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Synovial joint morphogenesis requires the chondrogenic action of *Sox5* and *Sox6* in growth plate and articular cartilage

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ABSTRACT

The mechanisms underlying synovial joint development remain poorly understood. Here we use complete and cell-specific gene inactivation to identify the roles of the redundant chondrogenic transcription factors Sox5 and Sox6 in this process. We show that joint development aborts early in complete mutants ($Sox5^{-/-}6^{-/-}$). *Gdf5* and *Wnt9a* expression is punctual in articular progenitor cells, but *Sox9* downregulation and cell condensation in joint interzones are late. Joint cell differentiation is unsuccessful, regardless of lineage, and cavitation fails. *Sox5* and *Sox6* restricted expression to chondrocytes in wild-type embryos and continued *Erg* expression and weak *Ihh* expression in $Sox5^{-/-}6^{-/-}$ growth plates suggest that growth plate failure contribute to this $Sox5^{-/-}6^{-/-}$ joint morphogenesis block. *Sox5/6* inactivation in specified joint cells and chondrocytes ($Sox5^{fl/fl}6^{fl/fl}Col2Cre$) also results in a joint morphogenesis block, whereas *Sox5/6* inactivation in specified joint cells only ($Sox5^{fl/fl}6^{fl/fl}Gdf5Cre$) results in milder joint defects and normal growth plates. $Sox5^{fl/fl}6^{fl/fl}Gdf5Cre$ articular chondrocytes remain undifferentiated, as shown by continued *Gdf5* expression and pancartilaginous gene downregulation. Along with *Prg4* downregulation, these defects likely account for joint tissue overgrowth and incomplete cavitation in adult mice. Together, these data suggest that synovial joint morphogenesis relies on essential roles for *Sox5/6* in promoting both growth plate and articular chondrocyte differentiation.

Introduction

Synovial joints allow vertebrates to display a great range of motions by articulating bones with one another. They consist of multiple highly specialized tissues. Articular cartilage coats bone surfaces and ensures pain-free and deformation-free movement. A fibrous capsule surrounds the joint and seals its cavity. A synovial membrane lines this capsule internally and produces a lubricating synovial fluid rich in nutrients for the avascular articular cartilage. Tendons allow articular motion by transmitting muscle force to bones, and ligaments stabilize joints by interconnecting bones. In addition, the knee features shock-absorbing menisci, cruciate ligaments, and space-filler fat pads. It is thereby the most complex synovial joint. Articular diseases occur in two main types. On one hand, inherited joint malformations are relatively rare, but present with a wide range of forms and severity, hinting to a complex genetic regulation of joint development (Ikegawa, 2006; Pauli, 2007). Of note, synovial joint malformations are often associated with other skeletal defects, mainly chondrodysplasias, suggesting important reciprocal cause/effect relationships. On the other hand, joint degeneration diseases, collectively called arthritis, are highly prevalent in humans, especially in the elderly (Goldring and Goldring, 2007; Hunter, 2007; Theis et al., 2007). They are caused by genetic defects as well as injuries and are aggravated by such conditions as obesity and joint overuse. They result in reduced mobility, pain and chronic disability. Yet, efficient treatments remain virtually nonexistent for many types of joint diseases. This can be explained at least in part by the fact that our understanding of the cellular and molecular mechanisms that underlie synovial joint development and adult homeostasis is still very limited. Therefore, it is requisite that these mechanisms be further defined if we want to be able to design new, efficient treatments for these diseases.

The first step in joint development is the specification of articular progenitor cells (Pacifici et al., 2005; Khan et al., 2007; Pitsillides and Ashhurst, 2008). It occurs in the embryo once skeletogenic mesenchymal cells have condensed into precartilaginous masses. Most cells in these masses commit to the growth plate chondrocyte fate, while specific subpopulations commit to the articular fate and go on to develop into one or another synovial joint cell type. These subpopulations are located in the outskirts of precartilaginous condensations as well as in a few discrete sites within these condensations. Milestone discoveries over the last decade have demonstrated that several signaling pathways work in a cascade to specify the fate of articular

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cells. TGFβ signaling, mainly triggered by the Tgfbr2 receptor, acts upstream of canonical Wnt/beta-catenin signaling (Seo and Serra, 2007; Spagnoli et al., 2007). The latter pathway is initiated by the Wnt9a (formerly named Wnt14), Wnt4, Wnt16, and possibly additional Wnt ligands (Hartmann and Tabin, 2001; Guo et al., 2004; Später et al., 2006). It results in the activation of the genes for the growth differentiation factor-5 (Gdf5) and other related bone morphogenetic proteins (BMPs), which contribute together to the specification of synovial joints (Storm and Kingsley, 1999; Settle et al., 2003). Articular progenitor cell specification prompts the downregulation of the gene for the master chondrogenic transcription factor Sox9, and the formation of a zone of condensed cells, called interzone. In addition to its specific expression of *Gdf5*, this zone is also readily identifiable by its expression of the gene for the Ets-domain transcription factor Erg (Iwamoto et al., 2007).

Joint morphogenesis follows the formation of joint interzones. This process consists in joint cavitation and concomitant differentiation of articular progenitors into articular and meniscal chondrocytes, synovial fibroblasts, fat pad cells, and cruciate ligament tenocytes (Rountree et al., 2004; Koyama et al., 2008). The mechanisms controlling each of these events and ensuring their exquisite coordination remain unclear. Skeletal movement, hyaluronan secretion, and shifts in extracellular matrix composition may contribute to inducing joint cavitation (Ito and Kida, 2000; Dowthwaite et al., 2003). Notch signaling, TGFβ signaling, and Erg may control articular chondrocyte specification and differentiation (Serra and Chang, 2003; Hardingham et al., 2006; Iwamoto et al., 2007). While most limb tendons and ligaments arise from cell populations distinct from those of the precartilaginous condensations, cruciate ligaments have a unique origin in the knee interzone (Schweitzer et al., 2001). Key roles have been identified for the helix–loop–helix transcription factor Scleraxis (*Scx*), and for FGF and TGFβ signaling in tenocyte fate determination (Tozer and Duprez, 2005; Murchison et al., 2007), but the mechanisms specifically involved in the development of cruciate ligaments are unknown. Finally, the developmental control of the meniscus, synovium, and fat pad remains elusive, as is the control of the few cell layers that line the joint surface of the articular cartilage and synovium and that distinguish themselves by producing the lubricin proteoglycan (encoded by *Prg4*; Rhee et al., 2005).

Sox5, Sox6 and Sox9 constitute a transcription factor trio that is essential for the development embryonic cartilage primordia and growth plates (Lefebvre and Smits, 2005; Akiyama, 2008). *Sox9* is expressed in pluripotent mesenchymal cells and becomes restricted to chondrocytes once these cells commit to specific lineages (Ng et al., 1997; Zhao et al., 1997). Chondrocytes express *Sox5* and *Sox6* along with *Sox9* from the precartilaginous condensation stage, and they turn off expression of the three genes as they undergo prehypertrophy in the growth plate (Lefebvre et al., 1998; Smits et al., 2001). *Sox9* is absolutely required for cell survival in precartilaginous condensations and for chondrocyte differentiation in cartilage primordia (Bi et al., 1999; Akiyama et al., 2002). In contrast to Sox9, Sox5 and Sox6 are not absolutely required for chondrocyte differentiation, but strongly potentiate Sox9's chondrogenic activity (Lefebvre et al., 1998; Smits et al., 2001; Han and Lefebvre, 2008). The two proteins are highly similar to one another and act in redundancy. They lack a transactivation domain, but efficiently bind to characteristic motifs present in cartilage-specific enhancers. They thereby allow Sox9 to bind to adjacent recognition sites and to use its potent transactivation domain to activate gene expression.

The chondrogenic *Sox* trio is expressed in articular cartilage in addition to cartilage primordia and growth plates, but its role in this tissue is unknown (Fukui et al., 2008). We started this study hypothesizing that it could have essential roles in the development of this tissue. Mice lacking *Sox9* in skeletogenic cells are not appropriate to test this hypothesis as chondrocytic cells fail to survive in precartilaginous condensations, thus precluding formation of presumptive joints. In contrast, *Sox5/6* double-null mice form normal precartilaginous condensations and, even though they are very deficient, cartilage primordia, growth plates, and endochondral bones do develop in these mice. We therefore used these mice along with conditional mutants lacking *Sox5/6* in specific cell populations to identify roles for Sox5/6 in joint development. Our studies show that *Sox5* and *Sox6* have essential cell-autonomous roles in articular cartilage and they also strongly suggest that their ability to promote cartilage primordia and growth plate formation is a non-cell-autonomous prerequisite for joint morphogenesis.

Materials and methods

Mice

Mice were used according to federal guidelines and as approved by the Cleveland Clinic Institutional Animal Care and Use Committee. They harbored *Sox5* and *Sox6* null alleles (Smits et al., 2001), *Sox5* and *Sox6* conditional null alleles (Dumitriu et al., 2006; Dy et al., 2008), the *Gt(ROSA)26Sor*^{tm1Sho} allele (referred to as *R26*^{lacZ}; Mao et al., 1999), and the *Prx1Cre* (Logan et al., 2002), *PrmCre* (O'Gorman et al., 1997), *Zp3Cre* (de Vries et al., 2000), *Gdf5Cre* (Rountree et al., 2004), or *Col2Cre* transgene (Ovchinnikov et al., 2000). *Sox5*$^{-/-}$*6*$^{-/-}$ embryos were obtained by mating *Sox5*$^{+/-}$*6*$^{+/-}$ males with *Sox5*$^{+/-}$*6*$^{+/-}$ females, or by mating *Sox5*$^{fl/fl}$*6*$^{fl/fl}$*PrmCre* males with *Sox5*$^{fl/fl}$*6*$^{fl/fl}$*Zp3Cre* females. The latter type of couples produced about 50% *Sox5*$^{-/-}$*6*$^{-/-}$ embryos (instead of 100%) and 25% control (*Sox5*$^{+/-}$*6*$^{+/-}$) littermates due to low efficiency of *PrmCre*-mediated gene recombination. All mice were on the 129xB6 background. Data were reproduced with at least two pairs of control and mutant littermates. The latter were sex-matched when analyzed postnatally.

Histological analysis

Mouse tissues were fixed with 4% paraformaldehyde and were embedded in paraffin using standard protocols. Postnatal samples were decalcified in 0.5 M EDTA pH 7.5 for up to 15 days after fixation. Sections were stained with Alcian blue and counterstained with nuclear fast red, or stained with Movats using standard procedures. Hyaluronan was detected using biotin-labeled HA-binding protein (Calbiochem, San Diego, CA) and fluorescently labeled (Alexa 488) streptavidin (de la Motte et al., 2003). X-gal staining was performed on whole embryos, which were then sectioned in paraffin, or on frozen sections (Hogan et al., 1994). Data were visualized with Leica DM2500 microscope, captured with Qimaging Micropublisher 5.0 RTV digital camera, and processed with Adobe Photoshop 7.0 software.

RNA in situ hybridization

Paraffin sections were hybridized with ^{35}S-labeled antisense RNA probes and were counterstained with the Hoechst 33258 nuclear dye (Smits et al., 2001). Pictures were taken in dark field with a red filter for RNA signals and under blue fluorescence to capture nuclei. RNA probes were as described: *Agc1*, *Col2a1*, *Col10a1*, *Gdf5*, *Ihh*, *Sox5*, *Sox6*, and *Sox9* (Smits et al., 2001), *Gli1* (Hui et al., 1994), *Prg4* (Rhee et al., 2005), *Scx* (Cserjesi et al., 1995), *Sox5* exon 5 (Dy et al., 2008), *Sox6* exon 2 (Dumitriu et al., 2006), and *Wnt9a* (Bergstein et al., 1997). The *Erg* probe was PCR amplified using published primers (Koyama et al., 2008).

Microcomputed tomography

Mouse skeletal anatomy pictures were generated using GE's eXplore Locus microcomputed tomography system (GE Healthcare, Piscataway, NJ). Whole-body skeletal volumes were acquired at 93 μm voxel resolution and rendered as isosurfaces using MicroView (GE Healthcare). Knee volumes were acquired at 26 μm (20 μm nominal) voxel

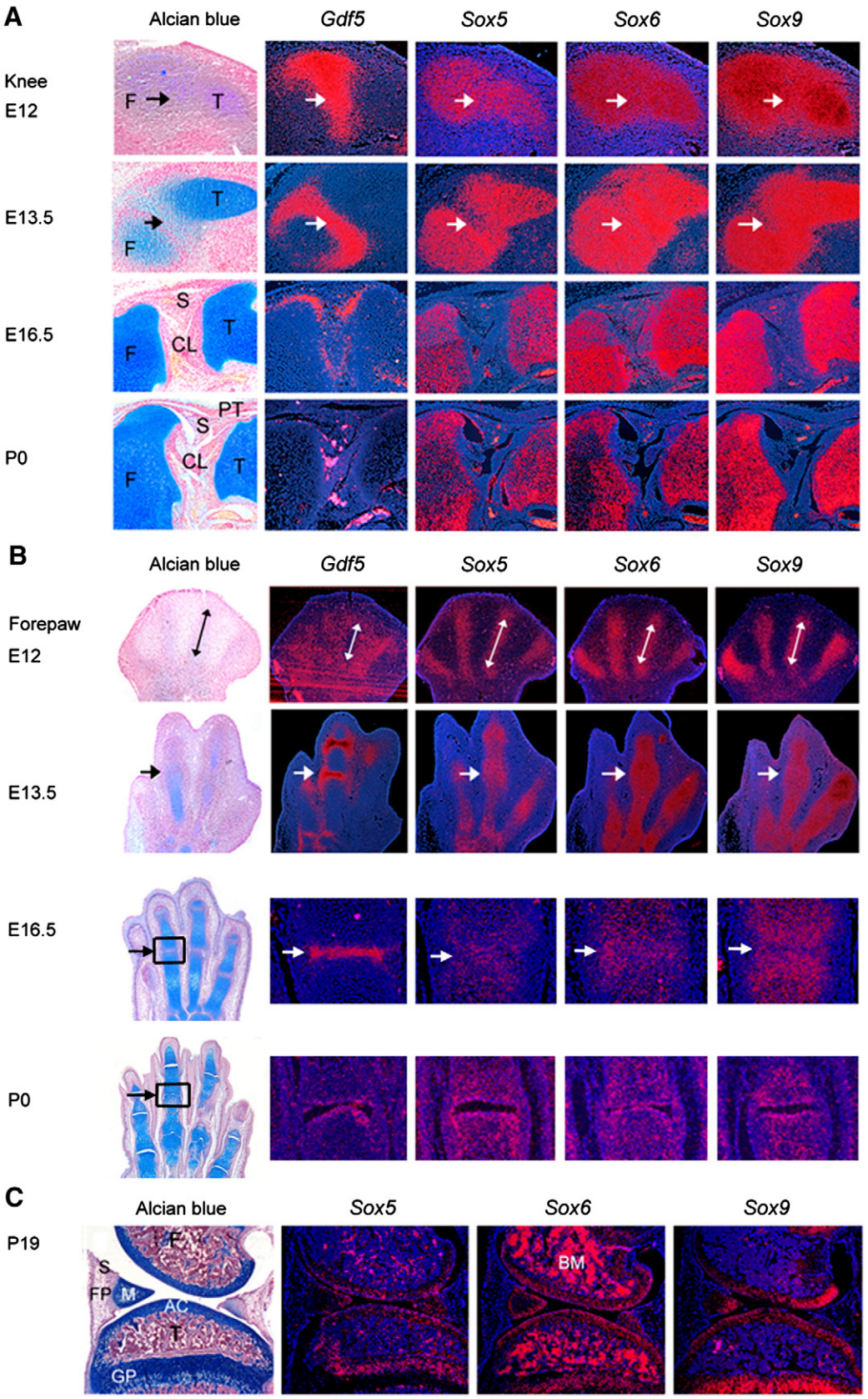


Fig. 1. Expression of *Sox5*, *Sox6*, and *Sox9* in synovial joints. Adjacent sections through the knee (A), forepaw (B), and meniscus (C) of wild-type mice at various ages were stained with Alcian blue or hybridized with *Gdf5* and *Sox* RNA probes, as indicated. The arrows in (A) point to the presumptive knee joint. The double-headed arrows in (B) mark digital condensations, whereas the arrows point to a developing phalangeal joint. The RNA expression data at E16.5 and P0 are shown as high-magnification pictures of the area boxed in the sections stained with Alcian blue. Note in (C) that *Sox6* is strongly expressed in bone marrow (BM). AC, articular cartilage; CL, cruciate ligament; F, femur; FP, fat pad; GP, growth plate. M, meniscus; T, tibia; S, synovium and fat pad; PT, patellar tendon.

resolution and opacity was rendered using VolSuite, 3D visualization and analysis software (Ohio Supercomputer Center, Columbus, OH). Images were inverted using AdobePhotoshop software.

Results

Sox5 and Sox6 are expressed in articular progenitor cells and chondrocytes

We started this study by determining when and where *Sox5* and *Sox6* are expressed in developing joints. We answered this question by hybridizing sections made through the knee and forepaw of wild-type mice at various stages of development with specific RNA probes. Articular progenitor cells in the prospective knee joint were specified by embryonic day 12 (E12), as shown by expression of *Gdf5* (Fig. 1A). *Sox5*, *Sox6* and *Sox9* expression was as strong in these cells as in adjacent precartilaginous condensations, and it started to be down-regulated in these cells around E13.5. By E16.5, expression of *Gdf5* and the three *Sox* genes remained active in presumptive articular cartilage, but was largely turned off in non-cartilaginous joint tissues, namely, the developing fat pad, synovium, and cruciate ligaments. At birth (postnatal day 0, P0), *Gdf5* expression was virtually undetectable in the knee region, and expression of the *Sox* trio was restricted to articular and growth plate cartilage. Similarly, the Sox trio was expressed in the precartilaginous digital rays of E12 embryos, while *Gdf5* was expressed in the condensation outskirts (Fig. 1B). At E13.5, *Gdf5* expression was beginning to mark incipient phalangeal joints, where the *Sox* genes were still expressed. At E16.5, *Gdf5* expression was restricted to the presumptive joints, where the three *Sox* genes remained largely expressed. At P0, phalangeal joints were cavitated. *Gdf5* was still expressed in the articular lining cells, and the three *Sox* genes remained expressed as strongly in presumptive articular chondrocytes as in overtly developed growth plate chondrocytes. At P19 and later on, the three *Sox* genes remained specifically expressed in articular and meniscal chondrocytes as well as in growth plate chondrocytes (Fig. 1C and data not shown). Altogether, these data demonstrate that *Sox5* and *Sox6*, like *Sox9*, are actively expressed in articular progenitor cells and in differentiated articular, meniscal, and growth plate chondrocytes. They thus suggest that the Sox trio may have important roles in synovial joint development and homeostasis.

Sox5 and Sox6 act in redundancy to ensure synovial joint development

To determine whether *Sox5* and *Sox6* control synovial joint development, we analyzed mice harboring null alleles for either or both genes. $Sox5^{-/-}$, $Sox6^{-/-}$, and $Sox5^{+/-}6^{+/-}$ (2-null-allele) mice were born with very mild skeletal dysplasia, as previously described (Smits et al., 2001), and had normal joints (Fig. 2A). $Sox5^{-/-}6^{+/-}$ and $Sox5^{+/-}6^{-/-}$ (3-null-allele) mice developed marked chondrodysplasia, also as described (Smits et al., 2004), and were born with mild joint defects (Fig. 2A). These defects included incomplete cavitation of the knee joint between the menisci and the tibia, and knee valgus deformation, i.e., misalignment of the femur and tibia. $Sox5^{-/-}6^{-/-}$ (4-null-allele) fetuses developed a very severe chondrodysplasia (Smits et al., 2001) and showed drastic synovial joint defects at E16.5 (Fig. 2B). While most joints were overtly developing and starting to cavitate in control littermates, the shoulder joint of $Sox5^{-/-}6^{-/-}$ littermates was missing, the elbow and knee joints were filled with unstructured mesenchyme, and the presumptive phalangeal joints could not be distinguished from underdeveloped precartilaginous digital rays on histology sections. To bypass the $Sox5^{-/-}6^{-/-}$ fetus lethality around E16.5 and determine whether synovial joints develop later on in the absence of *Sox5* and *Sox6*, we generated $Sox5^{fl/fl}6^{fl/fl}$*Prx1Cre* mice. In these mice, a *Cre* transgene allowed recombination of *Sox5* and *Sox6* conditional alleles into null alleles in the early limb bud mesenchyme (Logan et al., 2002). This mesenchyme included all precursor cells of the appendicular skeleton. $Sox5^{fl/fl}6^{fl/fl}$*Prx1Cre* mice were born alive with an extremely severe limb chondrodysplasia (Fig. 2C). Cartilage epiphyses, growth plates and endochondral bones were as rudimentary and as poorly developed as those of E16.5 $Sox5^{-/-}6^{-/-}$ fetuses. Knee joints also were similarly affected. Phalangeal joints could now be distinguished from underdeveloped cartilage primordia, but like other mutant joints, they were filled with unstructured mesenchyme. These data thus reveal that *Sox5* and *Sox6* have essential, redundant roles in synovial joint development.

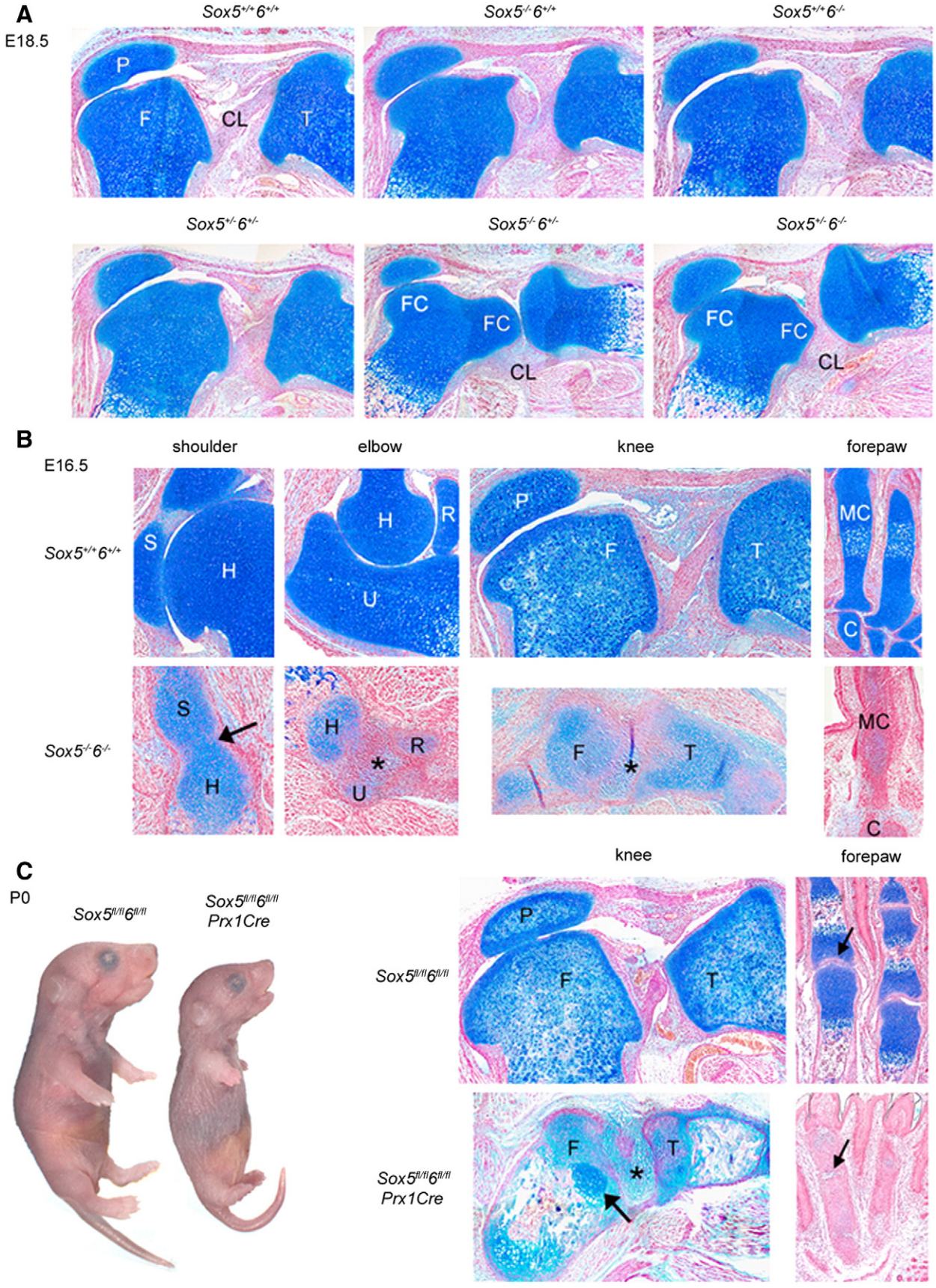
Sox5 and Sox6 are not required to specify articular progenitor cells, but they are required to form joint interzones in a proper and timely manner

Since *Sox5* and *Sox6* are expressed in articular progenitor cells, we asked whether they contribute to the specification of these cells. *Wnt9a* and *Gdf5* were expressed in the incipient knee of both control and $Sox5^{-/-}6^{-/-}$ embryos from E12.5 to E16.5, indicating that this presumptive joint region contained specified articular progenitors (Fig. 3A). However, the expression domain of each gene was loose rather than sharply delineated, and encompassed epiphyseal cells near the joint region. Moreover, the knees of control mice strongly increased *Gdf5* expression between E12.5 and E14.5 and concomitantly downregulated *Sox9* expression, whereas the knees of mutant littermates were still expressing *Gdf5* weakly and *Sox9* highly at E14.5. By E16.5, *Gdf5* was expressed at similar levels in control and mutant knees, and *Sox9* expression was now turned off in both control and mutant knees. The specification of the knee precursors thus started punctually in mutant embryos, but it took more time for these cells than for control cells to form characteristic interzones.

Since cruciate ligaments were absent in $Sox5^{-/-}6^{-/-}$ and $Sox5^{fl/fl}6^{fl/fl}$*Prx1Cre* knees (Figs. 2B and C) and since costal chondrocytes were previously shown to switch to a tenogenic fate in $Sox5^{-/-}6^{-/-}$ embryos (Brent et al., 2005), we asked whether $Sox5^{-/-}6^{-/-}$ limbs were expressing the tenogenic marker *Scx* properly (Schweitzer et al., 2001). We detected strong expression of *Scx* in all developing tendons and ligaments of E14.5 control and mutant limbs, and very low if any expression in precartilaginous condensations (Fig. 3A). This data thus ruled out a switch of chondrocytic cells to a tenogenic fate in the mutant limbs. At E14.5, the expression domain of *Scx* was highlighting the developing cruciate ligaments in the control knees, but it was very diffuse in the mutant knees, and by E16.5, *Scx* expression was no longer detected in the mutant knees. We failed to detect significant apoptosis in this region (data not shown) and thus conclude that the mutant cruciate ligament precursors likely lost their tenogenic fate.

We then examined synovial joint specification in developing digits. At E12.5, both control and $Sox5^{-/-}6^{-/-}$ embryos were expressing *Gdf5* in the cells bordering the digital precartilaginous condensations (Fig. 3B). It was previously suggested that phalangeal joints might develop by migration of *Gdf5*-expressing cells from the perichondrium region into precartilaginous condensations (Pacifici et al., 2006). Consistent with this report, *Gdf5*-expressing cells appeared to be migrating into presumptive phalangeal joint sites in E12.5 control embryos. However, there was no sign of possible relocation of such cells in mutant embryos. By E14.5, *Gdf5*-expressing cells were exclusively located in the incipient phalangeal joints in control embryos, whereas all *Gdf5*-expressing cells were still located around digital rays in mutant embryos. By E16.5, *Gdf5* expression had become very weak in mutant digits, but it was detectable in both digital ray outskirts and presumptive phalangeal joints. Digit articular progenitors thus appeared properly specified in $Sox5^{-/-}6^{-/-}$ early embryos, but strongly delayed in reaching their final location and partially losing their fate.

Together, these data indicate that *Sox5* and *Sox6* are not needed to specify articular progenitor cells, but they are needed to form joint interzones in a proper and timely manner.


A
E18.5
Sox5+/+ 6+/+
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Sox5+/+ 6-/-
Sox5+/- 6+/-
Sox5-/- 6+/-
Sox5+/- 6-/-
P
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E16.5
shoulder
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knee
forepaw
Sox5+/+ 6+/+
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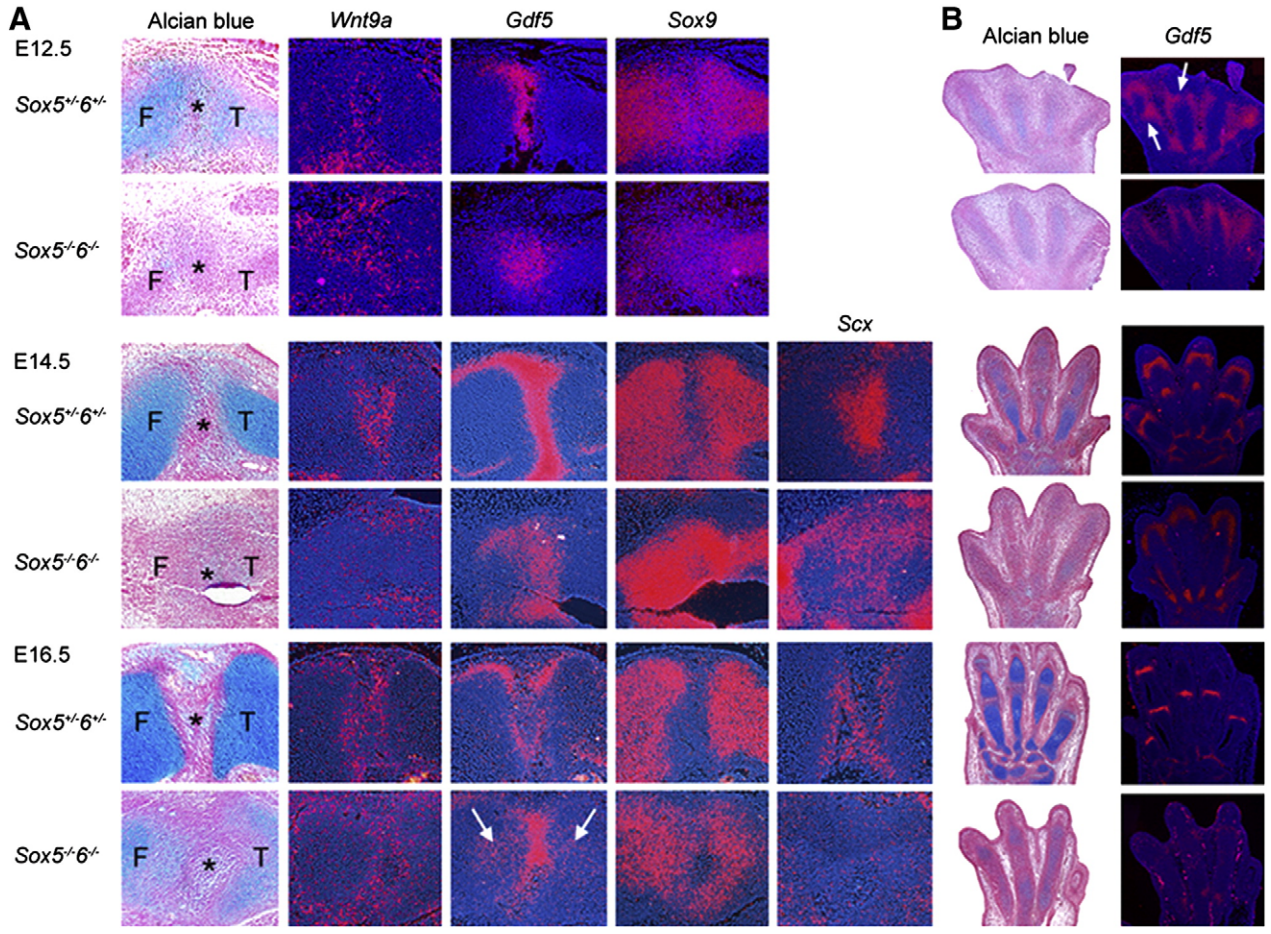


Fig. 3. Expression of genes involved in cell fate specification in $Sox5^{+/-}6^{+/-}$ control and $Sox5^{-/-}6^{-/-}$ mutant littermates. Adjacent sections through the knee (A) and forepaw (B) of embryos at various ages were stained with Alcian blue and hybridized with RNA probes, as indicated. F, femur; T, tibia; *, joint mesenchyme. The arrows in (A) point to ectopic expression of *Gdf5* in growth plate cartilage of E16.5 $Sox5^{-/-}6^{-/-}$ embryos. The arrows in (B) point to *Gdf5*-expressing cells appearing in presumptive phalangeal joints in E12.5 control embryos.

Sox5 and Sox6 are needed for articular cell differentiation and adequate morphogenesis of synovial joint structures

We determined whether joint cells are able to differentiate in *Sox5/6* mutants by testing marker gene expression. While *Gdf5* expression was virtually extinguished in the knee region of P0 control mice, it was still active in a large number of cells in the knee region of $Sox5^{fl/fl}6^{fl/fl}Prx1Cre$ littermates (Fig. 4). These cells were not expressing *Sox9* or *Agc1*, which encodes the panchondrocytic differentiation marker aggrecan. *Prg4*, which encodes the synovium and articular cartilage superficial cell marker lubricin, was expressed in most superficial cells in control mice, and *Tnd*, the gene for the differentiated tenocyte marker tenomodulin, was highly expressed in cruciate ligaments, joint capsule, and other tendons and ligaments in these mice. In $Sox5^{fl/fl}6^{fl/fl}Prx1Cre$ mutants, *Prg4* was expressed in a few cells in the knee joint, and *Tnd* was not expressed at all in the knee joint itself. However, both genes were ectopically expressed along the tibia shaft, suggesting that the knee capsule and synovium were misplaced in these mutants. This could have happened as a consequence of the failure of skeletal elements to grow in proportion with other limb structures. Together, these data indicated that *Sox5* and *Sox6* are needed for articular progenitor cell overt differentiation and for proper morphogenesis of all synovial joint structures.

Sox5 and Sox6 may control synovial joint development in part through promoting Ihh signaling but not through Erg expression or hyaluronan production

We next analyzed $Sox5^{-/-}6^{-/-}$ embryos for expression of genes previously shown or proposed to be involved in joint morphogenesis and articular cell differentiation. Ihh signaling was shown to be needed for phalangeal joint formation (St-Jacques et al., 1999; Koyama et al., 2007; Purcell et al., 2009). We therefore asked whether impaired Ihh signaling could explain the synovial joint phenotype of $Sox5^{-/-}6^{-/-}$ embryos. *Ihh* expression was readily detectable in

Fig. 2. Analysis of developing synovial joints in mice harboring *Sox5* and *Sox6* null alleles. (A) Mid-sagittal sections through the knees of E18.5 fetuses harboring various combinations of *Sox5* and *Sox6* null alleles. Note that although all sections are mid-sagittal, as indicated by the presence of the patella (P), the two femoral condyles (FC) are seen and cruciate ligaments (CL) are stretched in the $Sox5^{-/-}6^{+/-}$ and $Sox5^{+/-}6^{-/-}$ sections, reflecting valgus deformation. F, femur; T, tibia. (B) Sections through the shoulder, elbow, knee and forepaw of E16.5 wild-type and $Sox5^{-/-}6^{-/-}$ littermates. The arrow in the mutant shoulder points to the fusion point between the scapula and the humerus. C, carpal; H, humerus; MC, metacarpal; R, radius; S, scapula; U, ulna; *, joint mesenchyme. (C) Pictures of $Sox5^{fl/fl}6^{fl/fl}$ and $Sox5^{fl/fl}6^{fl/fl}Prx1Cre$ newborn littermates and histology sections through the knee and forepaw. The arrow in the mutant femur points to an island of growth plate chondrocytes with a wild-type histology aspect. These cells most likely escaped complete inactivation of the *Sox* conditional alleles. The arrows in the forepaw point to a phalangeal joint. All sections are stained with Alcian blue.

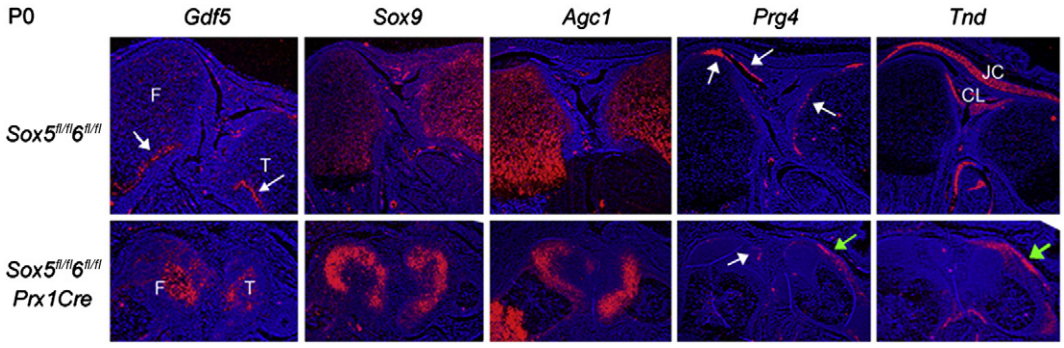


Fig. 4. Expression of joint cell specification and differentiation markers in *Sox5/6* mutants. Sections through the knees of $Sox5^{fl/fl}6^{fl/fl}$ and $Sox5^{fl/fl}6^{fl/fl}Prx1Cre$ newborns were stained hybridized with RNA probes as indicated. The arrows in the *Gdf5* control panel point to *Gdf5*-expressing cells underneath the perichondrium of the femur (F) and tibia (T) growth plates. The white arrows in the *Prg4* panel point to *Prg4*-expressing joint-lining cells, whereas the green arrow points to mutant cells ectopically expressing *Prg4* along the tibia shaft. In the *Tnd* panel, the green arrow points to mutant cells ectopically expressing *Tnd* along the tibia shaft. CL, cruciate ligaments; JC, joint capsule.

control digital rays at E12.5, but remained virtually undetectable in mutants through E16.5 (Fig. 5A). To test whether this weaker *Ihh* expression in the mutants resulted in a decrease in Ihh signaling, we tested expression of *Gli1*, a target and transcriptional mediator of Ihh signaling. We found *Gli1* expressed throughout the precartilaginous condensations of control digits at E12.5. Later, it became restricted to the perichondrium and was notably absent in presumptive joint regions. Interestingly, *Gli1* was expressed in the paws of E12.5 $Sox5^{-/-}6^{-/-}$ embryos, but in more narrow domains than in control paws, indicating that *Ihh* signaling was active, but weak in $Sox5^{-/-}6^{-/-}$ digital rays. At E14.5, *Gli1* expression became restricted to the perichondrium of $Sox5^{-/-}6^{-/-}$ digital rays, as seen in control digits. However, it started to be turned off at the level of presumptive joints only by E16.5. *Sox5* and *Sox6* may thus facilitate phalangeal joint development at least in part by allowing chondrocytes to differentiate up to the stage at which they express *Ihh*.

We then examined expression of *Erg* because this gene was described as an early marker and potentially important differentiation regulator of articular chondrocytes (Dhordain et al., 1995; Iwamoto et al., 2007). Interestingly, we found *Erg* expressed throughout the entire precartilaginous condensations of control and mutant embryos at E12.5, thus in the precursors of both articular and growth plate chondrocytes rather than, as previously described, in articular cells only (Fig. 5A). Growth plate chondrocytes were differentiated in control embryos by E14.5 and had turned off expression of *Erg*, whereas articular progenitor cells maintained *Erg* expression at least until birth (Figs. 5A and B). In contrast, *Sox5/6* mutant growth plate chondrocytes maintained *Erg* expression. These data indicate that *Erg* is a marker of both growth plate and articular chondrocyte precursors, and that *Sox5* and *Sox6* are needed directly or indirectly to repress *Erg* expression.

Hyaluronan was previously proposed to facilitate joint cavitation (Ito and Kida, 2000; Dowthwaite et al., 2003; Matsumoto et al., 2009). This glycosaminoglycan is very abundant in the synovial fluid, cartilage extracellular matrix, and other connective tissue matrices. It is produced by the hyaluronan synthase-2 (*Has2*) in joint superficial cells and in prehypertrophic chondrocytes, and $Has2^{fl/fl}Prx1Cre$ mice have cartilage and joint phenotypes very similar to those of $Sox5^{-/-}6^{-/-}$ and $Sox5^{fl/fl}6^{fl/fl}Prx1Cre$ mutants (Matsumoto et al., 2009). *Has2* expression was not detectable in precartilaginous condensations in E12 control and $Sox5^{-/-}6^{-/-}$ forepaws (Fig. 5A). At E14.5 and E16.5, it was detected in presumptive joints and perichondrium of control forepaws, and in the entire precartilaginous condensations of mutant littermates. By P0, *Has2* was similarly expressed in control and $Sox5^{fl/fl}6^{fl/fl}Prx1Cre$ mutant mice, that is, very weakly in the joint region and more strongly in prehypertrophic chondrocytes (Fig. 5B). Hyaluronan was nevertheless abundant in all expected tissues in $Sox5^{-/-}6^{-/-}$ mutants (except the missing phalangeal joint sites), and in the knee mesenchyme of $Sox5^{fl/fl}6^{fl/fl}Prx1Cre$ newborns (Fig. 5C). These data indicate that *Sox5* and *Sox6* are not needed for *Has2* expression and hyaluronan production, that hyaluronan production is not sufficient for joint development, and thus that the phenotype of *Sox5/6* mutants is unlikely due to lack of hyaluronan.

We conclude that *Sox5* and *Sox6* may control synovial joint development at least in part by allowing proper *Ihh* expression, but that they unlikely control this process through regulating *Erg* expression or hyaluronan production.

Sox5 and Sox6 have essential cell- and non-cell-autonomous roles in joint morphogenesis

Since some but not all articular cells express *Sox5* and *Sox6* in wild-type mice, the absence of overt synovial joint formation in *Sox5/6* mutants led to the possibility that *Sox5* and *Sox6* may have both cell- and non-cell-autonomous roles in joint morphogenesis, and that joint morphogenesis may fail in $Sox5^{-/-}6^{-/-}$ and $Sox5^{fl/fl}6^{fl/fl}Prx1Cre$ mice at least in part because of the severe chondrodysplasia of these mice. To test these possibilities, we compared the phenotypes of $Sox5^{fl/fl}6^{fl/fl}Col2Cre$ and $Sox5^{fl/fl}6^{fl/fl}Gdf5Cre$ mice. *Col2Cre* induces gene recombination in both chondrogenic cells (Ovchinnikov et al., 2000) and articular progenitors (Fig. 6A). In contrast to $Sox5^{-/-}6^{-/-}$ embryos, E13.5 $Sox5^{fl/fl}6^{fl/fl}Col2Cre$ embryos featured well-formed phalangeal and all other joint interzones, which were highly expressing *Gdf5*, and they had close-to-normal cartilage primordia (Fig. 6B). At birth, however, $Sox5^{fl/fl}6^{fl/fl}Col2Cre$ mice exhibited joint and other skeletal defects that were as severe as those of $Sox5^{-/-}6^{-/-}$ fetuses and $Sox5^{fl/fl}6^{fl/fl}Prx1Cre$ mice (Figs. 6C and D). This demonstrated that the synovial joint defects of *Sox5/6* mutant mice are not simply the result of failed or incomplete interzone formation, but are also the result of impaired chondrogenesis. *Gdf5Cre* induces gene recombination at a similar time as *Col2Cre*, but only in the cells of the presumptive joint regions (Rountree et al., 2004; Fig. 6E). Like $Sox5^{fl/fl}6^{fl/fl}Col2Cre$ mice, E13.5 $Sox5^{fl/fl}6^{fl/fl}Gdf5Cre$ embryos formed normal interzones and cartilage primordia (data not shown). $Sox5^{fl/fl}6^{fl/fl}Gdf5Cre$ newborn mice showed specific inactivation of *Sox5* and *Sox6* in articular cells (Fig. 6F). Their growth plates and endochondral bones were essentially normal, but their joints showed distinctive defects (Fig. 6G). Articular and meniscal chondrocytes were present, but undifferentiated, as reflected by lack of cartilage extracellular matrix surrounding them. The cells of the intra-articular fat pad were underdeveloped, as reflected by their small volume. Finally, cavitation was incomplete, resulting in fusion of the menisci with the tibial plateau. In contrast, the joint capsule and the cruciate ligaments appeared normal. The relative

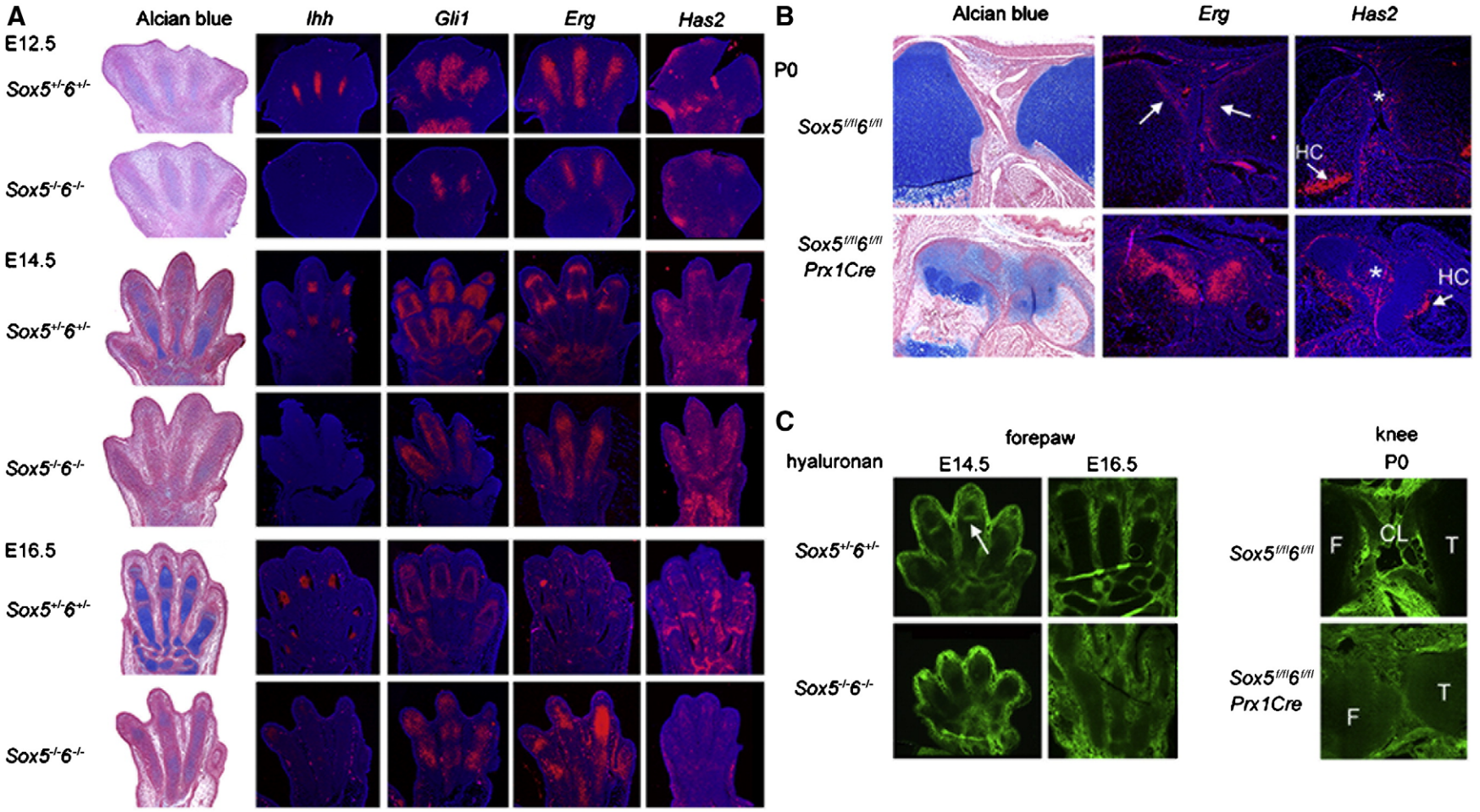
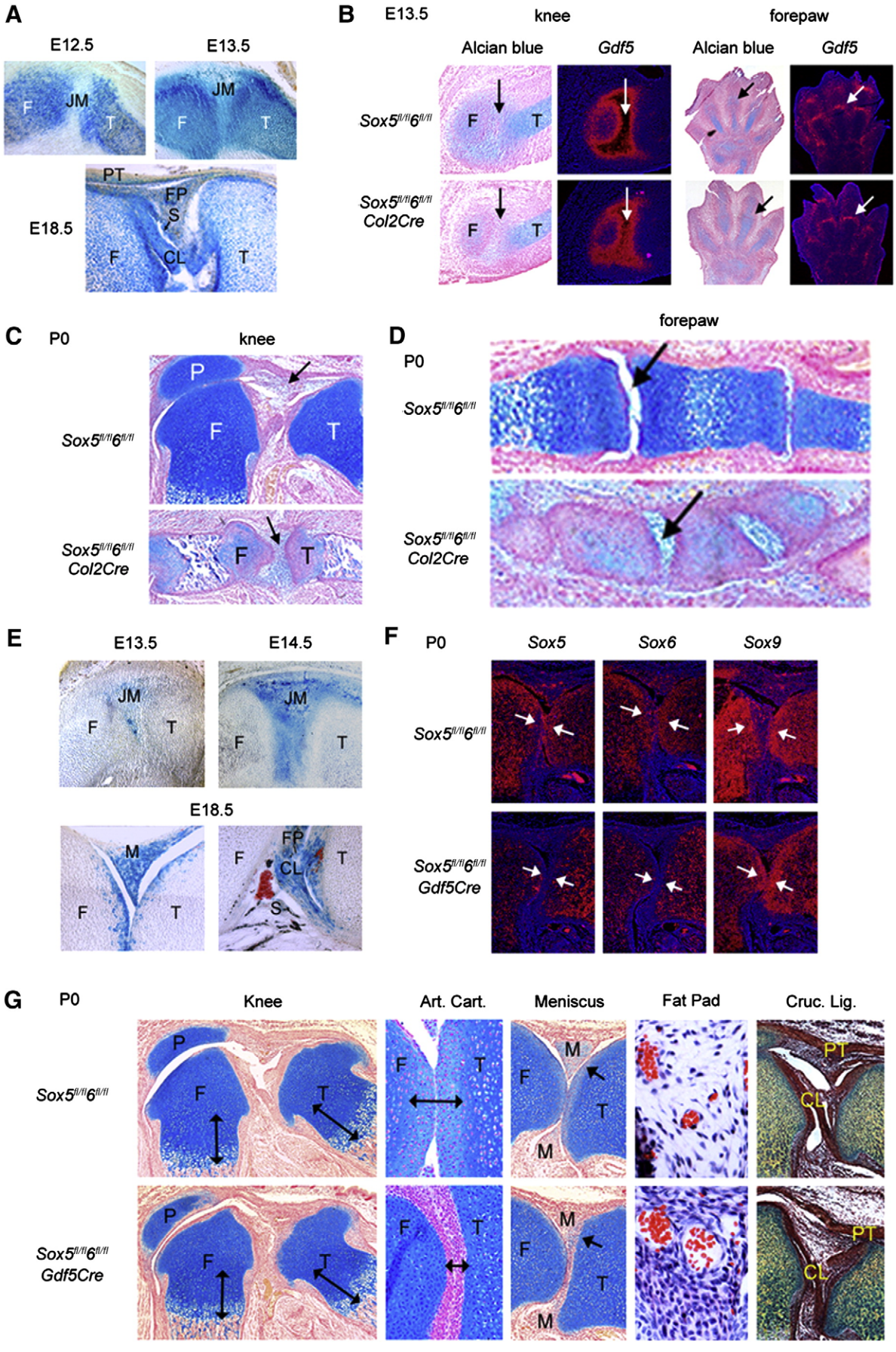


Fig. 5. Expression of genes involved in joint morphogenesis in *Sox5/6* mutants. Sections through the forepaw (A and C) and knee (B and C) of mice at various ages were stained with Alcian blue or for hyaluronan, or were hybridized with RNA probes, as indicated. In (B), the arrows in the *Erg* control panel point to *Erg*-expressing articular progenitor cells. The arrows in the *Has2* panels point to *Has2*-expressing hypertrophic chondrocytes (HC), whereas the star (*) points to *Has2*-expressing articular progenitors. The arrow in (C) points to a hyaluronan-positive presumptive phalangeal joint in the control forepaw. CL, cruciate ligaments; F, femur; T, tibia.


A
E12.5
E13.5
JM
F
T
PT
FP
S
E18.5
CL
B
E13.5
knee
forepaw
Alcian blue
Gdf5
Sox5fl/fl6fl/fl
Sox5fl/fl6fl/fl Col2Cre
C
P0
knee
P
D
forepaw
E
E13.5
E14.5
M
E18.5
F
CL
S
FP
F
Sox5fl/fl6fl/fl Gdf5Cre
Sox5
Sox6
Sox9
G
Knee
Art. Cart.
Meniscus
Fat Pad
Cruc. Lig.

mildness of this phenotype, compared to that of *Sox5*$^{fl/fl}$*6*$^{fl/fl}$*Col2Cre* and *Sox5*$^{fl/fl}$*6*$^{fl/fl}$*Prx1Cre* mice, strongly suggests that *Sox5* and *Sox6* are needed both cell-autonomously and non-cell-autonomously for joint morphogenesis.

Sox5 and Sox6 are needed for proper maturation of synovial joints

All *Sox5*$^{fl/fl}$*6*$^{fl/fl}$*Gdf5Cre* mice were viable and although they were often slightly smaller than control littermates, they looked otherwise normal and healthy. We therefore used them to determine whether *Sox5* and *Sox6* are needed for joint maturation, a process that occurs only postnatally. In control mice, the articular cartilage of the tibia and femur matured into superficial, middle and deep zones between P10 and P19, concomitantly with the formation of the secondary ossification centers in the femoral condyles and tibia epiphyses (Figs. 7A and B). These secondary ossification centers were developing normally in *Sox5*$^{fl/fl}$*6*$^{fl/fl}$*Gdf5Cre* P10 mice, whereas the articular cartilage continued to exhibit a severe extracellular matrix deficiency. In P19 mutant mice, the deep zone of the articular cartilage contained hypertrophic-like cells, as in control mice, but the other zones were highly disorganized. Their cellular content was irregular and their extracellular matrix was deficient. Furthermore, their joint surface was rough rather than smooth. Gene expression analysis showed that *Sox9* expression was unchanged, that expression of the genes for collagen type 2 *(Col2a1)* and aggrecan *(Agc1)* was lacking in the superficial and middle zones, and that expression of collagen type 10 (*Col10a1)* was also strongly downregulated in the deeper zone (Fig. 7C). In addition, *Sox5*$^{fl/fl}$*6*$^{fl/fl}$*Gdf5Cre* articular chondrocytes were still expressing *Gdf5*, were no longer expressing *Erg*, and they had not started to express *Prg4*. Articular chondrocytes are thus unable to overtly differentiate in the absence of *Sox5* and *Sox6*.

Microcomputed tomography revealed that the skeleton of *Sox5*$^{fl/fl}$*6*$^{fl/fl}$*Gdf5Cre* adult mice (P48, i.e., 2 months of age) was overall normal, but it was often slightly smaller than in control littermates and, interestingly, it was always lacking mineralized menisci (Figs. 7D and E). Histology analysis showed that the knees of these mice were severely affected (Fig. 7F). The menisci were underdeveloped, not showing signs of endochondral ossification, and they were still fused to the tibia. The articular cartilage was still disorganized. In addition, it was now projecting loose, fibrous outgrowths into the joint space. The fat pad/synovium was also underdeveloped and growing out fibrous tissue. Cruciate ligaments appeared normal. The same results were obtained in mice at 8 months of age (data not shown). These data thus demonstrate that *Sox5* and *Sox6* contribute important roles in the maturation of synovial joints.

Discussion

In this investigation, we used mice lacking the chondrogenic transcription factors *Sox5* and *Sox6* to increase understanding of the mechanisms underlying synovial joint development and maturation. Based on our results, we reached four major conclusions. First, *Sox5* and *Sox6* are not required to specify articular progenitor cells, but they are required to form joint interzones in a proper and timely manner. Second, *Sox5* and *Sox6* secure the differentiation of growth plate chondrocytes from bipotent articular/growth plate chondrocyte precursors. Third, growth plate development is required for joint morphogenesis. Fourth, *Sox5* and *Sox6* are necessary cell-autonomously for articular and meniscal chondrocyte differentiation and for joint proper maturation.

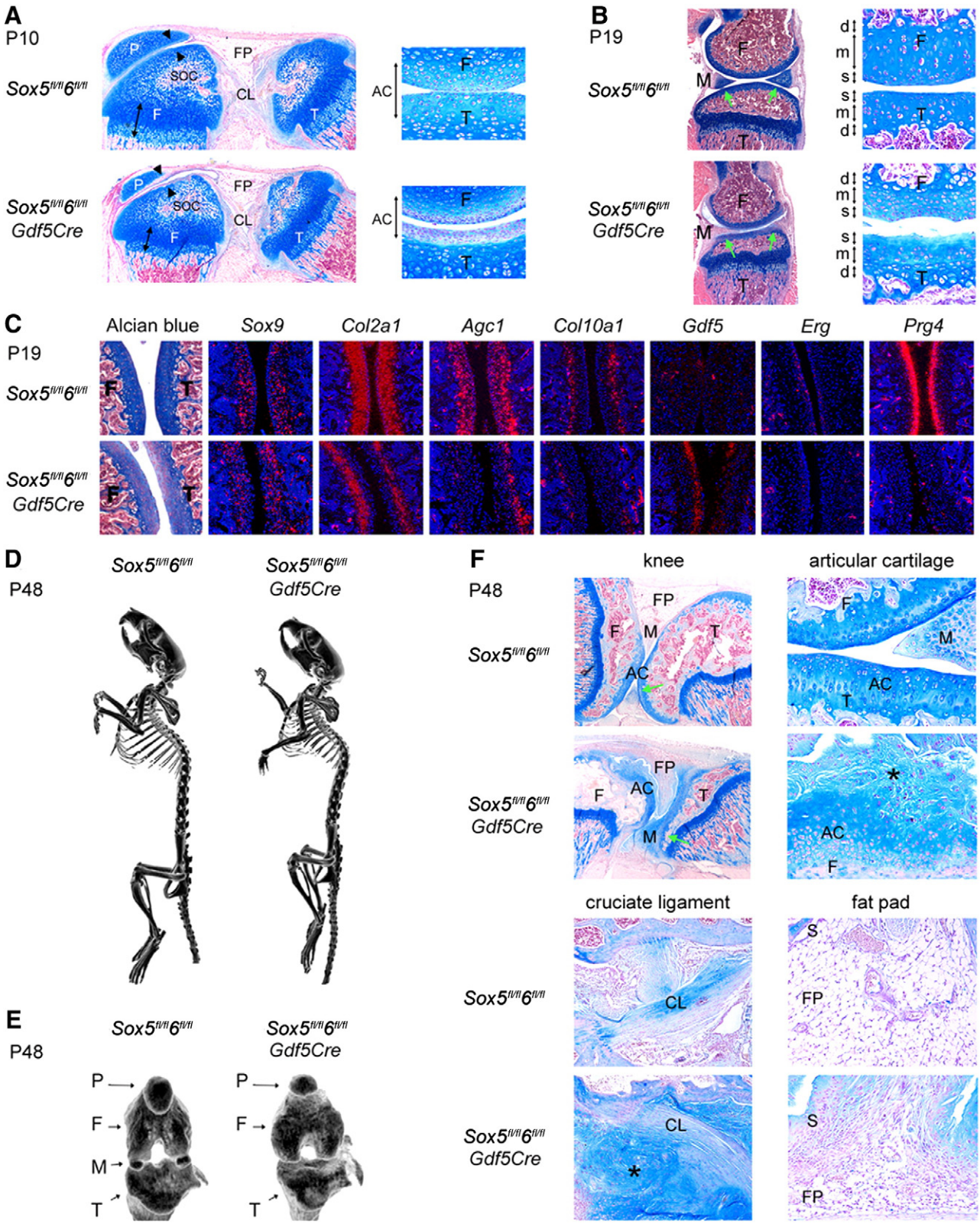
Commitment of skeletogenic cells to the articular fate was previously shown to be associated with downregulation of *Sox9* expression (Hartmann and Tabin, 2001). We showed here that it is also associated with downregulation of *Sox5* and *Sox6* expression. Since the latter two genes are expressed only in skeletogenic cells committed to chondrogenesis (Lefebvre et al., 1998; Smits et al., 2001), whereas *Sox9* is already expressed in multipotent mesenchymal cells (Ng et al., 1997; Zhao et al., 1997), this new finding adds weight to the notion that synovial joint progenitors arise from fully chondrogenic cells rather than from multipotent mesenchymal cells.

We demonstrated that *Sox5* and *Sox6* are dispensable to initiate joint fate specification by showing timely onset of *Wnt9a* and *Gdf5* expression (Storm and Kingsley, 1999; Hartmann and Tabin, 2001). This result is not entirely surprising since we have also shown that *Wnt9a* and *Gdf5* are expressed in the peripheral region of precartilaginous condensations before *Sox5* and *Sox6* allow overt chondrogenesis to occur. *Wnt9a* and *Gdf5* are thus simply re-expressed as chondrogenic cells turn off expression of the Sox trio in the joint interzone. More interestingly, while testing whether *Sox5* and *Sox6* are involved in the specification of articular progenitors, we found that the two genes are required in growth plate chondrocyte precursors to repress expression of *Erg*, a gene previously described as an articular chondrocyte marker (Iwamoto et al., 2007). We first interpreted our result as a change of the cells from the growth plate to the articular chondrocyte fate. Upon further investigation, however, we found that *Erg* is highly expressed in the entire core of wild-type precartilaginous condensations. This result is consistent with the finding that *Erg* may be a target of Gdf5 signaling (Iwamoto et al., 2007), since *Gdf5* is strongly expressed in the peripheral region of precartilaginous condensations. Importantly, this result identified *Erg* as a marker of undifferentiated chondrocytes in both presumptive growth plates and presumptive articular regions. We thus revised our data interpretation to propose that the continuous expression of *Erg* in *Sox5/6* mutant growth plates does not necessarily imply a switch of growth plate chondrocytes to the articular chondrocyte fate, but it at least strengthens the notion that *Sox5/6* are needed to promote growth plate chondrocyte differentiation. The role of *Erg* in the chondrocyte lineage remains elusive. It was shown that forced expression of *Erg* in chondrocytes in transgenic mice resulted in a delay of growth plate chondrocyte maturation (Iwamoto et al., 2007). It was therefore proposed that *Erg* might help maintain chondrocytes at a non-hypertrophic differentiation stage, as would be expected from an articular cartilage master factor. It was not shown, however, whether *Erg* overexpression had an effect on chondrocyte early differentiation, as could be expected from our observation that *Erg* is specifically expressed in precartilaginous condensations prior to chondrocyte early differentiation. Our data also suggest that *Sox5* and *Sox6* may promote growth plate chondrocyte differentiation not only by upregulating expression of cartilage extracellular matrix genes, but also by repressing *Erg*

Fig. 6. Analysis of *Sox5*$^{fl/fl}$*6*$^{fl/fl}$*Col2Cre* and *Sox5*$^{fl/fl}$*6*$^{fl/fl}$*Gdf5Cre* mice. (A) X-gal staining of longitudinal sections through the knee of E12.5, E13.5 and E18.5 *R26*^{lacZ}*Col2Cre* embryos. *R26*^{lacZ} confers on Cre-expressing cells and their daughters the ability to express *E. coli* beta-galactosidase and therefore to turn blue upon incubation with X-gal. CL, cruciate ligament; F, femur; FP, fat pad; JM, joint mesenchyme; PT, patellar tendon; S, synovium; T, tibia. (B) Alcian blue staining and *Gdf5* RNA in situ hybridization of knee and forepaw sections from E13.5 *Sox5*$^{fl/fl}$*6*$^{fl/fl}$ and *Sox5*$^{fl/fl}$*6*$^{fl/fl}$*Col2Cre* littermates. The arrows point to joint regions. (C and D) Alcian blue staining of knee (C) and forepaw (D) sections from control and *Sox5*$^{fl/fl}$*6*$^{fl/fl}$*Col2Cre* mice at P0. The arrows point to joint regions. P, patella. (E) X-gal staining of *R26*^{lacZ}*Gdf5Cre* knees at E13.5, E14.5, and E18.5. M, meniscus. (F) Hybridization of knee sections from P0 *Sox5*$^{fl/fl}$*6*$^{fl/fl}$ and *Sox5*$^{fl/fl}$*6*$^{fl/fl}$*Gdf5Cre* mice with *Sox5*, *Sox6*, and *Sox9* RNA probes. The *Sox5* and *Sox6* probes corresponded to the exons that are flanked with *loxP* sites in the conditional alleles. The arrows point to articular cartilage, where Cre-mediated recombination of *Sox5* and *Sox6* has occurred. (G) Histology analysis of the knees of P0 *Sox5*$^{fl/fl}$*6*$^{fl/fl}$ and *Sox5*$^{fl/fl}$*6*$^{fl/fl}$*Gdf5Cre* mice. Staining is with Alcian blue, except for the cruciate ligament sections, which are stained with Movats. The arrows in the low-magnification pictures of the knees show that the growth plates of the mutant are normal. The double-headed arrows in the high-magnification pictures of articular cartilage (Art. Cart.) show that this tissue is undifferentiated in the mutant. The arrows in the meniscus pictures point to the meniscus–tibia boundary, which is cavitated in the control and fused in the mutant mouse. The fat pad pictures show that the cells (with blue nuclei) are large in the control, but small in the mutant mouse. Red blood cells are seen in blood vessels. In the cruciate ligament pictures (Cruc. Lig.), the patellar tendon (PT) and cruciate ligaments (CL) are stained brown due to their high density of collagen fibers.

expression. They could fulfill the latter function by binding directly to *Erg* gene regulatory elements, or they could act indirectly by modulating the activity of signaling pathways leading to *Erg* expression.

Interestingly, we found lingering expression of *Wnt9a* and *Gdf5* in *Sox5/6* mutant epiphyseal chondrocytes throughout development and, by the time $Sox5^{fl/fl}6^{fl/fl}Prx1Cre$ mice were born, they were ectopically expressing *Gdf5* in a large portion of epiphyseal cells near the joint region. As indicated above, continued expression of *Erg* in *Sox5/6* mutant chondrocytes was not sufficient to conclude that growth plate chondrocytes were switching to an articular fate. However, since *Gdf5* is a specific marker of articular progenitors, the presence of *Gdf5*-expressing epiphyseal cells near the joint region strongly suggests that *Sox5* and *Sox6* are necessary to secure not only the differentiation, but also the fate of growth plate chondrocytes. This is a new concept



since we had previously shown that *Sox5* and *Sox6* upregulate expression of cartilage matrix genes, which are differentiation genes, but we had not identified gene expression changes in *Sox5/6* mutants that were indicative of a role for *Sox5* and *Sox6* in cell fate specification (Smits et al., 2001). This new concept is consistent with a previous report, in which it was proposed that *Sox5* and *Sox6* prevent rib chondrocytes from becoming tenogenic (Brent et al., 2005). In this previous report, no fate change was found in the limbs, and our results confirmed this observation. Together, these data suggest that *Sox5/6*-deficient chondrocytes maintain the flexibility to convert to different cell lineages based on environmental cues. These environmental cues could explain why only cells near the joint region, rather than all epiphyseal and growth plate chondrocytes, were expressing *Gdf5*. These cells, more than the others, could be under the influence of local factors directing cells to the joint fate. As *Gdf5* is expressed downstream of Tgfβ and canonical Wnt signaling in developing joints, this finding suggests that *Sox5* and *Sox6* might somehow contribute to block either or both pathways in epiphyseal chondrocytes adjacent to the joint region. *Sox9* has been shown to block canonical Wnt signaling by interacting with and inducing degradation of beta-catenin (Akiyama et al., 2004; Topol et al., 2009). Since *Sox9* is still expressed in *Sox5/6* mutant chondrocytes, our data suggest that Sox9 is not sufficient to fully block this pathway in chondrocytes. Like Sox9 and other Sox proteins (Sinner et al., 2007; Melichar et al., 2007), Sox5 and Sox6 may interfere with Wnt canonical signaling by binding to beta-catenin or to TCF1. Alternatively, they could directly control expression of genes involved in modulating TGFβ and Wnt signaling. These genes could be those for cartilage extracellular matrix components, many of which are known to be controlled by Sox5/6, since proteoglycans and extracellular proteins are capable of modulating signaling pathways by sequestering or interfering with the diffusion of ligands and ligand-interacting proteins (Kirn-Safran et al., 2004).

We showed that global inactivation of *Sox5* and *Sox6* in the mouse embryo severely impairs synovial joint morphogenesis beyond the joint interzone formation stage. Interestingly, all types of synovial joint cells were affected, not only articular and meniscal chondrocytes, the only joint cells still expressing *Sox5* and *Sox6* at the time of joint morphogenesis in wild-type animals. This global failure of joint morphogenesis suggested two possibilities: either *Sox5* and *Sox6* have cell-autonomous roles in synovial joint progenitor cells, and these roles have a major impact on joint morphogenesis at a later stage; and/or *Sox5* and *Sox6* have cell-autonomous roles in articular and growth plate chondrocytes, and these roles have an important, non-cell-autonomous impact on the development of the synovial joint structures that no longer express *Sox5* and *Sox6*. We tested these possibilities by analyzing $Sox5^{fl/fl}6^{fl/fl}$*Gdf5Cre* and $Sox5^{fl/fl}6^{fl/fl}$*Col2Cre* mice. We showed that *Sox5/6* inactivation in the presumptive joint region after specification ($Sox5^{fl/fl}6^{fl/fl}$*Gdf5Cre* mice) affected the development of articular and meniscal chondrocytes, but did not seriously affect the formation of other joint structures and growth plates, whereas *Sox5/6* inactivation in specified articular cells and developing chondrocytes ($Sox5^{fl/fl}6^{fl/fl}$*Col2Cre* mice) resulted in the same joint morphogenesis and cartilage growth plate defects as those observed in $Sox5^{-/-}6^{-/-}$ global mutants. These data thus provide strong support to the concept that proper development of cartilage primordia and growth plates is necessary for synovial joint morphogenesis. To our knowledge, these data are the first ones to convincingly substantiate this concept. Several other mouse mutants were previously shown to exhibit joint morphogenesis failure along with severe growth plate defects. They include mice lacking the transcription factor Hif-1α (Amarilio et al., 2007; Provot et al., 2007; Pitsillides and Ashhurst, 2008), Ihh (St-Jacques et al., 1999; Koyama et al., 2007), or hyaluronan synthase 2 (Matsumoto et al., 2009). The notion that severe growth plate malformation could explain joint morphogenesis failure was put forward in the case of *Hif1α* mutants, but was not tested. In the case of *Has2* mutants, in contrast, it was proposed, but also without demonstration, that hyaluronan is directly required for joint morphogenesis. However, since *Has2* expression and hyaluronan production still occur in *Sox5/6* mutants, it is possible that the two types of mutants develop similar joint defects largely as a consequence of their similar growth plate deficiencies. We showed here and previously (Smits et al., 2001) that *Ihh* expression is weak and delayed in *Sox5/6* global mutants, but that Ihh signaling eventually occurs normally. This finding suggests that defects in Ihh signaling could contribute to, but not entirely explain the growth plate and joint defects of *Sox5/6* mutants. In addition, this finding suggests that the lack of joints in *Ihh* mutants may be due to direct actions of Ihh signaling in either or both the joint region and the growth plate. Experiments in which *Has2* and Ihh signaling genes will be specifically inactivated in the joint region will be needed to definitively demonstrate whether *Has2* and *Ihh* are needed cell-autonomously or non-cell-autonomously for joint development. Cartilage could impact joint development in several ways. First, its expansion could be required to force the joint mesenchyme to condense into interzones and to subsequently differentiate and cavitate. Second, chondrocytes could secrete various types of factors required for joint morphogenesis, such as growth factors, extracellular matrix components, and matrix-remodeling factors. Third, the cartilage matrix could be required to properly distribute such factors between the cartilage and the joint region. Fourth, the alignment of collagen fibers parallel to the joint surface in the superficial zone of the articular cartilage could help delineate the sites of cavitation. Finally, the swelling pressure of the cartilage matrix, along with movement induced by muscle contraction, recently shown to be required for joint development (Kahn et al., 2009), could also help move factors between the cartilage and the developing joint region.

Since *Sox5* and *Sox6* are required for chondrocyte differentiation in cartilage primordia and growth plates and are expressed in articular and meniscal chondrocytes throughout life, our finding that they are required for articular and meniscal chondrocyte differentiation was certainly expected, but nonetheless required demonstration. Additionally, we made the unpredicted finding that *Sox5* and *Sox6* are necessary for the differentiation of the superficial cells of synovial joints. While the general consensus is that the cells that line the surface of articular cartilage belong to the articular chondrocyte lineage, the specific expression of *Prg4* in these cells and in the cells that line the surface of the synovium suggests that the superficial cells of both tissues could represent a distinct cell lineage. We showed that these cells remain undifferentiated in *Sox5/6* mutants, expressing *Gdf5* but neither *Sox9* nor *Prg4*. The question arises as whether *Prg4* could be a direct target of the chondrogenic Sox trio. However, *Prg4* is strongly expressed in synovial lining cells, where the Sox trio is not expressed, and it is not expressed in the middle and deep zone of articular cartilage and in growth plate cartilage, where the Sox trio is

Fig. 7. Analysis of $Sox5^{fl/fl}6^{fl/fl}$ and $Sox5^{fl/fl}6^{fl/fl}$*Gdf5Cre* littermates postnatally. (A) Alcian blue staining of knee sections at P10. The left pictures show that growth plates (arrow) are slightly smaller than normal but well organized in the mutant. The secondary ossification centers (soc) and cruciate ligaments (CL) are normal, but articular cartilage (arrowheads) and fat pad (FP) are underdeveloped in the mutant. P, patella; T, tibia. The right pictures show the femur and tibia articular cartilage (double-headed arrows) at higher magnification. Articular chondrocytes (AC) are surrounded by an abundant amount of extracellular matrix in the control, but by much less matrix in the mutant. (B) Alcian blue staining of knee sections at P19. The left pictures show that the secondary ossification centers are fully formed in control and mutant mice. Meniscal cartilage (M) is fully developed in the control, but not in the mutant, where it remains attached to the tibia (green arrows). The right pictures show that the articular cartilage has acquired its definitive zonal organization into superficial (s), middle (m), and deep (d) zones in the control mouse, but that these zones are poorly organized in the mutant. (C) Alcian blue staining and RNA in situ hybridization of sections through the femur and tibia articular cartilage in P19 mice. (D) Low-resolution microcomputed tomography of P48 mice. (E) High-resolution microcomputed tomography of the knees of the same mice. F, femur; M, meniscus; P, patella; T, tibia. (F) Histology analysis of the knees of P48 mice. The upper-left panels are low-magnification pictures, whereas the other panels are high-magnification pictures of specific areas. S, synovium. The green arrows show that the meniscus is still fused to the tibia in the mutant knee. The stars (*) point to articular tissue outgrowths with a highly variable aspect.

actively expressed. Moreover, *Prg4* was ectopically expressed along the tibia shaft of *Sox5/6* mutants. While not ruling out the possibility, these arguments nevertheless strongly suggest that *Prg4* is not a direct target of the Sox trio.

Since *Sox5* and *Sox6* control the expression of cartilage extracellular matrix genes and since deficiencies in cartilage extracellular matrix components lead to osteoarthritis (Spector and MacGregor, 2004), we were expecting that inactivation of *Sox5* and *Sox6* in articular cells would lead to precocious osteoarthritis. We observed that the articular cartilage of $Sox5^{fl/fl}6^{fl/fl}Gdf5Cre$ mice featured extracellular matrix deficiency and clusters of proliferating chondrocytes, which are early signs of osteoarthritis, but we did not see cartilage fissures and erosion, which are more advanced disease features, even when mice were eight months of age. Instead, we saw anarchic outgrowth of fibrous tissue in $Sox5^{fl/fl}6^{fl/fl}Gdf5Cre$ joints from early adulthood. This result was also surprising considering that chondrocyte proliferation is virtually abolished in the growth plates of $Sox5^{-/-}6^{-/-}$ fetuses (Smits et al., 2001, 2004). However, the most plausible explanation for this phenotype is the lack of production of the proteoglycan lubricin (encoded by *Prg4*) in superficial articular cells in $Sox5^{fl/fl}6^{fl/fl}Gdf5Cre$ mutants. $Prg4^{-/-}$ mice indeed develop similar overgrowths postnatally (Rhee et al., 2005), as do humans with camptodactyly-arthropathy-coxa vara-pericarditis (CACP), a syndrome caused by mutations in *PRG4* (Marcelino et al., 1999). This abnormal tissue, referred to as hypertrophic synovitis, causes joint failure in CACP patients, but not osteoarthritis. It is thus possible that this abundant fibrous tissue protects the unfit underlying cartilage of $Sox5^{fl/fl}6^{fl/fl}Gdf5Cre$ mice from rapid degeneration.

Erg was previously reported to be expressed in mouse articular cartilage postnatally, but the data were provided only at one week of age, when articular cartilage is still immature (Iwamoto et al., 2007). No data were provided at later stages, after formation of secondary ossification centers and maturation of articular cartilage. It remained thus unknown whether *Erg* is also a marker of mature articular chondrocytes. We were unable in this study to detect *Erg* expression in mature articular cartilage of weanling wild-type animals. We thus propose that *Erg* is a marker of prospective rather than differentiated articular chondrocytes. Similarly, we showed that *Erg* is a marker of prospective but not differentiated growth plate chondrocytes. Our data thus suggest that Erg may not be a transcription factor that dictates the distinctive fate of articular chondrocytes, as previously proposed (Pacifici et al., 2006), but a marker of articular and growth plate chondrocyte precursors. To our knowledge, there is thus no evidence, as of today, that unique transcription factors direct the fate and differentiation of articular chondrocytes. Although the importance of Sox9 in these cells remains to be demonstrated, the data presented here for Sox9's essential partners in chondrogenesis and for *Sox9*'s expression pattern make it likely that the Sox5/6/9 trio presides over the fate and differentiation of both articular and growth plate chondrocytes.

In conclusion, our data provide a better understanding of the cellular and molecular mechanisms underlying cell fate decisions and differentiation during synovial joint embryonic development and postnatal maturation. *Sox5* and *Sox6* appear to secure the fate and proper differentiation of chondrocytes in both growth plate and articular cartilage and they most likely work in concert with *Sox9*. Through these cell-autonomous functions, they are also needed non-cell-autonomously for joint morphogenesis. These dual roles help put forward the concept that the development and homeostasis of synovial joints rely on complex interactions between the molecular and cellular mechanisms that regulate their multiple tissues.

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