On the development of the patella
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ABSTRACT
The current view of skeletal patterning fails to explain the formation of sesamoid bones. These small bones, which facilitate musculoskeletal function, are exceptionally embedded within tendons. Although their structural design has long puzzled researchers, only a limited model for sesamoid bone development has emerged. To date, sesamoids are thought to develop inside tendons in response to mechanical signals from the attaching muscles. However, this widely accepted model has lacked substantiation. Here, we show that, contrary to the current view, in the mouse embryo the patella initially develops as a bony process at the anteriodistal surface of the femur. Later, the patella is separated from the femur by a joint formation process that is regulated by mechanical load. Concurrently, the patella becomes superficially embedded within the quadriceps tendon. At the cellular level we show that, similar to bone eminences, the patella is formed secondarily by a distinct pool of Sox9- and Scx-positive progenitor cells. Finally, we show that TGFβ signaling is necessary for the specification of patella progenitors, whereas the BMP4 pathway is required for their differentiation. These findings establish an alternative model for patella development and provide the mechanical and molecular mechanisms that underlie this process. More broadly, our findings that activation of a joint formation program can be used to switch between the formation of bony processes and of new auxiliary bones provides a new perspective on plasticity during skeletal patterning and evolution.

KEY WORDS: Sesamoid bone, Patella, Skeletogenesis, Morphogenesis, Mouse, Joint formation, Mechanoregulation, Progenitor cell, Sox9, Scleraxis, TGFβ, BMP4

INTRODUCTION
Over the past two centuries, the development of the skeletal system has been extensively studied (Hall, 2005; Lefèbvre and Bhattaram, 2010; Olsen et al., 2000; Owen, 1848), resulting in a substantial body of knowledge. Nevertheless, the development of a group of bones known as sesamoid bones has largely been neglected. Named after their morphological resemblance to the sesame seed, these small and flat bones share the unique property of being superficially embedded within tendons, notably tendons that wrap around joints. Sesamoid bones are estimated to have evolved 200 million years ago (Carter et al., 1998) and are integral to the skeletons of many vertebrates, such as frogs (anurans) (Ponssa et al., 2010), scaled reptiles (squamates) (Jerez et al., 2009; Maisano, 2002) and placental mammals (eutherians) (Bizarro, 1921; Sarin et al., 1999).

The most recognized and studied sesamoid bone is the patella, also known as the kneecap. It is the largest sesamoid in the human body and is consistent in shape and appearance in almost all mammalian taxa (Pearson and Davin, 1921b). The patella is part of the patellofemoral joint, one of two joints composing the knee. It has a crucial effect on the mechanics and stability of the knee, facilitating hindlimb function and locomotion (Sutton et al., 1976). The patella increases the distance between the quadriceps muscle and the knee and thereby increases the moment arm of the muscle, enhancing its extension force by up to 50% (Schindler and Scott, 2011). It is also speculated that the patella protects the quadriceps tendon and the underlying patellofemoral cartilage from erosion (Mottershead, 1988). Congenital pathologies such as patella aplasia and hypoplasia, as well as surgical removal of the patella (patellectomy), may result in severe pain, reduced ability to walk and run, difficulties in ascending and descending stairs, and even acute knee instability (Bongers et al., 2005; Braun, 1978; Sutton et al., 1976).

In a recent study, we identified a mechanism for the development of bone eminences (Blitz et al., 2013), which are superstructures on the bone surface that serve as insertion points for tendons (Gray, 1918; Hill, 1964). Interestingly, these superstructures were shown to be added modularly onto the main structure of the bone. Furthermore, we showed that bone eminences originate in a distinct pool of progenitors, which uniquely express both tenocyte (SCX) and chondrocyte (Sox9) marker genes. We also showed that TGFβ signaling controls specification of these progenitors, whereas the SCX/BMP4 pathway mediates their differentiation to eminence cells.

In this study, we show that the patella develops initially as part of the femur. Moreover, similar to bone eminences, the patella originates from a distinct pool of Sox9- and Scx-positive progenitors that is regulated by the same molecular mechanism. Finally, we provide compelling evidence that patella progenitors are separated from the femur by the application of a joint formation program under regulation of mechanical load, as the patellofemoral joint is formed.

RESULTS
The patella arises as part of the femur from a distinct pool of Sox9- and Scx-positive progenitors
Patella development has not been documented in detail yet. In order to observe the initiation of the patella, we analyzed histologically the hindlimbs of wild-type mouse embryos (Fig. 1). Sagittal sections from knees at embryonic day (E) 16.5 revealed a cartilaginous patella that was superficially embedded within the quadriceps tendon, whereas dorsally it was separated from the femur by the patellofemoral joint (Fig. 1A,A’). Having identified the patella, we were able to track earlier stages of its development. Surprisingly, at E14.5, although the patella was superficially embedded within the tendon, it appeared linked to the femur by a population of flat and elongated cells, referred to hereafter as ‘boundary cells’ (Fig. 1B,B’). One day earlier (E13.5), the patella could not be distinguished from the femur, as the boundary cells were not observed; moreover, the mature tendon could not be

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Fig. 1. The patella initially develops as part of a distinct pool of Sox9- and Scx-positive progenitors. (A-C') Sagittal sections through the patella from hindlimbs of wild-type embryos stained with Alcian Blue and Fast Red to highlight cartilage cells (A-C) or with Hematoxylin and Eosin (A-C'). Below each panel is an enlargement of the rectangular area (outlined above). (A,A') At E16.5, the patella (pa) appears as a distinct cartilaginous element superficially embedded within the quadriceps tendon (qt) and separated from the femur (fe) by the patellofemoral joint (arrows). (B,B') At E14.5, although the patella (asterisk) and femur are distinguishable, the patellofemoral joint is missing. The boundary between the two cartilaginous elements is occupied by cells with distinct flat and elongated morphology (arrow). (C,C') At E13.5, an aggregation of chondrogenic cells is seen at the presumable location of the patella (dashed circles) that appears to be part of the femur, as the boundary cells are absent (arrows). (D-F) In situ hybridization for Sox9 and Scx in hindlimb sections shows that at E13.5, patella progenitors (red arrow in D) are part of the femur, whereas the quadriceps tendon primordial cells (red arrows in E,F) begin to mature. (G-G') Fluorescence in situ hybridization using digoxigenin- and fluorescein-labeled antisense RNA probes for Sox9 and Scx is shown in sagittal sections through the patellae of wild-type embryos. At E13.5, cells that express both Sox9 and Scx are observed at the presumable patella location (enlarged in the inset). Scale bars: 200 µm.

Patella arises from a distinct pool of Sox9- and Scx-positive progenitors

To further substantiate that the Sox9- and Scx-double-positive cells were indeed patella progenitors, we followed the lineages of two cell populations. First, we followed the descendants of Scx-expressing cells using a Cre line driven by Scx promoter crossed with a Rosa26-tdTomato reporter. Our result clearly indicated that both patella progenitors, examined at E13.5 (Fig. 2A), and patella cells, examined at E15.5 (Fig. 2B), were labeled. Next, we used the CreERT2 system driven by a Sox9 promoter (Soeda et al., 2010) to follow the descendants of Sox9-expressing cells. This system allows for temporally controlled activation of Cre recombinase by administration of tamoxifen. Tamoxifen was administered at E13.5 to label patella progenitors and the embryos were harvested at E16.5. Examination of sagittal knee sections clearly showed that patella cells were indeed labeled, suggesting that they originated from the previously labeled progenitors (Fig. 2C). Taken together, both lineage experiments confirm that the patella develops from the Sox9- and Scx-positive progenitors.

As mentioned previously, we recently reported that the progenitors of bone eminences co-express Sox9 and Scx (Blitz et al., 2013). Finding that patella progenitors are initially part of the femur and co-express Sox9 and Scx led us to hypothesize that the patella forms by the same mechanism as do bone eminences. Because we showed that eminence progenitors are specified secondarily to the progenitors of the bones from which the eminence protrudes, we proceeded to examine whether patella

detected (Fig. 1C,C'). These results suggest that the patella develops initially as part of the femur.

To validate this hypothesis, we examined the expression of Sox9, a marker for chondroprogenitors and chondrocytes (Zhao et al., 1997). In situ hybridization on hindlimb sections showed that, at E13.5, cells at the presumed patella site expressed Sox9 (Fig. 1D). The expression domain of Sox9 was extended continuously across both femur and patella cell populations, thus supporting our hypothesis that patella progenitors are initially part of the femur.

Previous studies suggest that sesamoids develop from within functional tendons (Hall, 2005). We therefore compared the timeline of tendon development with that of patella formation by studying the expression of scleraxis (Scx), a tendon-specific marker (Schweitzer et al., 2001). Results showed that, at E13.5, only primordial tendon cells were seen, whereas the mature tendon was visible only at E14.5 (Fig. 1E,F), indicating that patella initiation preceded tendon maturation. Intriguingly, double fluorescence in situ hybridization for Sox9 and Scx in E13.5 hindlimb sections showed that, unlike chondrocytes, which solely expressed Sox9, and tenocytes, which solely expressed Scx, cells at the presumptive site of the patella co-expressed both genes (Fig. 1G-G'). Collectively, these results indicate that the patella starts to develop as part of the femur before the maturation of the quadriceps tendon and suggest that it is formed by a distinct Sox9- and Scx-positive progenitor population.
and differentiation (Blitz et al., 2013, 2009). We therefore examined the role of TGFβ and BMP4 signaling pathways in patella development. To do this, Tgfb2 or Bmp4 were specifically knocked out (cKO) from early limb mesenchyme, using Prxl (also known as Prx1 – Mouse Genome Informatics)-Cre as a deleter mouse (Prxl-Bmp4; Prxl-Tgf-BRII) (Chytil et al., 2002; Liu et al., 2004; Logan et al., 2002; Selever et al., 2004). To verify the efficiency of gene ablation, we first measured by qRT-PCR the expression levels of Tgfb2 or Bmp4 genes in limbs of embryos harvested at E13.5. The results showed a drastic decrease in expression levels in the mutants, when compared with control littersmates (supplementary material Fig. S1). Next, examination of E17.5 skeletons revealed that the patella was completely absent in both mutant strains (Fig. 3A-D), which was verified by histological analysis (Fig. 3A′-D′).

To further decipher the role of TGFβ-BMP signaling in patella development, we examined the amount and spatial distribution of the Sox9/Scx co-expressing progenitors during the early patterning phase, at E13.5. Then, we examined progenitor differentiation at E14.5 by observing collagen production, using antibodies against SOX9 and COL2A1. In Prxl-Tgf-BRII embryos, the Sox9/Scx double-positive cell population was missing (Fig. 3E-F′). Correspondingly, at E14.5, no patella cells were observed in the mutants (Fig. 3G-H′). Interestingly, in E13.5 Prxl-Tgf-BRII limbs, Scx expression was lost in patella progenitors but not in tendon progenitors, where it was clearly evident. These results suggest that, at this stage, TGFβ signaling is necessary for specification of the Sox9/Scx double-positive patella progenitors.

To uncover the role of BMP4 signaling, we analyzed Prxl-Bmp4 limbs by the same method. As seen in Fig. 3I-J′, at E13.5 we detected a population of Sox9/Scx double-positive patella progenitors. This result suggests that, unlike TGFβ signaling, BMP4 does not play a role in specification of patella progenitors. Alternatively, Bmp2 might have compensated for the loss of Bmp4 in this process (Bandyopadhyay et al., 2006). However, at E14.5, although control patella cells have already differentiated to chondrocytes and expressed both Sox9 and Col2a1, in the Prxl-Bmp4 mutants, the cells expressed only Sox9 and failed to express Col2a1 (Fig. 3K-L′). These results suggest that BMP4 is required for the differentiation of patella progenitors. Collectively, the results support our hypothesis that, similar to bone eminences, patella development is regulated by the TGFβ-BMP signaling pathway.

The patella is separated from the femur by joint formation

Our finding that, similar to bone eminences, the patella first emerges as part of the femur raised the issue of the mechanism by which it is eventually separated. One mechanism that is known to facilitate separation of a skeletal element into two bones is the process of joint formation. During this process, cells that are specified as joint progenitors, dubbed ‘interzone cells’, lose their typical chondrocyte appearance and adopt a more flat and elongated shape. At the molecular level, these cells stop expressing chondrogenic genes and instead express a new set of genes, such as Gdf5, Tppp3, Wnt9a and Wnt4, which specify them as joint cells (Guo et al., 2004; Hartmann and Tabin, 2000, 2001; Mitrovic, 1977; Später et al., 2006; Staverosky et al., 2009; Storm et al., 1994). As mentioned, at E14.5 the boundary cells between the patella and femur exhibited a flat and elongated morphology (Fig. 1B,B′). This observation suggested that the separation of the patella from the femur was mediated by a process of joint formation.

To test this hypothesis, we initially examined the expression of Sox9 and Col2a1 at E14.5. Our results showed that, while...
expression of Sox9 and Col2a1 by patella and femur cells was maintained, it was downregulated in the boundary cells (Fig. 4A, B). Further examination showed that, in contrast to Sox9 and Col2a1, Gdf5 was upregulated in the boundary cells (Fig. 4C). Moreover, examination of the expression patterns of Tppp3, Wnt9a and Wnt4 revealed that these genes were also expressed by the boundary cells (Fig. 4D-F). Together, these results establish that the boundary cells acquired a joint-forming identity and that they were in fact interzone cells.

Fig. 3. Patella progenitors are regulated by TGFβ-BMP signaling. (A-D) Conditional knockout of either Tgfb2 or Bmp4 in mesenchymal cells resulted in patellar aplasia (black arrows). (A-D) Skeletal preparations of hindlimbs from E17.5 Prx1-Cre, Tgfbr2ff (B) and Prx1-Cre, Bmp4ff (D) mutants and control littermates (A, C). (A’-D’) Sagittal knee sections stained with Safranin O/Fast Green. (E-L) Sagittal sections through the patellae of Prx1-Cre, Tgfbr2ff and Prx1-Cre, Bmp4ff mutant embryos and control littermates. White arrows demarcate patella cells or their presumable location. (E-F’, J-J’): Fluorescence in situ hybridization using digoxigenin- and fluorescein-labeled antisense RNA probes for Sox9 and Scx at E13.5. (G-H, K-L) Immunofluorescence staining with antibodies against SOX9 and COL2A1 at E14.5. At E13.5, unlike in control littermates (E-E’), the Scx/Sox9 double-positive cells are absent in Prx1-Cre, Tgfbr2ff mutants (F-F’), suggesting failed specification. Consequently, at E14.5, the patella fails to form in these mutants (H-H’). In Prx1-Cre, Bmp4ff mutants, these cells are present at E13.5 (J-J’) but fail to differentiate by E14.5 (L-L’).

Fig. 4. The patella is separated from the femur by joint formation. Sagittal sections through the patella from hindlimbs of wild-type embryos at E14.5 were analyzed by in situ hybridization using digoxigenin-labeled antisense RNA probes for various cartilage and joint markers. Red arrows demarcate patella cells, black arrows demarcate boundary cells. (A, B) Whereas expression of Sox9 and Col2a1 by patella and femur cells is maintained, it is downregulated in the boundary cells. (C) Gdf5 is upregulated in the boundary cells. (D-F) Joint markers Tppp3, Wnt9a and Wnt4 are also expressed by boundary cells, indicating that they are interzone cells. Scale bar: 200 µm.
cells (as referred to hereafter). Furthermore, these results strongly support the hypothesis that the patella is separated from the femur by the formation of a patellofemoral joint.

**Mechanical load is required for formation of the patellofemoral joint**

Previous studies have suggested a central role for mechanical load in the initiation of sesamoid development (Abdala and Ponsa, 2011; Barnett and Lewis, 1958; Kim et al., 2009; Mikic et al., 2000; Roddy et al., 2011). Our finding that the patella initially forms as part of the femur and is later separated by joint formation led us to revisit the issue of mechanical contribution to patella development. To achieve this, we studied patella development in mutant embryos devoid of contracting muscles, as a result of the naturally occurring autosomal recessive mutation muscular dysgenesis (mdg; Cacna1s). These mice lack excitation-contraction coupling and, consequently, their skeletal muscles lack contractility (Pai, 1965a,b).

Histological analysis of sagittal sections of hindlimbs identified at E13.5, in both mutant and control littermates, the existence of patella progenitors, which were part of the femur (Fig. 5A,A′). However, at E14.5, whereas in the control we observed joint formation between the patella and the femur, in mdg mutants this process was not detected (Fig. 5B,B′). Consequently, at E16.5 the mutant patella was still part of the femur (Fig. 5C,C′). These results suggest that, although muscle contraction does not affect patella initiation, it plays a crucial role later during the separation phase. Interestingly, at all stages chondrocyte morphology and the matrix around them appeared different, when compared with control littermates. This observation might indicate an additional role for mechanical load in chondrocyte differentiation.

To gain molecular insight into the effect of muscle contraction on patella development, we analyzed expression of tissue-specific markers in control and mdg mutants during this process. At E13.5, double fluorescence in situ hybridization for Sox9 and Scx confirmed the presence of patella progenitors co-expressing these genes at the presumable patella location in both mutant embryos and control littermates (Fig. 6A-C′), reinforcing the notion that patella initiation is independent of muscle contraction. In situ hybridization for Gdf5 also showed comparable expression patterns (Fig. 6D,D′).

Given the known involvement of muscle contraction in joint formation, we next examined its effect on the formation of the patellofemoral joint. At E14.5, unlike in the control, downregulation of Sox9 or Coll2a1 was not observed in the patellofemoral interzone cells of mutant embryos (Fig. 6E-F′). Furthermore, upregulation of the joint markers Tppp3 and Gdf5 was also not observed in the mdg mutants, in contrast to control embryos (Fig. 6G-H′). These results suggest a failure in joint formation in the mdg mutants. Moreover, they support the possibility that the development of a joint between patella and femur requires muscle contraction.

Finally, we have previously reported that muscle contraction is necessary to maintain joint progenitor cell fate (Kahn et al., 2009). To examine whether muscle contraction is also required for specification of patellofemoral joint progenitors, we performed genetic lineage experiments. To achieve this, Gdf5-Cre mice crossed with Rosa26-YFP reporter mice, which were used to label joint progenitor cells and their descendants (Rountree et al., 2004), were mated with either wild-type or mdg mice. Analysis at E17.5 showed that, in control embryos, patellofemoral joint cells were labeled (Fig. 7A-A′). In the mdg mice, although the patellofemoral joint failed to form, YFP-positive cells could be observed at the presumptive joint area (Fig. 7B-B′). These results strongly suggest that, although joint progenitors were initially specified, as indicated by the activation of the YFP reporter, in the absence of muscle load these cells eventually lost their fate and remained as chondrocytes, as indicated by Sox9 expression. This resulted in arrested joint formation and in a patella that remained fused to the femur. Furthermore, these results highlight the similarity in mechanoregulation between the patellofemoral joint and other long bone joints (Kahn et al., 2009).

**DISCUSSION**

Traditionally, the developmental program of sesamoid bones has been regarded as an exception, as no canonical skeletogenic program could suitably explain their unique property of being separate from the main skeleton and integrated with a tendon. In this work, by studying the development of the mouse patella as a model system, we provide both the cellular and molecular foundations for a model that describes patella development. First, histological, cellular and molecular analyses of this developmental process led us to conclude that the patella forms by a distinct pool of progenitors specified, as indicated by the activation of the YFP reporter, in the absence of muscle load these cells eventually lost their fate and remained as chondrocytes, as indicated by Sox9 expression. This resulted in arrested joint formation and in a patella that remained fused to the femur. These results strongly suggest that, although joint progenitors were initially specified, as indicated by the activation of the YFP reporter, in the absence of muscle load these cells eventually lost their fate and remained as chondrocytes, as indicated by Sox9 expression. This resulted in arrested joint formation and in a patella that remained fused to the femur. Furthermore, these results highlight the similarity in mechanoregulation between the patellofemoral joint and other long bone joints (Kahn et al., 2009).
as the ‘detachment’ model, suggested that sesamoids arise from bony processes that detach from the skeleton through a process that was then unknown (Pearson and Davin, 1921a,b). For over 50 years, neither model predominated; yet, later biomechanical studies tilted the balance and the intratendinous model has been widely adopted (Hall, 2005). Our finding that the patella initially forms as part of the femur fits well with the theoretical model raised by Pearson. Moreover, we provide the missing piece in Pearson’s theory in the form of cellular and molecular indications for joint formation between the rudimentary patella and femur. This process takes place concurrent with the maturation of the quadriceps tendon, which eventually embeds the patella superficially.

With regards to the contribution of mechanical stimuli, using paralyzed embryos we show that the induction of patella development is independent of muscle-induced mechanical load. However, we establish that muscle contraction is fundamental for joint cavitation and thus for patella separation from the femur. These findings are in agreement with our previous study, where we showed that muscle contraction is necessary for maintenance of joint progenitors in some of the limb joints. In its absence, the progenitors lose their fate of joint-forming cells and remain chondrocytes, resulting in the loss of joint formation (Kahn et al., 2009).

In a previous study, we suggested a model for the development of bone eminences (Blitz et al., 2013). The foundations of this model included a distinct pool of progenitors that uniquely expresses both Sox9 and Scx, development of the eminence as a separate module that is added onto the bone shaft, and a regulatory mechanism involving TGFβ and BMP signaling. Here, we expand this model by providing compelling evidence that these three features are also found in sesamoid development. Our finding of Sox9- and Scx-positive patella progenitors is in line with a previous study showing that patella cells are descendants of Scx-positive cells and that patella development is arrested when Sox9 expression is blocked in Scx-positive cells (Sugimoto et al., 2013). In addition, we show that, as in bone eminences, patella development is regulated by the TGFβ-BMP signaling pathway. Taken together, these findings suggest a common developmental mechanism for bone eminences and sesamoid bones such as the patella.

From an evolutionary viewpoint, modular pools of progenitors increase the plasticity in skeletal patterning and morphogenesis. Modularity also provides an efficient mechanism for adaptation of the musculoskeletal system to environmental changes and pressures, as modules can be altered or created without the need to rewrite the entire skeletogenic program. This strategy allows for the formation of secondary structures on the bone surface through a designated developmental program (Fig. 8A,B), as we previously proposed (Blitz et al., 2013). Here, we suggest that the incorporation of a joint formation program into the strategy adds another layer of developmental plasticity, as it allows secondary structures to detach from the skeleton and form new auxiliary bones (Fig. 8C).

An interesting issue that remains unresolved regards the level of uniformity in the mechanism that regulates sesamoid development. Sesamoid bones are a large family, the members of which exhibit variations in morphology, anatomical architecture and function (Bizarro, 1921). It is therefore interesting to study whether these variations are reflected by variations in the developmental programs of different sesamoid bones. For example, in contrast to patella development, Mikic et al. reported that the plantar tarsal sesamoids do not form in paralyzed chicks (Mikic et al., 2000).
Moreover, there is a growing body of evidence suggesting that early patterning genes, such as Hox9 (Fromental-Ramain et al., 1996), Hox10 (Carpenter et al., 1997), Hox11 (Koyama et al., 2010; Small and Potter, 1993) and Tbx4 (Bongers et al., 2004) affect other aspects of sesamoid development such as their number, distribution and size.

MATERIALS AND METHODS

Animals
All animal experiments were pre-approved and supervised by the Institutional Animal Care and Use Committee of the Weizmann Institute (IACUC). Mice heterozygous for the mutation muscular dysgenesis (mdg) (Pai, 1965a) were kindly provided by G. Kern (Innsbruck Medical University, Innsbruck, Austria). To create mdg mutant mice, animals heterozygous for the mutations were crossed; heterozygous embryos were used as a control. For genetic lineage analysis of patella cells, Sox9-CreERT2 mice (Soeda et al., 2010) were crossed with Rosa26-lacZ reporter mice (Soriano, 1999). Induction of Cre recombinase was performed at various pregnancy stages by administration of 1 mg tamoxifen by oral gavage (stock concentration was 20 mg/ml).

Fig. 7. Specified joint-forming cells lose their fate in the absence of contracting muscles. Sagittal sections through the patella from hindlimbs of E17.5 wild-type and mdg embryos, in which joint progenitor cells and their descendants were labeled by Gdf5-Cre-driven Rosa26-YFP reporter line. Sections were analyzed by immunofluorescence antibodies against SOX9 and YFP. (A-A”) In control embryos, the patella (pa) has separated from the femur (fe) and the patellofemoral joint is established. Descendants of Gdf5-expressing cells line the forming joint and, to some extent, contribute to the patella cell population. (B-B”) In mdg mutant embryos, the patellofemoral joint has failed to develop. However, a large population of descendants of Gdf5-expressing cells is detected at the presumable joint area, suggesting that the joint fate of these cells has been lost and that they remained chondrocytes. Bottom left corners in A”, B” show magnifications of the boxed areas. Scale bar: 200 µm.

Fig. 8. Bone eminences and sesamoid bones share a common developmental strategy. We propose that modular pools of chondrogenic progenitor cells expressing both Sox9 and Scx are specified in proximity to the existing cartilage. (A) These cells are regulated by both TGFβ and BMP4 signaling. (B) After differentiation, these modules often become an integral part of the skeleton and serve as an attachment site for tendons. (C) However, under different environmental conditions and pressures, these modules can be separated from the existing cartilage by formation of a joint between them. This leaves the module superficially embedded within the tendon, thus creating a sesamoid bone.
These mice were crossed with Rosa26-telTomato mice obtained from The Jackson Laboratory (007905). For genetic lineage analysis of patellofemoral joint cells, Gdf5-Cre heterozygous mice (Rountree et al., 2004) were crossed with homozygous Rosa26-YFP mice (Srinivas et al., 2001). Limbs from Gdf5-Cre, Rosa26-YFP embryos were then compared with limbs from Gdf5-Cre, Rosa26-YFP, mdf−/− mice. The generation of floxed-Tgfbr2 (Chytli et al., 2002), Prx1-Cre (Logan et al., 2002) and floxed-Bmp4 (Liu et al., 2004; Selever et al., 2004) has been described previously. To create Prx1-Tgfbr2 and Prx1-Bmp4 mutant mice, floxed-Tgfbr2 or floxed-Bmp4 mice were mated with either Prx1-Cre-Tgfbr2 or Prx1-Cre-Bmp4, respectively. As a control, Prx1-Cre-negative embryos were used.

In all timed pregnancies, plug date was defined as E0.5. For harvesting of embryos, timed-pregnant females were sacrificed by cervical dislocation. The gravid uterus was dissected out and suspended in a bath of cold phosphate-buffered saline (PBS) and the embryos were harvested after amniocentomy and removal of the placenta. Tail genomic DNA was used for genotyping.

**Histological analysis and in situ hybridization**

For histology and in situ hybridization, embryos were harvested at various ages, dissected and fixed in 4% paraformaldehyde (PFA)/PBS at 4°C overnight. After fixation, tissues were dehydrated in 100% ethanol and embedded in paraffin. The embedded tissues were cut to generate 7 µm sections and mounted onto slides. Hematoxylin and Eosin staining and Safranin O/Fast Green staining were performed following standard protocols. For Alcian Blue staining, sections were incubated in Alcian Blue solution (pH 2.5) for 10 min, washed and then counterstained with Nuclear Fast Red solution (Sigma, N3020) for 5 min. Section in situ hybridizations were performed as described previously (Murtaugh et al., 1999; Riddle et al., 1993). All probes were transcribed from plasmids.

**Immunofluorescence staining**

For immunofluorescence staining for SOX9 and collagen type II α1 (COL2A1), 7-µm paraffin sections of embryo limbs were deparaffinized and rehydrated to water. Antigen was then retrieved in 10 mM citrate buffer (pH 6.0), boiled and cooked for 10 min in a microwave oven. In order to block non-specific binding of immunoglobulin, sections were incubated with 7% goat serum, 1% BSA dissolved in PBST. Following block, sections were incubated overnight at 4°C with primary anti-SOX9 antibody (1:200; AB5535, Millipore). Sections were then washed in PBST and incubated with Cy2-conjugated secondary fluorescent antibodies (1:200; 711-225-152, Millipore). Sections were then washed in PBST and fixed in 4% PFA at room temperature for 10 min. Slides were then incubated with protein kinase (Roche), washed and post-fixed again in 4% PFA. Next, sections were washed and incubated overnight at 4°C with primary anti-COL2A1 antibody (1:100; DSHB). The next day, sections were washed in PBST and incubated with Cy3-conjugated secondary fluorescent antibodies (1:200; 115-165-003, Jackson Laboratories). Slides were mounted with Immuno-mount aqueous-based mounting medium (Thermo).

For immunofluorescence staining for SOX9 and YFP, 10-µm cryostat sections of embryo limbs were air-dried for 30-45 min prior to staining (preparation of OCT-embedded samples is described below). Antigen was then retrieved by washing twice in PBST for 5 min. In order to block non-specific binding of immunoglobulin, sections were incubated with 7% goat serum and 1% BSA dissolved in PBST. Following block, sections were incubated overnight at 4°C with a mixture of primary anti-SOX9 antibody (1:200; AB5535, Millipore) and primary (biotin) anti-GFP antibody (1:50; Ab6658, Abcam). The next day, sections were washed twice in PBST and incubated with a mixture of secondary Cy3 fluorescent antibodies (1:200; 111-165-045, Jackson Laboratories) for staining of SOX9 and Cy2-streptavidin antibody (1:200; 016-220-084, Jackson Laboratories) for staining of GFP. Slides were mounted with Immuno-mount aqueous-based mounting medium (Thermo).

**Section β-galactosidase staining**

For β-gal staining, harvested embryos were dissected and fixed in 1% PFA/PBS and 2 mM MgCl2 overnight at 4°C. Fixed embryos were then dehydrated gradually, first in 15% sucrose and 2 mM MgCl2 for 4-6 h at room temperature and then in 30% sucrose and 2 mM MgCl2 overnight at 4°C. Next, hindlimbs were dissected and soaked in 15% sucrose/50% OCT for 30-60 min and then embedded in OCT. Frozen samples were immediately sectioned at 10 µm and mounted onto slides. Prior to staining, slides were air-dried for 30-45 min, fixed in 1% PFA/PBS and 2 mM MgCl2 for 10 min, and washed twice with PBS for 5 min. To stain the samples, slides were immersed in staining solution containing rinse buffer (0.01% deoxycholate, 0.02% NP-40, 2 mM MgCl2 and 5 mM EGTA) supplemented with 1 mg/ml X-gal, 5 mM K3[Fe(CN)6] and 5 mM K4[Fe(CN)6]. The slides were left in staining solution overnight at 37°C, gently agitated. Following staining, slides were washed once with double-distilled water for 1 h at 37°C and then twice with PBS for 5 min at room temperature. Samples were then counterstained with Nuclear Fast Red solution (Sigma) for 5 min, dehydrated and cleared in xylene.

**Quantification of X-gal-stained cells**

Quantification of X-gal-stained cells was performed on sections collected from E16.5 embryos following a single tamoxifen administration, either at E10.5 or at E13.5. For each time point, three embryos from three different litters were harvested, dissected, sectioned and stained with X-gal. From each embryo, four sections were used for statistical analysis (n=12). In each section, the number of stained cells in either femur or patella was counted within a constant area of 25,000 µm2. The prevalence of marked cells in patella is presented relative to their prevalence in the femur, defined as 1. Statistical significance was determined as P<0.05, using Student’s t-test.

**Quantitative real-time (qRT-) PCR**

Total RNA was purified from whole limbs of E13.5 mutant and control embryos using the RNasy Kit (Qiagen). Reverse transcription was performed with High Capacity Reverse Transcription Kit (Applied Biosystems) according to the manufacturer’s protocol. RT-PCR was performed using Fast SYBR Green master mix (Applied Biosystems) on the StepOnePlus machine (Applied Biosystems). Values were calculated using the StepOne software (version 2.3), according to the relative standard curve method. Data was normalized to TBP-box binding protein (Tbp) in all cases. Primer sequences can be found in supplementary material Table S1. Statistical significance was determined using Student’s t-test and P<0.05 was considered significant.

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**Competing interests**

The authors declare no competing or financial interests.

**Author contributions**

S.E. conceived and jointly designed the study, conducted the experiments, analyzed and interpreted the data, and wrote the manuscript. E.B. jointly designed the study. R.S. analyzed and interpreted the data, E.Z. jointly designed the study, supervised the project, analyzed and interpreted the data, and wrote the manuscript.

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**Supplementary material**

Supplementary material available online at http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.121970/-/DC1
References


Supplementary Figure 1. Quantitative real-time (qRT-) PCR shows a drastic decrease in expression levels of Tgfbr2 or Bmp4 genes. Total RNA was purified from whole limbs of E13.5 Prx1-Cre, Tgfbr2\textsuperscript{f/f} (Purple bars) and Prx1-Cre, Bmp4\textsuperscript{f/f} (Red bars) mutants and control littermates (Green and Blue bars, respectively). Then, the efficiency of gene ablation was measured by qRT-PCR. Data was normalized to TATA-box binding protein (Tbp) in all cases. The results showed a drastic decrease in expression levels in the mutants, as compared with control littermates. Statistical significance was determined by Student’s t-test as P<0.05.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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<tbody>
<tr>
<td>Tbp</td>
<td>5’ – GCAGCCTCAGTACAGCAATCA – 3’</td>
<td>5’ – GGTGCAGTGTCAGAGTTTGA – 3’</td>
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<tr>
<td>TgflβR2</td>
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<tr>
<td>Bmp4</td>
<td>5’ – GGCAGCCATGCTAGTTTTGATACT – 3’</td>
<td>5’ – AGCCCCAACATCTGCAGAAGTGC – 3’</td>
</tr>
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Table S1. List of primer sequences used for the qRT-PCR analysis.