD, current therapies do not lead to tissue regeneration. Here we show that homeobox protein Mohawk (Mkx) is a key transcription factor that regulates AF development, maintenance and regeneration. Mkx is mainly expressed in the outer AF (OAF) of humans and mice. In Mkx−/− mice, the OAF displays a deficiency of multiple tendon/ligament-related genes, a smaller OAF collagen fibril diameter and a more rapid progression of IVD degeneration compared with the wild type. Mesenchymal stem cells overexpressing Mkx promote functional AF regeneration in a mouse AF defect model, with abundant collagen fibril formation. Our results indicate a therapeutic strategy for AF regeneration.

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The spine is composed of vertebral bodies connected by intervertebral discs (IVDs) and plays a central role in human movement, as it enables standing, twisting and bending positions. Rupture of the exterior section of the IVD known as the annulus fibrosus (AF), especially by degenerative changes in the outer AF (OAF), can cause IVD herniation, which is a common and severe disease associated with pain and disability. Although standard discectomy provides pain relief, this technique reduces the mechanical property of the AF and accelerates progression to IVD degeneration and degenerative spondylosis. To date, tissue regenerative therapy of the IVD has not been achieved, to IVD degeneration and degenerative spondylosis. To date, tissue regenerative therapy of the IVD has not been achieved, for instance postnatal week 8 in mice). Conversely, Venus expression in the IAF decreased gradually as it approached the NP region (Fig. 1c). We also evaluated the expression of Mkx in human lumbar discs (Fig. 1d (details of regions between the OAF and IAF); Supplementary Table 1). Consistent with the observations in mice, more Mkx-positive cells were observed in the OA compared with the IAF (Fig. 1d,f). These results suggest that Mkx is expressed mainly in the OA.

Mkx deficiency affects development of AF. We examined the function of Mkx in the OAF using Mkx+/+ and Mkx−/− mice. In haematoxylin and eosin (HE) staining, AF cell number did not differ between Mkx+/+ and Mkx−/− mice at 10 weeks. For Mkx+/+ mice, the mean cell number was 213. For Mkx−/− mice, the mean cell number was 217. The P value was 0.51. However, HE staining revealed that AF collagen fibres were thinner in Mkx−/− mice than they were in Mkx+/+ mice (Fig. 2a,b). An electron microscopy-based analysis confirmed that the diameter of collagen fibrils was significantly narrower in Mkx−/− mice than in Mkx+/+ mice at 10 weeks (Fig. 2c–e). This difference in phenotype was also confirmed at postnatal day 1 (P1; Supplementary Fig. 1). We next examined the effect of Mkx on gene expression levels using OA-F cells isolated from the tail discs (C1/2-8/9) of 10-week-old Mkx+/+ or Mkx−/− mice (Supplementary Fig. 2). Quantitative real-time reverse transcription PCR (qRT–PCR) analyses revealed the downregulation of (i) a set of collagen genes, including Coll1a1 and Colla2, which were the main components of the OA; (ii) various small leucine-rich proteoglycan (SLRP) family genes (especially Biglycan (Bgn), which is expressed mainly in the OA and plays an essential role in the maintenance of OA homeostasis) and (iii) the tendon-related transcription factor Scleraxis (Sca) in Mkx−/− compared with Mkx+/+ cells (Fig. 3a). The P value of Elastin (Eln) was 0.084 and a significant difference between Mkx+/+ mice and Mkx−/− mice at 10 weeks was not seen. Thus, we judged that Mkx was not a definitive contributor to Eln expression. In IHC, Bgn and Col14 protein expression were decreased in Mkx−/− mice compared with Mkx+/+ mice at 10 weeks (Fig. 3c). We also investigated the knockdown effect of MKX in human AF (HAf) primary cultured cells and found that the expression of tendon/ligament-related genes, such as those encoding Scx, Tnmd, C0114a1, Tnc, Bgn and Tnxb, was significantly downregulated, whereas expression of the cartilage-related genes Sox9 and Acan was upregulated (Fig. 3b). Western blot analysis confirmed the downregulation of Bgn and upregulation of Sox9 following Mkx knockdown in HAF cells (Fig. 3d). Taken together, in mice and human, Mkx has an important role in the expression of some collagen and SLRP genes in the OA. These results also indicate a role for Mkx in collagen fibril formation during development, including adulthood.

Results

Mkx is mainly expressed in the OAF of humans and mice. To investigate the expression of Mkx in IVD, we used Mkx-Venus knock-in mice. The details about these mice were previously reported. The endogenous expression of Mkx in the IVD, as determined by ISH, shadowed the Venus expression observed in Mkx-Venus knock-in mice by IHC at E14.5. This suggests that the expression of Mkx-Venus is consistent with the endogenous expression pattern of Mkx in the IVD (Fig. 1a). In IHC, Mkx-Venus was strongly expressed at E14.5 in the OAF of the somite, and its expression was continued even at the later stages (Fig. 1a,b). Importantly, the expression of Venus was specifically localized in the OAF, even after disc development was completed, in 10-week-old mice (disc formation is completed by approximately postnatal week 8 in mice). Conversely, Venus expression in the IAF decreased gradually as it approached the NP region (Fig. 1c). We also evaluated the expression of Mkx in human lumbar discs (Fig. 1e (details of regions between the OAF and IAF); Supplementary Table 1). Consistent with the observations in mice, more Mkx-positive cells were observed in the OAF compared with the IAF (Fig. 1d,f). These results suggest that Mkx is expressed mainly in the OAF.
Next, to test whether the \( \text{Mkx}^{-/-} \) phenotype has an effect on the IVD degeneration that occurs during the aging process, we analysed \( \text{Mkx}^{+/+} \) and \( \text{Mkx}^{-/-} \) mice using HE staining and Safranin O fast green staining at various stages. The OAF collagen fibres in \( \text{Mkx}^{-/-} \) mice were thinner than those in \( \text{Mkx}^{+/+} \) mice at all ages (Supplementary Fig. 3a). In terms of IVD degeneration, there were no clear differences between \( \text{Mkx}^{+/+} \) and \( \text{Mkx}^{-/-} \) mice at 10 weeks and 6 months; however, at 12 and 21 months, \( \text{Mkx}^{-/-} \) mice showed high levels of degenerative changes in lumbar discs, whereas the lumbar discs of \( \text{Mkx}^{+/+} \) mice did not show changes from those observed at 10 weeks. At 12 and 21 months, small round cells, morphologically resembling chondrocytes, were observed in the NP and IAF of \( \text{Mkx}^{-/-} \) mice (Fig. 4a; Supplementary Fig. 3b). The NP and the IAF were stained more strongly with Safranin O, indicating degenerative changes. To further analyse IVD in \( \text{Mkx}^{-/-} \) mice, we performed IHC for Venus, Coll1, CD24 and KRT18 using 21-month-old \( \text{Mkx}^{+/+} \) mice and \( \text{Mkx}^{-/-} \) mice. Coll1 is a main component of the AF and Venus shadows Mkx expression. CD24 and KRT18 are well characterized as NP cell markers\(^{23-27}\). As expected, Venus expression is restricted at AF of 21-month-old \( \text{Mkx}^{-/-} \) mice. Coll1 expression was observed in the AF of both 21-month-old \( \text{Mkx}^{+/+} \) and \( \text{Mkx}^{-/-} \) mice, but not in the NP region of \( \text{Mkx}^{+/+} \) and \( \text{Mkx}^{-/-} \) mice (Supplementary Fig. 4a). KRT18 was observed in the NP region of both 21-month-old \( \text{Mkx}^{+/+} \) and \( \text{Mkx}^{-/-} \) mice. On the contrary, CD24-positive cells were observed only in the NP of 21-month-old \( \text{Mkx}^{+/+} \) mice but not in \( \text{Mkx}^{-/-} \) mice (Supplementary Fig. 4b). These results suggested that the cells at NP region of 21-month-old \( \text{Mkx}^{-/-} \) mice do not possess characters of neither normal AF cells nor normal NP cells.

In microcomputerized tomography views of the lumbar spine, the disc height index between L6 and S1 was measured in \( \text{Mkx}^{+/+} \) and \( \text{Mkx}^{-/-} \) mice\(^{28}\). As a result, there was no significant difference between the two groups at 10 weeks, but disc height index was clearly reduced in \( \text{Mkx}^{-/-} \) mice at 21 months (Supplementary Fig. 5a–c). Moreover, at 21 months, bone spur formations were observed in the lumbar spine of \( \text{Mkx}^{-/-} \) mice (Fig. 4b; Supplementary Fig. 5d). This phenotype could be related to the instability of vertebrae in \( \text{Mkx}^{-/-} \) mice\(^{29}\).
The degenerative histological score of \( \text{Mkx}^{-/-} \) mice was higher than that of \( \text{Mkx}^{+/+} \) mice at 12 and 21 months (Fig. 4c). These degenerative changes were also observed in the cervical–thoracic and sacral–caudal junctions in \( \text{Mkx}^{-/-} \) mice. It was predicted that the mechanical stress on IVD was related to the degenerative change of IVD of \( \text{Mkx}^{-/-} \) mice because the changes did not occur in all IVDs. These results indicate the essential role of \( \text{Mkx} \) in collagen fibril formation in the OAF during developmental stages, and its function as a stabilizer in IVDs.

The ability of \( \text{Mkx} \) for the differentiation of MSC. Cell transplantation has been proposed as an effective strategy to treat IVD degeneration. Among the various cell sources available, MSCs either from the bone marrow or adipose tissue have been proved to facilitate IVD repair in animal models. To test whether MSCs with \( \text{Mkx} \) induction are a therapeutic option for IVD regeneration, we overexpressed Venus-Mkx in C3H10T1/2 cells using retroviruses (C3H10T1/2 cells induced by Venus-Mkx are referred to as C3H10T1/2-VM or Venus-Mkx, and C3H10T1/2 cells induced only by Venus are referred to as C3H10T1/2-V or...
Venus). Venus-Mkx overexpression markedly induced the differentiation of C3H10T1/2 cells into a spindle-shaped cell type, with the upregulation of multiple ligament-related genes, such as those encoding Scx, Col1a1, Colla2, Col1a4a1, Tnc and Dcn (Fig. 5a,b). The downregulation of cartilage- and fat-related genes was also observed (Fig. 5c,e), whereas the expressions of bone-related genes were not much changed (Fig. 5d). Western blot analyses of Col1, Dcn and Sox9 were consistent with the results of the qRT–PCR (Fig. 5f). We also confirmed the above data observed with C3H10T1/2 using mouse bone marrow-derived MSCs (Supplementary Fig. 6). These data indicate that Mkx promotes differentiation of MSCs to tendon/ligament-like cells in gene expression levels, consistent with the previous reports.14,15

To gain insight into the molecular mechanisms underlying the determination of cell lineage by Mkx, we performed microarray analyses of these C3H10T1/2 cells. In addition to the dynamic upregulation of multiple tendon/ligament-related markers and downregulation of cartilage-related markers, transforming growth factor-β (TGFβ)-related genes were upregulated and bone morphogenetic protein (BMP)-related genes were downregulated in C3H10T1/2 cells (Supplementary Fig. 7a,b). Previous reports revealed that TGFβ functions in IVDs to promote the differentiation of AF from the sclerotome and to prevent chondrocyte differentiation in the presumptive IVD.22 Other reports suggested that disc cells continue to respond to TGFβ signalling during postnatal growth33 and that BMP signalling was related to IVD degeneration.34 However, the role of Mkx in these pathways remains unclear. Therefore, further analyses were performed. qRT–PCR revealed that Smad2, Smad3, TGFβr1 and TGFβr2 were upregulated (Fig. 6a), whereas Smad1, Bmpr1a and Bmpr2 were downregulated (Fig. 6c). Western blot analysis also showed that Smad3 and p-Smad2/3 were increased, whereas Smad1 and p-Smad1/5/8 were decreased (Fig. 6b,d). Luciferase assay using BMP-responsive luciferase reporter revealed that C3H10T1/2-VM exhibited reduced luciferase activity with BMP2 stimulation compared with C3H10T1/2-V (Fig. 6e). These results suggest that changes in the Mkx-dependent Smads ratio in C3H10T1/2 account for the promoted signal responses to TGFβ and the reduced responsiveness to BMP signals. Furthermore, these changes in signal responsiveness may partly explain the promotive function in the IVD, the ligament differentiation of MSC,36,37 and the suppression of differentiation into other lineages and IVD degeneration.22 So we examined the mesodermal-lineage induction in C3H10T1/2-V and C3H10T1/2-VM using BMP2. In C3H10T1/2-VM cells, the induction of differentiation into chondrocytes and osteocytes was severely disrupted (Fig. 6f–i). qRT–PCR analyses showed either very little or no expression in multiple cartilage- and bone-related markers compared with C3H10T1/2-V cells (Fig. 6j,k). Adipogenic differentiation was also inhibited by Mkx overexpression (Supplementary Fig. 8). These results suggest that Mkx act positively on the property maintenance of the AF cells and suggest that MSCs overexpressing Mkx can be effective as transplanted cells.
The possibility of Mkx for therapeutic tools of OAF. To test whether MSCs overexpressing Mkx are capable of producing collagen fibres, which can be used for OAF regeneration in vivo, we first performed an in vitro three-dimensional culture experiment using a chemical gel without animal collagen. After 8 weeks, we performed IHC for Col1a1 and found that C3H10T1/2-VM cells had a higher capacity for type I collagen synthesis compared with C3H10T1/2-V cells (Supplementary Fig. 9). On the basis of these results, we next examined whether C3H10T1/2-VM cells inserted in a type I collagen scaffold, with greater rigidity than a chemical gel, embedded subcutaneously in dorsal skin pockets of mice had the potential to form collagen fibrils (Fig. 7a–c,e). The control group was set to the group of C3H10T1/2-V cells. After 8 weeks, the gels were excised and evaluated. HE staining and IHC revealed that, although the reconstruction of the structure of lamella was not confirmed, abundant tissue was synthesized in the OAF even in an environment different from the OAF.

Transmission electron microscopy (TEM) revealed that the diameter of collagen fibrils of the C3H10T1/2-VM scaffold was clearly larger compared with that of the C3H10T1/2-V scaffold (Fig. 7d,f). TEM also revealed that collagen fibrils were embedded subcutaneously in dorsal skin pockets of mice had the potential to form collagen fibrils (Fig. 7a–c,e). The control group was set to the group of C3H10T1/2-V cells. After 8 weeks, the gels were excised and evaluated. HE staining and IHC revealed that, although the reconstruction of the structure of lamella was not confirmed, abundant tissue was synthesized in the OAF even in an environment different from the OAF.

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Figure 4 | Mkx−/− mice develop early onset disc degeneration. (a,b) Histological and computerized tomography (CT) changes with age between Mkx+/+ and Mkx−/− mice. Safranin O fast green staining of sagittal sections of L3/4 (a) and sagittal CT images of the lumbar spine (b) at 10 weeks, and 6, 12 and 21 months. Scale bars, 300 μm. (c) Histological degenerative scores of intervertebral discs (IVDs) in Mkx+/+ and Mkx−/− mice. Error bars represent s.e.m. **P < 0.01, ***P < 0.001. Statistical differences were assessed with Student’s t-test.

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Next, we investigated whether MSCs overexpressing Mkx have the capacity to repair AF defects. A solution of liquefied collagen gel containing C3H10T1/2-V or C3H10T1/2-VM cells was placed in the cavity of the AF (Fig. 8a–d). After 8 weeks, the discs were excised and evaluated. HE staining and IHC revealed that, although the reconstruction of the structure of lamella was not confirmed, abundant tissue was synthesized in the OAF transplanted with C3H10T1/2-V cells (Fig. 8f–i). TEM analysis revealed that there were also abundant collagen fibrils in the tissues that were transplanted with C3H10T1/2-VM cells in the entire field, even at low magnification; in contrast, there were few collagen fibrils in the tissues that were transplanted with C3H10T1/2-V cells (Fig. 8j,k). In addition, the diameter of the collagen fibrils of C3H10T1/2-VM tissues was larger than that of C3H10T1/2-V tissues (Fig. 8l). Moreover, the diameter of these fibrils was almost equal in size to those of intact OAF of C3H1/HeScI mice (Fig. 8l). Although the detailed mechanism underlying these observations remains unknown, these results suggest that the environment around the transplant contributes to the regulation of collagen fibril formation. Subsequently, to examine whether the newly formed tissue had sufficient physical property against mechanical stress and could inhibit IVD degeneration, we performed a modified tail-looping model, in which the uneven mechanical loading of the IVD was applied to the transplanted site in this experiment (Fig. 8c).
the degenerative changes of the whole AF. Wild-type C3H/HeSlc mice with tail-looping were used as a control group. After 4 weeks, the discs were excised and evaluated. In Safranin O fast green staining, the AF of C3H10T1/2-V group was severely disrupted and the red area, which stained aggrecan in the NP, was reduced (Fig. 8m,n). In contrast, the AF of C3H10T1/2-VM group was not significantly different and it was maintained at a high level. The histological grading of the AF by Nishimura system showed that the control group and the C3H10T1/2-VM group were not significantly different and it was maintained at a high level. These results indicate the newly synthesized tissue of C3H10T1/2-VM group was nearly equal physical property of an intact AF against compression force.

Discussion

The biomechanical properties of the IVD are produced by the structure in which AF surrounds the NP to provide enough flexibility and alleviate the pressure. Extensive mechanical stress or aging could be a cause of IVD degeneration including disc herniation with serious clinical symptoms, such as lower back pain and leg pain, which restricts the patients’ quality of life. To prevent degeneration of IVD and to develop regenerative therapies for IVD, it is necessary to understand the molecular program of IVD development and homeostasis. The developmental molecular mechanisms of NP and IAF have recently been revealed to some extent. Sonic Hedgehog (Shh) and transcription factors Fox1a and Fox2a are essential for the formation of the NP in mouse embryos. Sox5, 6 and 9 also play important roles in the formation and maintenance of NP and IAF. There are also some reports regarding the maintenance of NP: the basic fibroblast growth factor was required for the maintenance of the properties of NP cells, and inhibition of tumour necrosis factor α production positively enhanced NP tissue growth.

In contrast, there has been very limited information about the molecular network regulating OAF development. Here we reveal that Mkx was expressed specifically in the AF, and mainly in the OAF from embryonic stages throughout life (Fig. 1a–c). In addition, we observed severe degenerative phenotype of the AF in Mkx−/− mice. In vitro analyses in both mouse and human AF cells revealed that Mkx regulates the expression of some collagen and SLRPs genes (Fig. 3a,b), the main components of OAF tissues. Scx expression was downregulated in Mkx−/− mice (Fig. 3) and was promoted by Mkx overexpression in C3H10T1/2 cells and bone marrow-derived MSCs (Fig. 5a,b). Scx expression in C3H10T1/2 cells was significantly decreased in the presence of siMkx (Fig. 5a). These results indicate the newly synthesized tissue of C3H10T1/2-VM cells has nearly equal physical property of an intact AF against compression force.
MKX expression was decreased with age in the human anterior cruciate ligament. It is of interest to analyse Mkx expression in adult IVD tissues. Scale bars, 200 μm. (h) Mucopolysaccaride qRT–PCR analyses of mesodermal gene expression in C3H10T1/2-V and C3H10T1/2-VM cells in the presence or absence of induction with BMP2. mRNA levels in C3H10T1/2-VM cells without induction were normalized to 1. Error bars represent s.e.m. **P < 0.01. Statistical differences were assessed with Student’s t-test. (i) Alcian blue staining of C3H10T1/2-V and C3H10T1/2-VM cells after induction of chondrocyte differentiation. (j) Alizarin red staining of C3H10T1/2-V and C3H10T1/2-VM cells after induction of osteocyte differentiation. Scale bars, 200 μm. (k) Luciferase assay to determine responsiveness to BMP2. Id1-Bre luciferase was overexpressed in C3H10T1/2 cells downregulated the expression of Mkx in knockdown OAF cells (Fig. 3b). In addition, Mkx overexpression in C3H10T1/2 cells downregulated the expression of Sox9 (Fig. 5c, h). Consistent with this observation, it is reported that Sox9 expression is downregulated in OAF cells during the differentiation of OAF cells from the Sox+/Sox9 cells. These results suggest that multilayer composition of OAF and IAF might be partly regulated by reciprocal regulation between Mks and Sox9.

Furthermore, Mks-induced C3H10T1/2 acquired greater responsiveness to TGFβ, but reduced responsiveness to BMP (Fig. 6a–e). As TGFβ has a function not only to promote the differentiation of AF from the sclerotome but also to prevent chondrocyte differentiation in the presumptive IVD, the gradient is shadowing the differences between expression levels of Mks and Sox9 in AF. In Mks−/− mice, this transition between IAF and OAF layers is disturbed and the expression of Sox9 was upregulated in Mks knockout OAF cells (Fig. 3b). In addition, Mks overexpression in C3H10T1/2 cells downregulated the expression of Sox9 in AF cells (Fig. 5c, i). Consistent with this observation, it is reported that Sox9 expression is downregulated in OAF cells during the differentiation of OAF cells from the Sox+/Sox9 progenitor cells. These results suggest that multilayer composition between OAF and IAF might be partly regulated by reciprocal regulation between Mks and Sox9.
The collapse of either AF or NP may cause the tissue degeneration of the whole IVD. The collapse of NP and loss of NP property will cause a decrease of mechanical resistance of the AF, followed by the degeneration of the entire IVD. To regain normal NP property, introduction of several growth factors or MSCs into NP has been reported in animal models and in clinical trials. Conversely, in this report, after the initial degradation of AF in Mkx<sup>−/−</sup> mice, we also observed the following degenerative changes of the NP tissue, where Mkx is not expressed (Fig. 1c). This phenotype may be explained as secondary changes due to insufficient mechanical property of the AF, as previous reports showed. The abolishment of critical matrix molecules in the AF of mice, such as BGN<sup>19,21</sup> and secreted protein acidic rich in cysteine (SPARC)<sup>61</sup>, have been shown to cause the whole IVD degeneration. In these knockout mice, including Mkx<sup>−/−</sup> mice, morphologically resembling chondrocytes were seen in the degenerative NP, the origin of which is unclear. Further cell...
**Figure 8 | Mkk-induced mesenchymal stem cells promote functional AF regeneration.** (a) Strategy for assessing type I collagen synthesis of C3H10T1/2 cells induced by Venus (C3H10T1/2-V or Venus) and Venus-Mkk (C3H10T1/2-VM or Venus-Mkk) after outer AF (OAF) transplantation. (b) OAF before creating a transplantation site. Scale bar, 200 μm. (c) OAF after creating a transplantation site. Scale bar, 200 μm. (d) Cut surface of intervertebral disc (IVD) after creating a transplantation site. Yellow arrow, transplantation site. Scale bar, 200 μm. (e) Schema of the modified tail-looping model. Black arrow, transplanted site. (f,h) Haematoxylin and eosin staining of the transplantation sites of the OAF (f: C3H10T1/2-V; h: C3H10T1/2-VM). * The area in which the collagen gel was dissolved. ** Newly synthesized tissue. *** OAF. Scale bars, 50 μm. (g,i) IHC of these products (g: C3H10T1/2-V; i: C3H10T1/2-VM). Blue, Hoechst; green, anti-GFP. Scale bars, 50 μm. (j,k) Images of transmission electron microscopy (j: C3H10T1/2-V; k: C3H10T1/2-VM). Scale bars, 500 nm. (l) The calculated mean of the diameter of 100 collagen fibrils from C3H10T1/2-V and C3H10T1/2-VM cells. The mean diameter of 100 collagen fibrils was calculated: for C3H10T1/2-V cells, mean diameter = 53.72 nm (s.d. = 7.281 nm); for C3H10T1/2-VM cells, mean diameter = 83.54 nm (s.d. = 16.34 nm); for the intact OAF of C3H/HeSlc mice, mean diameter = 77.74 nm (s.d. = 24 nm). Error bars represent the s.d. **P < 0.01. (**m,n) Representative images of IVDs in each group. Safranin O fast green staining. (m) Whole images of each group. Transplantation sites of C3H10T1/2-V or C3H10T1/2-VM cells are on the left side in the images. Scale bars, 300 μm. (n) Images with a focus on the transplanted side of the AF. Scale bars, 100 μm. (o) Histological grading score of the AF using the Nishimura system. The mean average grade: control = 2.4 (s.d. = 0.5164); Venus = 4 (s.d. = 0.4082); Venus-Mkk = 2.2 (s.d. = 0.7528). **P < 0.01. Statistical differences were assessed with Student’s t-test. GFP, green fluorescent protein.
Three-dimensional culture. To perform the three-dimensional cell culture, cells were trypsinized and suspended in α-MEM and then mixed with PanaceaGel (NeuroNure, USA) at a volume ratio of 1:2. Then, one micro-liter suspension of the cells in the hydrogel was 2 × 10^5 cells per ml. The cells were grown in α-MEM plus 10% FBS and 1% PS (Invitrogen), and incubated in 5% CO₂ at 37°C. The medium was replaced with fresh medium every 2–3 days. The gels containing cells were subsequently used for HIC.

Knockdown of MKX in HAF cells. Fifty micrometres of small interfering RNA for MKX (Silencer Select, s49084 and s49085, Thermo Fisher) or negative control (Silencer Select Negative Control No.1, 4390843, Thermo Fisher) was transfected into HAF cells (8 × 10⁶ cells per well) using 8 μl of Lipofectamine RNAiMAX (Invitrogen) in six-well plates. After 72 h, total RNAs and cell lysates were extracted and used for qRT–PCR and western blotting, respectively.

Retrovirus infection. Venus or Venus-Mkx coding sequences were inserted into the pMIGR vector (Addgene). The pMIGR-Venus construct (as a control) or the pMIGR-Venus-Mkx construct was transfected into PLAT-E cells using FugeneHD (Promega). Forty-eight hours later, media were collected, filtered and transferred to the CHI10T1/2 stable cell line in a medium containing puromycin (5 μg ml⁻¹).

Mkx knockdown using a cDNA shRNA cassette11. Male C57BL/6N and C3H/HeSlc mice were purchased from the Sankyo Laboratory (Tokyo, Japan). This animal study was approved by the Tokyo Medical and Dental University ethical committee.

Methods
Animals. Venus knock-in Mkx heterozygous mutant mice in a C57BL/6N background carried an insertion of a Venus cassette that inactivates the Mkx gene. We inactivated the Mkx gene by homologous recombination in embryonic stem cells using a targeting vector to replace the Mkx gene from the translation start site to the end of exon 2 with the Venus gene and PKG-neomycin-resistance (PGKneo) cassette.11 Male C57BL/6N and CHI/HeSlc mice were purchased from the Sankyo Laboratory (Tokyo, Japan). This animal study was approved by the Tokyo Medical and Dental University ethical committee.

Human samples. The human samples (nine discs from five human donors; Supplementary Table 1) were obtained from the organ banks through the commercial source (Cosmo Bio USA, Carlsbad, CA). The informed consent was obtained from families of donors through the organ bank.

Cell culture. CHI10T1/2 cells were grown in α-MEM with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin (PS; all Sigma-Aldrich). PLAT-E cells were grown in DMEM plus 10% FBS (all Sigma-Aldrich). HAF cells (Articular Engineering) were grown in DMEM: Nutrient Mixture F-12 HAM (DMEM:F12 HAM) plus 20% FBS, 1% PS and 20 μg ml⁻¹ of ascorbate. Mouse OAF cells were isolated as follows. Mkx⁻/⁻ or C57BL/6N mice were killed by cervical dislocation and tail discs were dissected (T1/2-7/8, 10 mice per group). Using a clean bench, the discs were trimmed and pieces of OAF tissues were obtained. These tissues were incubated with Triple Express (Gibco) for 30 min and 0.25% Liberase (Roche) for 75 min (Supplementary Fig. 2a–c). Isolated cells were grown in α-MEM with 20% FBS and 1% PS (Invitrogen) until day 5, at which point the medium was changed to α-MEM plus 10% FBS and 1% PS (Invitrogen). Cells were retrieved in passage 1 and processed for qRT–PCR analysis. The purity of AF cells was confirmed by examining the number of Venus-positive cells in the isolated Mkx⁻/⁻ cells (Supplementary Fig. 2d). All cells were cultured at 37°C in 5% CO₂.
anti-Collagen 14 antibody (1:100, LS-C119470, LSBio), anti-Bgn antibody (1:100, LS-C241858, LSBio), anti-CD24 antibody (1:100, 553262, Bioscience) and anti-KRT18 antibody (1:500; Life Technologies) or Hoechst 33342 (1:2000; Life Technologies) for 1 h. The sections for Collagen 14, Bgn, CD24 and KRT18 were mounted with VECTASHIELD (VECTOR Laboratories) or Hoechst 33342 (1:2000; Life Technologies) for 1 h. Confirmable signals in 10 × views were recorded in three fields per view, and the sum and percentage of Hoechst-positive MKX-positive cells were calculated.

**In situ hybridization.** Mouse embryos were obtained after euthanasia by cervical dislocation (E14.5 of MxK+/− mice). The samples were fixed in 4% PFA/PBS and processed for frozen sectioning (16 μm). Sections were treated with 10 μg/ml−1 proteinase K (Roche) for 10 min at room temperature (RT) and then acetylated with acetylation solution for 20 min at RT. Pre-hybridization (in × 5 SSC (pH 4.5), 50% formamide, 1% SDS, 50 μg ml−1 yeast tRNA (Roche) and 50 μl ml−1 heparin (Nacalai Tesque)) was performed at 68°C for 2 h; then, a DIG- RNA probe (500 ng ml−1) was added and hybridized for 14 h at 68°C. Subsequently, sections were subjected to a series of post-hybridization washes in wash buffers containing formamide, SSC, SDS and 0.05% CHAPS. After blocking with 2% BM Blocking reagent (Roche) containing 0.1% Tween 20 (TBST) for 30 min at RT, embryos were incubated with anti-DIG-AP Fab antibody fragments (Roche) and 1% sheep serum in TBST for 3 h at RT. After a series of washes with TBST, embryos were equilibrated with NTMT (5 M NaCl, 1 M Tris–HCl (pH 9.5), 1 M MgCl2, and 0.1% Tween 20). Colour development reactions were performed at 4°C or RT with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (Roche).

**Electron microscopy analysis.** Lunbar discs (L4/5 level) from MxK−/− or wild-type mice (10 weeks of age, male, one per group of littersmates) and tail discs from 8-week postoperative C3H/HeSlc mice (18 weeks of age, n = 3 per group) were dissected and fixed in 2.5% glutaraldehyde solution for 2 h and 1% osmium solution (Sigma–Aldrich) for 1 h. After fixation, tissues were processed for sectioning (1 μm).

For scanning electron microscopy, the specimens were dried in a critical point drying apparatus (HCP-2; Hitachi, Tokyo, Japan) with liquid CO2 and were then acetylated with acetic acid buffer at 98–100°C for 10 min in a microwave oven. The sections were then blocked with Blocking One solution (Nacalai Tesque) for 60 min and incubated with a rabbit anti-human MKX antibody (1:500, LS-C36267; LifeSpan Biosciences) overnight at 4°C. The sections were then incubated with a rabbit Alexa Fluor 488 donkey anti-rabbit antibody (1:500; Life Technologies) and Hoechst 33342 (1:2000; Life Technologies) for 1 h. The scan was done in air.

**Western blotting.** Whole-cell proteins were extracted from C3H10T1/2-V and C3H10T1/2-VM cells, and HAF cells. Cellular proteins collected in SDS–PAGE sample buffer were electrophoresed on 4–12% SDS-bis-ris gels and transferred to polyvinylidene difluoride membranes. Membranes were blocked in Blocking One solution (Nacalai Tesque) for 30–60 min. After blocking, the membranes were incubated overnight at 4°C with dilutions of anti-Smad1 (1:500, #9743; Cell Signaling Technology), anti-phospho-Smad1/5/8 (1:500, #9511; Cell Signaling Technology), anti-Smad2/3 (1:500 BD #610843), anti-phospho-Smad3 (1:1000 Abcam Ab52903), anti-Sox9 (1:1000 Millipore AB5535), anti-Bgn (1:1000 LS-C341858), anti-Coll (1:250 Abcam ab21386), anti-Dcn (1:250 Abcam ab137501), anti–β-actin (1:5000; MAB374; Millipore) antibodies. Rinsed membranes were incubated for 1 h with ECL mouse or rabbit IgG, horseradish peroxydase-Linked Whole antibody (GE Healthcare). The enhanced chemiluminescence immunoblotting detection system (Thermo Scientific) was used to detect the antigen–antibody complexes. Extracts were prepared from three independent samples. Full scans of the key immunoblots are indicated to Supplementary Fig. 12.

**Luciferase assay.** C3H10T1/2-V or C3H10T1/2-VM cells were seeded at 1 × 104 cells per well in 96-well plates (Cellstar; Greiner Bio-One). On the following day, the ID1-Bre luciferase construct (Addgene 25) was transfected using FuGENE HD (Promega). Twenty-four hours later, the medium was changed to 10% MEM plus 0.5% FBS and 1% FBS. Twelve hours later, BMP2 at 100 ng ml−1 was added to the medium. Twenty-four hours later, the luciferase activity was quantitated by using the luciferase assay kit (AKO3, Cosmo Bio). Osteogenic differentiation was performed as follows. Trypsinized cells were resuspended in ζ-MEM (Sigma–Aldrich) at a concentration of 2 × 103 cells per ml−1, and a 20 μl drop of this cell suspension was placed in the centre of a well in a 12-well plate (Cellstar; Greiner Bio-One). The cells were allowed to adhere for 2 h at 37°C and 5% CO2 and 1 ml of ζ-MEM (Sigma–Aldrich) containing BMP2 (100 ng ml−1) was added to the culture. Osteogenic differentiation was performed by adding asialic blue staining, acidic mucopolysaccharide quantification and qRT–PCR after 9 days of culture. Acidic mucopolysaccharide quantification was performed using the Mucopolysaccharide Assay kit (AKO3, Cosmo Bio). Osteogenic differentiation was performed as follows. Trypsinized cells were resuspended in ζ-MEM (Sigma–Aldrich) at a concentration of 8 × 103 cells per well in a 12-well plate (Cellstar; Greiner Bio-One). The cells were incubated for 24 h, after which the medium was replaced with the induction medium (10% ζ-MEM, 10% FBS, 100 μM ascorbic acid, 10 mM 0 β-glycerophosphate and 100 ng ml−1 of BMP2). Osteogenic differentiation was analysed by alizarin red staining, calcification evaluation and qRT–PCR after 14 days of culture. Calcification evaluation was performed using the Calcification Evaluation set (CSR-ARD-SE; Cosmo Bio). PBS-washed cells were incubated with 4% PFA/PBS and fixed with 2% Alcian Blue staining (CSR-ARD-A1; Cosmo Bio). After washing the cells, the alizarin red dye was extracted with Calciﬁed Nodule Extraction Solution (CSR-ARD-E1; Cosmo Bio) and the absorbance at 450 nm was determined using a microplate reader.
Scoring system of IVD degeneration. We used the IVD degenerative histological score to evaluate the whole IVD of ageing wild-type and Mx−/− mice. Two blinded orthopedic surgeons performed each image evaluation and each scoring. To evaluate the AF of modified tail-looping model mice, we used the Nishimura system, a histological grading system of degenerative changes in the AF. This system is classified into six grades (grade 0: normal structure; grade 1: mildly serpentine with rupture; grade 2: moderately serpentine with rupture; grade 3: severely serpentine with mildly reversed contour; grade 4: severely reversed contour; grade 5: indistinct).

Statistical analysis. Data are presented as the mean ± s.e.m. The Student’s t-test was used to evaluate differences between groups. A one-tailed t-test was performed for Figs 6b and 8b, while a two-tailed t-test was performed for the other figures. P values <0.05 were considered significant. *P<0.05. **P<0.01. ***P<0.001.

Data availability. Microarray data that support the findings of this study have been deposited in GEO with the primary accession code GSE12776. The authors declare that all other data supporting the findings of this study are available within the article and its files.

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Author contributions
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