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Tendon-bone attachment unit is formed modularly by a distinct pool of *Scx*- and *Sox9*-positive progenitors

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SUMMARY

The assembly of the musculoskeletal system requires the formation of an attachment unit between a bone and a tendon. Tendons are often inserted into bone eminences, superstructures that improve the mechanical resilience of the attachment of muscles to the skeleton and facilitate movement. Despite their functional importance, little is known about the development of bone eminences and attachment units. Here, we show that bone eminence cells are descendants of a unique set of progenitors and that superstructures are added onto the developing long bone in a modular fashion. First, we show that bone eminences emerge only after the primary cartilage rudiments have formed. Cell lineage analyses revealed that eminence cells are not descendants of chondrocytes. Moreover, eminence progenitors were specified separately and after chondroprogenitors of the primary cartilage. Fields of *Sox9*-positive, *Scx*-positive, *Col2a1*-negative cells identified at presumable eminence sites confirm the identity and specificity of these progenitors. The loss of eminences in limbs in which *Sox9* expression was blocked in *Scx*-positive cells supports the hypothesis that a distinct pool of *Sox9*- and *Scx*-positive progenitors forms these superstructures. We demonstrate that TGFβ signaling is necessary for the specification of bone eminence progenitors, whereas the SCX/BMP4 pathway is required for the differentiation of these progenitors to eminence-forming cells. Our findings suggest a modular model for bone development, involving a distinct pool of *Sox9*- and *Scx*-positive progenitor cells that form bone eminences under regulation of TGFβ and BMP4 signaling. This model offers a new perspective on bone morphogenesis and on attachment unit development during musculoskeletal assembly.

KEY WORDS: Musculoskeletal system, Skeleton, Cartilage, Tendons, Attachment unit, Modularity, Progenitors, Specification, Patterning, SOX9, SCX, TGFβ, BMP4, Mouse

INTRODUCTION

The musculoskeletal system provides the body with form, stability and mobility. This requires precise and tightly coordinated assembly of tendons, muscles and bones into one functional system. Although the development of each of these components has been extensively studied, the process of musculoskeletal assembly has remained poorly understood.

A fundamental step in the assembly process is the development of an attachment unit between a bone and a tendon. The term 'tendon-bone attachment unit' (referred to herein as AU) describes a complex structure that includes the tip of the tendon and the part of the bone into which it is inserted. Tendons are often inserted into specific sites named bone eminences. These are superstructures that grow on the surface of the bone, exhibiting a variety of shapes and sizes depending on bone type and organism (Gray, 1918; Hill, 1964). Bone eminences are vital for the functionality of the musculoskeletal system. They provide stable anchoring points for muscles, which are inserted into the skeleton via tendons, and dissipate the stress exerted on the skeleton by contracting muscles. This effect improves the mechanical resilience of muscle attachment and facilitates movement (Benjamin et al., 2002; Biewener et al., 1996; Thomopoulos et al., 2011; Thomopoulos et al., 2010). Additionally, because of their prominence in the bone landscape, these structures largely contribute to the three-dimensional morphology of bones (Gray, 1918). Obviously, deciphering the process by which bone eminences develop is vital for the understanding of tendon-bone AU formation during the assembly of the musculoskeletal system.

The development of the appendicular skeleton is initiated when a subset of mesenchymal cells, originating in the lateral plate mesoderm, amasses and is specified as chondroprogenitors. *Sox9* is the earliest known marker for these progenitor cells and is necessary for their condensation and differentiation (Akiyama, 2008; Akiyama et al., 2002). Interestingly, lineage studies on *Sox9-Cre* mice suggest that *Sox9*-expressing cells also serve as progenitors for osteoblasts, tenocytes and synovial cells (Soeda et al., 2010; Akiyama et al., 2005). As development proceeds, chondroprogenitors differentiate into chondrocytes, expressing markers such as type II collagen, and form cartilaginous templates that prefigure the future bones (Karsenty et al., 2009; Lefebvre and Smits, 2005; Provot and Schipani, 2005).

Previously, we showed that bone eminences are formed during the cartilaginous phase of skeletal development, in a process that involves both molecular and mechanical signals. We showed that the basic helix-loop-helix (bHLH) transcription factor scleraxis (SCX) drives the expression of bone morphogenetic protein 4 (*Bmp4*) at the tendon-skeleton junction to induce formation of cartilaginous bone eminences. Blockage of either *Scx* or *Bmp4* expression in the limb arrested eminence development (Blitz et al., 2009).

Despite this progress, we still lack basic understanding of many aspects of AU development, such as the origin of eminence cells and the molecular mechanisms that control eminence progenitors. In this work, we identify a novel mechanism that regulates AU formation. We show that an external, distinct pool of progenitors that express both *Sox9* and *Scx* form eminences, which are modularly added onto the developing bone. Moreover, we show that TGFβ signaling controls specification of these progenitors,

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whereas the SCX/BMP4 pathway mediates their differentiation to eminence cells.

MATERIALS AND METHODS

Animals

The generation of *Scx*$^{-/-}$ (Murchison et al., 2007), *R26R-lacZ* reporter (Soriano, 1999), floxed *Tgf-βRII* (Chytil et al., 2002), floxed *Bmp4* (Liu et al., 2004; Selever et al., 2004), floxed *Sox9* (Akiyama et al., 2002), *Prx1-Cre* (Logan et al., 2002), *Col2a1-CreER* (Nakamura et al., 2006) and *Sox9-CreER* (Soeda et al., 2010) mice have been described previously. *Scx-Cre* transgenic mice were generated by and obtained from R. Schweitzer (Shriners Hospital for Children Research Division, Portland, OR, USA) and R. L. Johnson (The University of Texas MD Anderson Cancer Center, Houston, TX, USA). To create *Scx*$^{-/-}$ mice, animals heterozygous for the mutation were crossed; as a control we used heterozygous *Scx* embryos. To create *Scx-Sox9*, *Prx1-Tgf-βRII* and *Prx1-Bmp4* mutant mice, floxed *Sox9*, floxed *Tgf-βRII* and floxed *Bmp4* mice were mated with *Scx-Sox9*, *Prx1-Tgf-βRII* and *Prx1-Bmp4* mice, respectively. As a control, we used embryos that lack *Cre* alleles. To create *Col2a1-CreER, R26R-lacZ* and *Sox9-CreER, R26R-lacZ* reporter mice, floxed *R26R-lacZ* mice were mated with *Col2a1-CreER* and *Sox9-CreER* mice, respectively.

In all timed pregnancies, plug date was defined as embryonic day (E) 0.5. For harvesting of embryos, timed-pregnant females were sacrificed by cervical dislocation. Tail genomic DNA was used for genotyping by PCR.

Skeletal preparations

Cartilage and bones in whole mouse embryos were visualized after staining with Alcian Blue and Alizarin Red S (Sigma) and clarification of soft tissue with potassium hydroxide (McLeod, 1980).

Tamoxifen induction

Adult females were administered 6.5 mg tamoxifen (TM) in corn oil by oral gavage or 6 mg 4-hydroxy-TM in 1:10 (v/v) ethanol:sunflower oil (from a stock of 20 mg/ml) by intraperitoneal injection at the indicated time points.

Immunofluorescence staining

For immunofluorescence, embryo limbs were fixed overnight in 4% paraformaldehyde (PFA) at 4°C, embedded in paraffin and sectioned at 7 μm. Paraffin sections were collected on Fisherbrand Superfrost Plus slides, de-paraffinized and rehydrated to water. Antigen was retrieved in 10 mM citrate buffer, pH 6, using a microwave. In order to block non-specific binding of immunoglobulin, sections were incubated with 7% goat serum. Following blockage, sections were incubated overnight at 4°C with primary antibody anti-SOX9 (1:200; AB5535, Chemicon). Then, sections were washed in 0.1% Tween 20 in PBS (PBST) and incubated with Cy2-conjugated secondary fluorescent antibodies (1:200; Jackson Laboratories). After staining for SOX9, slides were washed in PBS and fixed in 4% PFA at room temperature for 10 minutes. Then, slides were incubated with protein kinase (P2308, Sigma), washed and post-fixed again in 4% PFA. Next, sections were washed and incubated overnight at 4°C with primary antibody anti-collagen II [1:100; Developmental Studies Hybridoma Bank (DSHB), University of Iowa, USA]. The next day, sections were washed in PBST and incubated with Cy3-conjugated secondary fluorescent antibodies (1:200; Jackson Laboratories). Slides were mounted with Immuno-mount aqueous-based mounting medium (Thermo).

Whole-mount X-gal staining

For pulse-chase experiments on *Col2a1-CreER, R26R-lacZ* heterozygous embryos, whole-mount X-gal staining was carried out. Embryos were fixed for 1 hour in 4% PFA at 4°C, washed three times in rinse buffer (PBS containing 0.01% deoxycholate, 0.02% NP-40, 2 mM $MgCl_2$ and 5 mM EGTA) at room temperature and stained for 5 hours at 37°C in rinse buffer supplemented with 1 mg/ml X-gal, 5 mM $K_3Fe(CN)_6$ and 5 mM $K_4Fe(CN)_6$. For histological examination, stained whole-mount limbs were fixed in 4% PFA overnight, dehydrated, embedded in paraffin and sectioned at 7 μm. Sections were then collected on Fisherbrand Superfrost Plus slides, dehydrated and cleared in xylene. Fast Red (Sigma) was used for counter-staining.

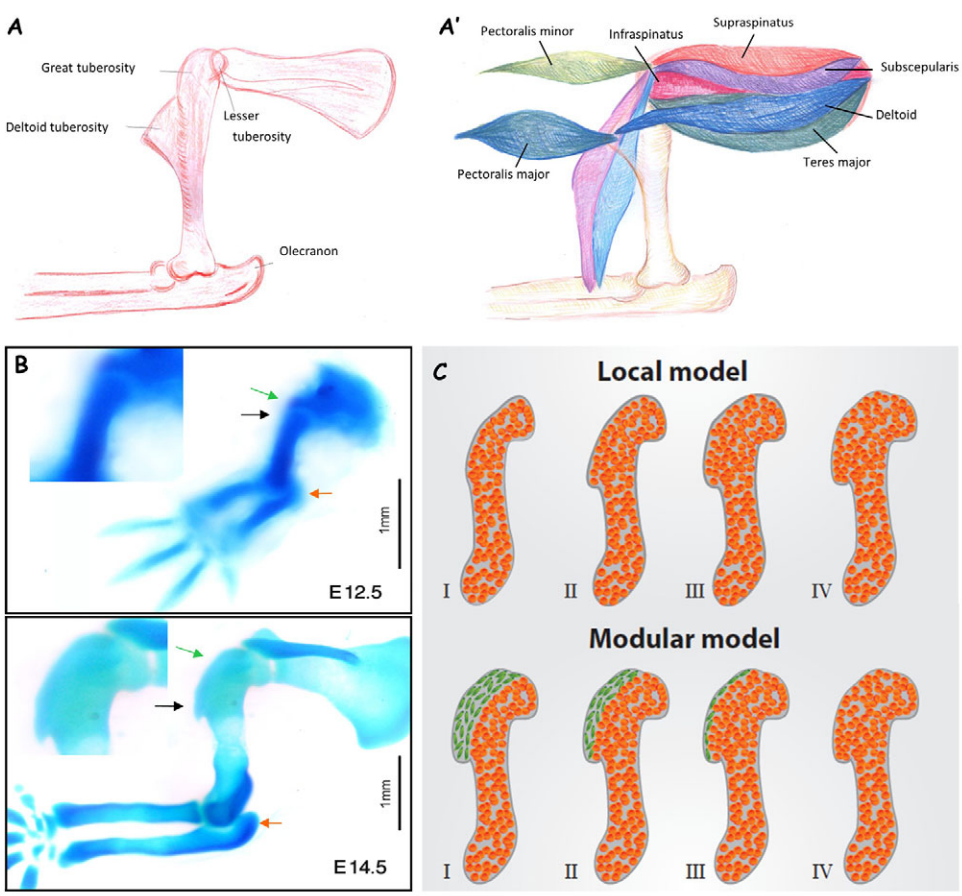

Fig. 1. Bone eminence formation. (**A**,**A′**) Anatomical sketches of forelimb bones showing the outlines of major eminences and their contribution to the final morphology of each bone (A). Muscles that are inserted into bone eminences are illustrated (A′). (**B**) Skeletal preparations from E12.5 and E14.5 wild-type embryos demonstrate morphological changes in the cartilage template during development. Arrows indicate eminences on the forelimb bones: deltoid tuberosity (black arrow) and great tuberosity (green arrow) of the humerus and the olecranon of the ulna (red arrow). (**C**) Two alternative models for the genesis of a bone eminence. Local model: the eminence is formed by local growth of the primary cartilaginous elements. Modular model: the eminence is derived from a distinct pool of progenitor cells located outside the primary cartilaginous elements. During development, progenitor cells of the external pool differentiate to form a cartilaginous eminence. Orange circles indicate differentiated cartilage cells; green ovals indicate eminence progenitor cells.

Slide X-gal staining

For pulse-chase experiments on *Sox9-CreER, R26R-lacZ* heterozygous embryos, we used slide X-gal staining. Cryostat sections were cut at a thickness of 10 μm and dried for 1 hour at 37°C. Then, sections were fixed for 10 minutes in 4% PFA, washed three times in rinse buffer containing 0.01% deoxycholate, 0.02% NP-40, 2 mM $MgCl_2$ and 5 mM EGTA at room temperature. Slides were stained overnight at 37°C in rinse buffer supplemented with 1 mg/ml X-gal, 5 mM $K_3Fe(CN)_6$ and 5 mM $K_4Fe(CN)_6$. For histological examination, stained slides were counter-stained with Fast Red solution (Sigma), dehydrated and cleared in xylene. To quantify the prevalence of *lacZ*-positive chondrocytes, regional density was approximated by counting the number of X-Gal-stained cells in sampled rectangles of equal area in images of the humerus, femur and calcaneus. Manual marking of cells was followed by automatic enumeration using in-house software. At least five sections were analyzed for each of the *Sox9-CreER, R26R-lacZ* littermate embryos at E15.5. Statistical significance was determined by Student's *t*-test.

In situ hybridization

Section *in situ* hybridizations were performed as described previously (Murtaugh et al., 1999; Riddle et al., 1993). Double fluorescent *in situ* hybridizations on paraffin sections were performed using fluorescein- and DIG-labeled probes (see supplementary material Table S1 for probe sequences). After hybridization, slides were washed, quenched and blocked. Probes were detected by incubation with anti-fluorescein-POD and anti-DIG-POD (Roche; 1:300), followed by Cy3- or Cy2-tyramide labeled fluorescent dyes (according to the instructions of the TSA Plus Fluorescent Systems Kit, Perkin Elmer).

Micro-CT analysis

Harvested limbs were fixed overnight in 4% PFA in PBS, dehydrated to 100% ethanol and then soaked in 2% iodine solution. Samples were scanned using a microfocus X-ray tomographic system (Micro XCT-400, Xradia), at 40 kV and 200 lA. A thousand projection images at a total integration time of 5 mseconds with a linear magnification of ×4 were taken. The final pixel size was 2.3μm. The volume was reconstructed using a back projection filtered algorithm (XRadia). Following reconstruction, 3D image processing and analysis were carried out using MicroView (MicroView software version 5.2.2, GE Healthcare). Bone and cartilage tissues were manually segmented and segmented structures were rendered as 3D surfaces.

RESULTS

Eminence cells are not descendants of chondrocytes that form the primary cartilaginous elements

Despite their functional importance (Fig. 1A,A′), little is known about bone eminence development. Examination of the chronological sequence of long bone morphogenesis revealed that different eminences, such as the deltoid tuberosity and great

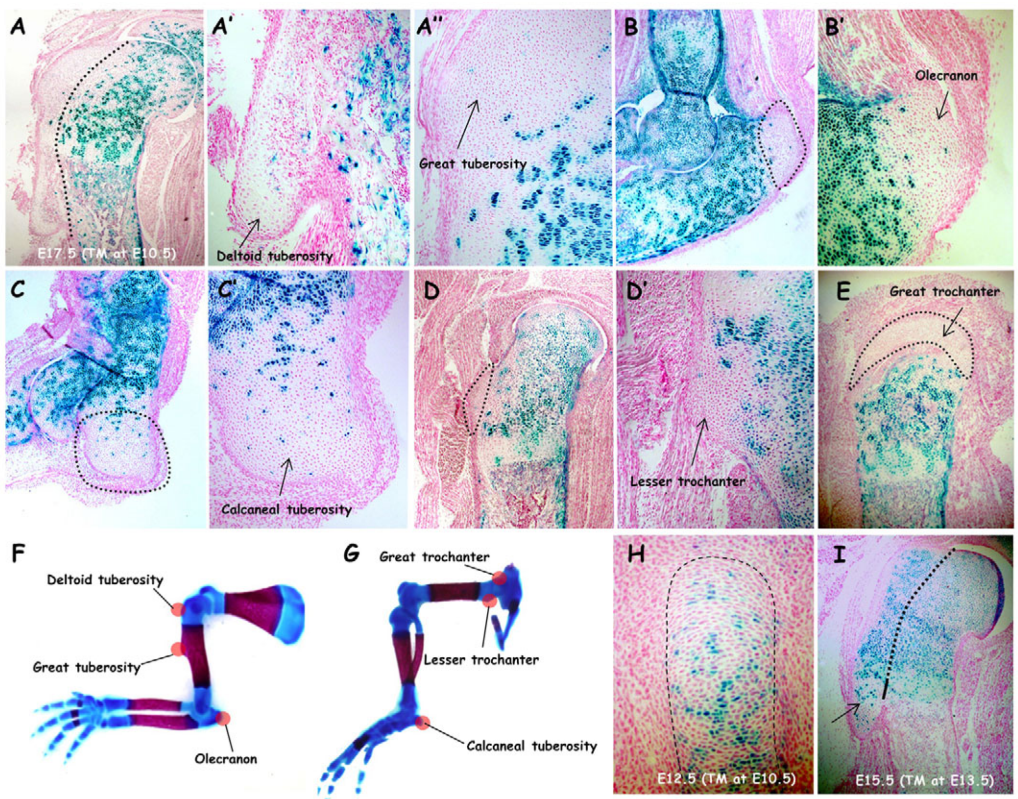

Fig. 2. Eminence-forming cells are not descendants of chondrocytes that establish the primary cartilage element. (**A-E**) A pulse-chase cell lineage experiment using *Col2-CreER* mice demonstrates that bone eminence cells are not derived from the main cartilage template of the long bone. Cartilage-forming cells were marked at E10.5 by tamoxifen (TM) administration and their descendants were followed at E17.5. Dotted lines demarcate eminence areas and black arrows indicate the various eminences: deltoid tuberosity of the humerus (A,A′), great tuberosity of the humerus (A,A″), olecranon process of the ulna (B,B′), calcaneal tuberosity (C,C′), lesser trochanter of the femur (D,D′) and great trochanter of the femur (E). A′ and A″, B′, C′ and D′ are enlargements of the marked areas in A, B, C and D, respectively. (**F**,**G**) Skeletal preparations showing the location of the above-mentioned bone eminences on the forelimb (F) and hindlimb (G). (**H**) To verify the effectiveness of the Col2-CreER system, cartilage-forming cells were marked at E10.5 and their descendants were followed at E12.5, before cartilaginous eminences form; only the main cartilage anlage was marked. Dashed line highlights the borders of the cartilage anlage. (**I**) Cartilage-forming cells were marked at E13.5, the initiation day of the deltoid tuberosity, and their descendants were followed at E15.5. *lacZ*-positive cells are seen inside the humeral eminences. Dotted line demarcates deltoid and great tuberosity cartilage. Arrow indicates the deltoid tuberosity.

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tuberosity of the humerus or the olecranon process of the ulna, could first be observed at E14.5. However, rudimentary cartilaginous elements were visible already at E12.5 (Fig. 1B). This observation prompted us to study the mechanism that regulates the formation of eminences on the surface of developing bones.

We examined two possible scenarios for the genesis of bone eminences (Fig. 1C). The first, referred to as the 'local model', suggests that eminences form by outgrowth from the pre-existing primary cartilage template. According to this model, eminence-forming cells are descendants of chondrocytes that form the primary cartilage. An alternative hypothesis, referred to as the 'modular model', suggests that the bone eminence is derived from a pool of cells located externally to the primary template.

To determine which of these two models is correct, we performed a pulse-chase cell lineage experiment using the Cre-ER system, in which the expression of Cre recombinase is activated at different stages of development by tamoxifen administration. By crossing mice that express Cre-ER under control of the collagen type II promoter (*Col2a1-CreER*) with R26R reporter mice (Nakamura et al., 2006; Soriano, 1999), we were able to mark chondrocytes of the primary cartilage and follow their descendants at later stages. Administration of tamoxifen at E10.5 to *Col2a1-CreER, R26R-lacZ* heterozygous embryos revealed at E17.5 *lacZ* expression along the main axis of long bones. Interestingly, there was no X-Gal staining in various eminences (Fig. 2A-G), suggesting that eminence cells do not originate from chondrocytes of the primary template.

To exclude the possibility that the lack of staining was due to inefficiency of the Cre-ER system to mark the entire primary template, we administered tamoxifen to *Col2a1-CreER, R26R-lacZ* heterozygous embryos at E10.5 and followed their descendants at E12.5. Examination showed that the initial induction of the system was effective, as most of the primary cartilaginous template cells were X-Gal-positive and equally distributed (Fig. 2H). To verify that *lacZ* expression can be induced in eminence cells, we administered tamoxifen to *Col2a1-CreER, R26R-lacZ* heterozygous embryos at E13.5 and followed their descendants at E15.5. The results showed that the Cre-ER system was active in bone eminence cells, as X-Gal-positive cells were detected in the deltoid tuberosity (Fig. 2I).

These results strongly imply that eminence-forming cells are not descendants of chondrocytes that compose the primary cartilage.

Bone eminences originate from a field of progenitor cells located outside the primary cartilage

Our findings that bone eminences appear after the primary template is established (Fig. 1B) and that cells that form eminences are not descendants of primary template chondrocytes (Fig. 2A-E) suggest that bone eminences develop from an external pool of cells. To examine this possibility, we analyzed the differentiation state of cells at the presumable locations of three eminences: the deltoid, the great and the lesser tuberosities of the humerus. To that end, we performed immunofluorescence staining with antibodies for SOX9 as a marker for chondrocyte progenitors and for collagen type II alpha 1 (COL2A1) as a marker for differentiated cartilage. At E12.5, although cells in the primary template have already differentiated to chondrocytes and expressed both *Sox9* and *Col2a1*, we identified at presumable eminence sites cells that expressed only *Sox9* (Fig. 3A). From E13.5 to E14.5, cells in these domains underwent differentiation and expressed *Col2a1* in addition to *Sox9* (Fig. 3A′,A″). Examination of presumable locations of other eminences in the forelimb and hindlimb also revealed cells that

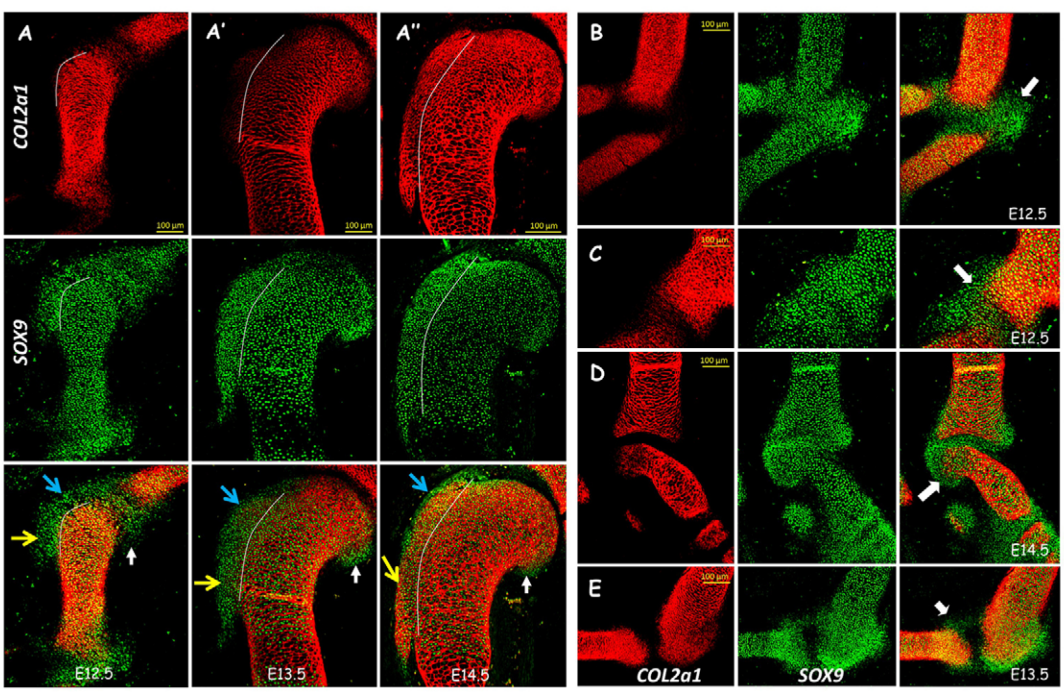

Fig. 3. Bone eminence cells differentiate from an external pool of progenitors. (**A-E**) Immunofluorescence staining of long bone sections from wild-type mice using anti-collagen II (COL2A1) and anti-SOX9 antibodies, showing the presence of an external pool of *Sox9*-positive, *Col2a1*-negative cells at presumable sites of different eminences. (A-A″) Humeral sections from E12.5 (A), E13.5 (A′) and E14.5 (A″) embryos demonstrate differentiation of eminence progenitors to chondrocytes. White lines demarcate a field from which the deltoid and great tuberosities develop; blue arrows indicate the great tuberosity, yellow arrows indicate deltoid tuberosity and white arrows indicate the lesser tuberosity. (B-E) White arrows mark the olecranon, located at the end of the ulna (B), the coracoid process of the scapula (C), the calcaneal tuberosity (D) and the medial condyle of the tibia (E).

expressed only *Sox9* (Fig. 3B-E). Finding fields of *Sox9*-positive, *Col2a1*-negative cells at presumable locations of bone eminences at a stage when primary template cells have already differentiated suggests that eminences develop from external pools of progenitors, thus supporting the modular model (Fig. 1C).

Bone eminences are specified as a secondary pool of progenitor cells

Our finding that bone eminences originate from an external pool of *Sox9*-positive progenitor cells raised a question about their specification. During limb development, eminence progenitors can be specified as part of a common pool of progenitor cells that will later form both the eminence and the primary cartilaginous element. In this scenario, the common pool of progenitors differentiates to chondrocytes in two steps, first to form the primary cartilage and then to form the eminence (Fig. 4A). Alternatively, specification of a distinct pool of eminence progenitors might be a particular event that is secondary to the specification of primary cartilage progenitors (Fig. 4A′).

In order to distinguish between these two possibilities, we again performed a pulse-chase cell lineage experiment using the Cre-ER system. By crossing mice that express Cre-ER under control of the *Sox9* promoter (*Sox9-CreER*) (Soeda et al., 2010) with R26R reporter mice, we marked *Sox9*-positive progenitors and followed their descendants. Examination of E15.5 *Sox9-CreER, R26R-lacZ* heterozygous embryos that had been administered tamoxifen at E9.5-E10.5 revealed *lacZ* expression by chondrocytes in different skeletal elements; by contrast, cells in various eminences were poorly stained (Fig. 4B-D,F). As a control, tamoxifen administration at E12.5-E13.5 induced *lacZ* expression by all cells, including in eminences (Fig. 4E). Quantification of X-Gal-positive cells in the eminence field relative to marked cells in the primary structure of cartilage confirmed these observations (Fig. 4G; supplementary material Fig. S1). These results indicate that eminence progenitors are specified separately from and secondary to the pool of cells that gives rise to the primary cartilage (Fig. 4A′). This implies that during limb development, a distinct and previously unknown pool of progenitors is involved in the formation of bone eminences, which are part of the tendon-bone AU.

Eminence progenitors express both *Sox9* and *Scx*

The specification of eminence-forming cells as a secondary pool of progenitor cells prompted us to examine what distinguishes this population from the progenitor pool of the primary cartilage. Double fluorescence *in situ* hybridizations on sections of E11.5 forelimbs showed that, unlike cells of the primary cartilage, the *Sox9*-positive

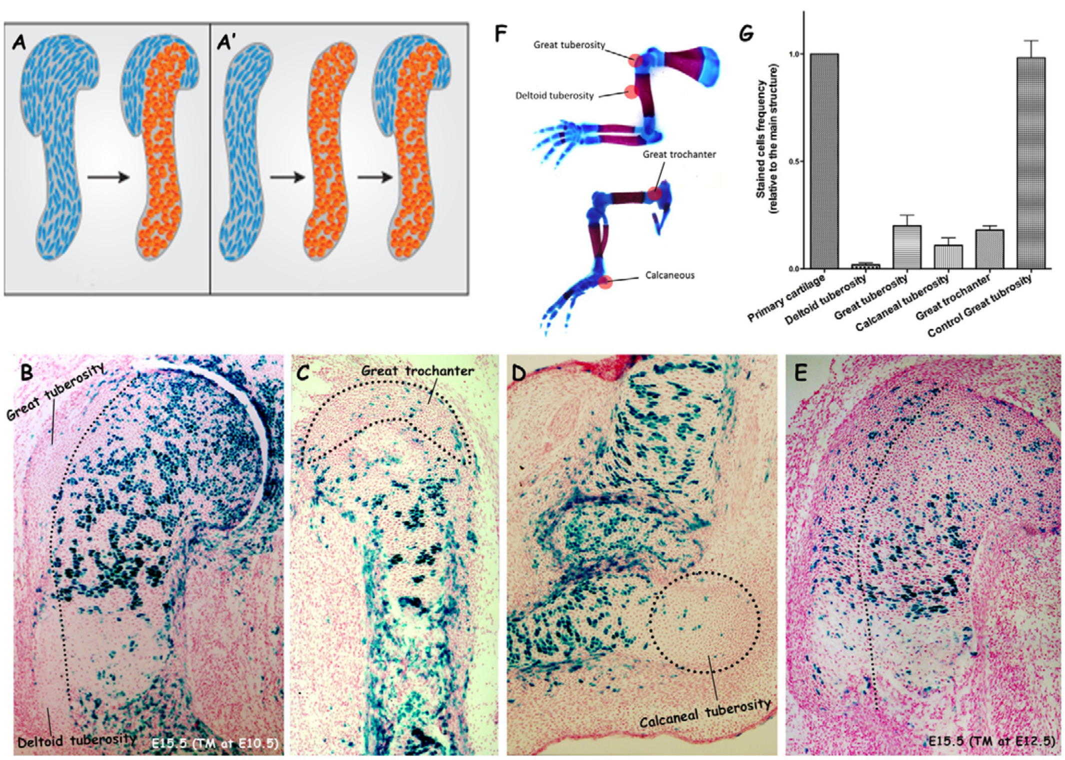

Fig. 4. Specification of eminence progenitors is secondary to that of the primary cartilage. (**A**,**A′**) Two alternative models for the specification sequence of progenitors of the primary cartilage and eminences. (A) All progenitors are specified as one pool of cells that differentiate in two phases, first to form the primary cartilage and then the eminence. (A′) Alternatively, primary cartilage and eminence progenitors are specified separately as two distinct pools of cells. First, primary cartilage progenitors are specified and differentiate to chondrocytes, and only then a secondary pool of eminence progenitors is specified. Orange circles indicate differentiated cartilage cells; blue ovals indicate *Sox9*-positive progenitor cells. (**B**-**E**) A pulse-chase cell lineage experiment using *Sox9-CreER* mice demonstrates that bone eminence cells are specified from a secondary pool of progenitors. *Sox9*-positive progenitor cells were marked by tamoxifen (TM) administration at E9.5-E10.5 (B-D) and at E12.5-E13.5 as a control (E) and their descendants were followed at E15.5. Dotted lines demarcate eminence areas. (**F**) Skeletal preparations indicate the bone eminences shown in B-E. (**G**) Prevalence of marked cells measured in eminences from E15.5 embryos, relative to their prevalence in the primary structure of cartilage, defined as 1. Control staining (TM administration at E12.5-E13.5) is shown for the great tuberosity; for other eminences, see supplementary material Fig. S1. Deltoid tuberosity: 0.01899±0.008791; great tuberosity: 0.2003±0.04917; calcaneal tuberosity: 0.109±0.03516; great trochanter: 0.1801±0.02033; control great tuberosity: 0.9819±0.08007; $P<0.0001$. Error bars represent the s.e.m.

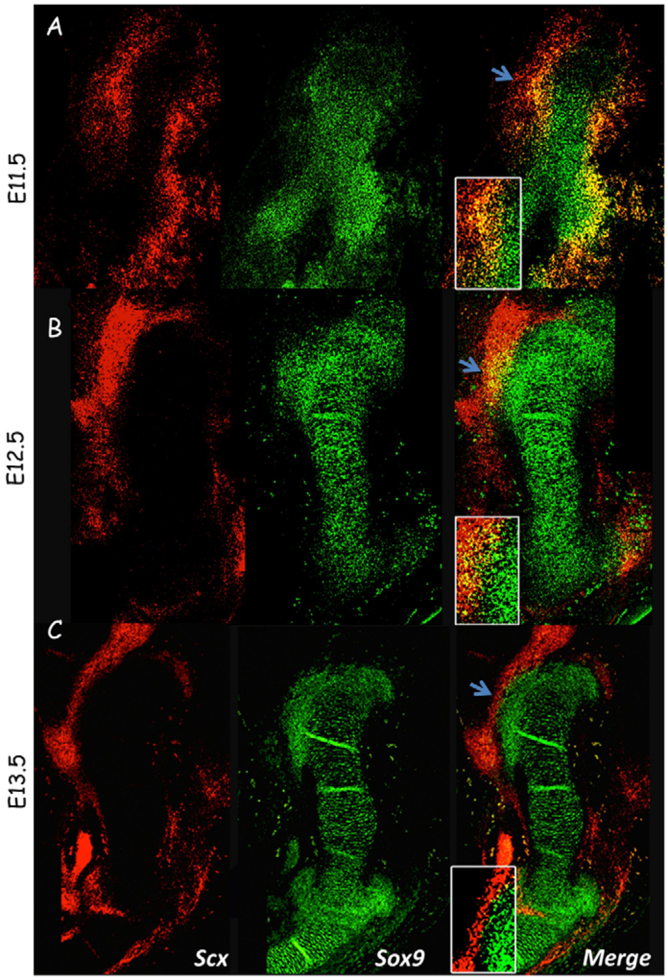

Fig. 5. Bone eminence progenitors co-express *Sox9* and *Scx*. (**A-C**) Double fluorescence *in situ* hybridization of sagittal humerus sections from E11.5-E13.5 wild-type mice, using antisense complementary RNA probes for *Sox9* and *Scx*. Blue arrows demarcate a field from which the deltoid and great tuberosities develop; box shows enlargement of eminence progenitors expressing both *Sox9* and *Scx*.

eminence progenitors also expressed *Scx* (Fig. 5A). As development proceeded, the *Scx* expression domain in the forming AU became more restricted, concomitantly with tendon patterning (Fig. 5B). By E13.5, *Scx* and *Sox9* expressions were mutually exclusive as the former was expressed only in forming tendons (Fig. 5A-C). To support this observation, we studied the *Scx* lineage in the limb using the *Scx-Cre* mouse crossed with the R26R reporter mouse. As expected, tendons and various eminences were positive for X-Gal, supporting the notion that AU progenitors express both *Scx* and *Sox9* (supplementary material Fig. S2). Interestingly, our analysis revealed that some cells in different skeletal elements were X-Gal-positive as well, most prominently in the proximal humeral head and the distal femoral head. This result agrees with a previous report of overlapping *Scx* and *Sox9* expression during initial stages of limb development (Asou et al., 2002).

Next, we sought to evaluate the contribution of the *Scx*- and *Sox9*-positive progenitors to bone eminence formation. For that, we targeted *Sox9* in eminence progenitors using the *Scx-Cre* mouse as a deleter (*Scx-Sox9*). Given the results of the *Scx* lineage analysis, it was important to determine which cells lost *Sox9* expression. Immunofluorescence staining with antibodies for SOX9 on sections from *Scx-Sox9* conditional knockout (cKO) embryos revealed loss of *Sox9* expression only in eminence progenitors (Fig. 6A), whereas in other skeletal elements it was maintained. This result strongly supports our expression analysis results, indicating that bone eminence progenitors are indeed *Sox9* and *Scx* positive.

Examination of skeletons of *Scx-Sox9* mutants revealed loss of bone eminences (Fig. 6B), suggesting that expression of *Sox9* by *Scx*-positive cells is necessary for eminence formation. Finally, because previous lineage studies suggest that some tenocytes originate in *Sox9*-expressing progenitors (Soeda et al., 2010), we examined in *Scx-Sox9* cKO mutants tendon development upon the loss of corresponding bone eminences using tendon markers such as *Scx*, *Tnmd* and *Col1a1*. As shown in Fig. 6C, tendon development was comparable between *Scx-Sox9* cKO and control embryos.

Together, these results imply that bone eminences develop from distinct pools of progenitor cells that co-express *Sox9* and *Scx*.

The SCX/BMP4 pathway regulates differentiation of eminence progenitors

Next, we explored the molecular mechanism that regulates the *Sox9*- and *Scx*-positive bone eminence progenitors. The expressions of *Scx* by eminence progenitors motivated us to examine its role in this mechanism. Analysis of forelimbs from E12.5 $Scx^{-/-}$ embryos revealed *Sox9*-positive progenitors at the presumptive eminence site (Fig. 7A). This result indicates that SCX is not involved in the specification of eminence progenitors.

Previously, we showed that BMP4, produced under regulation of SCX, mediates bone eminence formation (Blitz et al., 2009). We therefore examined the involvement of this pathway in the regulation of eminence progenitors. Examination of forelimbs from E12.5 mutants in which *Bmp4* was ablated from limb mesenchyme (*Prx1-Bmp4*) revealed the presence of *Sox9*-positive eminence progenitors (Fig. 7B), similarly to $Scx^{-/-}$ mice. However, as development proceeded, these cells maintained *Sox9* expression and failed to express *Col2a1*. That these cells remained in their progenitor state implies that the SCX/BMP4 pathway is necessary for differentiation of eminence progenitors (Fig. 7C,D).

TGFβ signaling is necessary for specification of eminence progenitors

Having found that the SCX/BMP4 pathway regulates differentiation of eminence progenitors, we proceeded to explore their specification. Previously, it was shown that in mice in which *Tgfbr2* was ablated in limb mesenchyme (*Prx1-Tgf-βRII*), prominent bone eminences were absent (Blitz et al., 2009; Seo and Serra, 2007; Spagnoli et al., 2007). This phenotype was attributed to the loss of mechanical load exerted by muscle contraction (Seo and Serra, 2007) or to the loss of tendon formation (Blitz et al., 2009). The observation of *Tgfbr2* expression by AU progenitors at E12.5 (supplementary material Fig. S3A) raised the possibility that TGFβ signaling has a direct role in their specification and/or differentiation. To test this hypothesis, we examined the presence of *Sox9*-positive progenitor cells in *Prx1-Tgf-βRII* mutants (Baffi et al., 2004; Logan et al., 2002). Our results showed that at E13.5, whereas control embryos exhibited *Sox9*-positive, *Col2a1*-negative eminence progenitors, the mutants lacked these cells (Fig. 8A,B). To verify this result we studied the expression of *Scx* in the limbs of these mutants and, as expected, it was dramatically reduced (supplementary material Fig. S3B). These results indicate that TGFβ signaling is necessary

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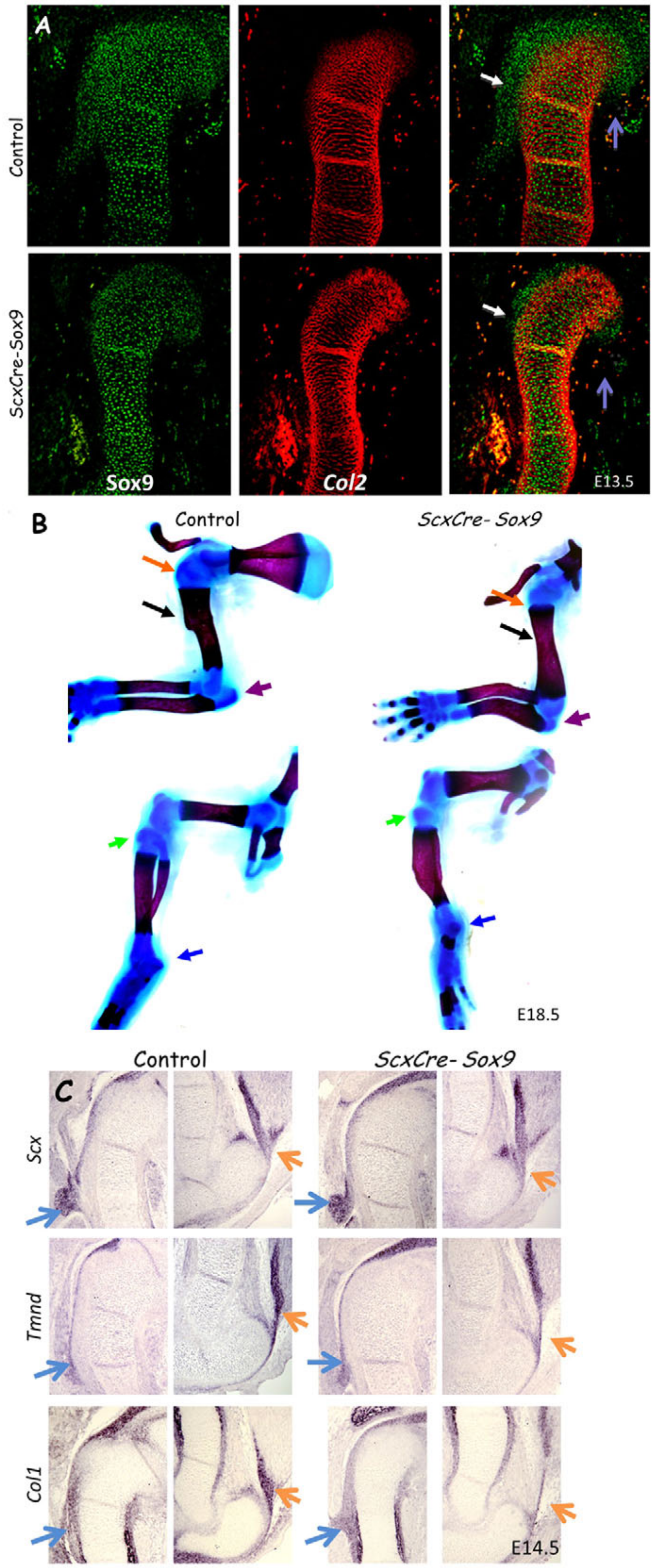

Fig. 6. Co-expression of *Sox9* and *Scx* in eminence progenitors is necessary for their formation. (**A**) Immunofluorescence staining of humeral sections from control mice and *Scx-Sox9* mutants using anti-collagen II (COL2A1) and anti-SOX9 antibodies demonstrates the loss of eminence progenitors in the mutants. White arrows indicate deltoid tuberosity and great tuberosity field and purple arrows indicate lesser tuberosity. (**B**) Skeletal preparations of E18.5 control and *Scx-Sox9* mutant embryos. The lack of various eminences in the mutant indicates that the *Sox9*- and *Scx*-expressing progenitor cells are essential for their formation. Arrows indicate bone eminences: deltoid tuberosity (black arrows), great tuberosity (orange arrows), olecranon (purple arrows), calcaneal tuberosity (blue arrows), medial condyle of the tibia (green arrows). (**C**) *In situ* hybridization analysis of sagittal humerus sections from E14.5 control and *Scx-Sox9* mutants using antisense complementary RNA probes for tendon markers scleraxis (*Scx*), tenomodulin (*Tnmd*) and collagen type I (*Col1a1*) mRNA indicates tendon formation in *Scx-Sox9* mutants. Arrows indicate tendon insertion into bone eminences: deltoid tuberosity (blue arrows) and olecranon (orange arrows).

for specification of bone eminence progenitors. Moreover, the formation of a primary cartilaginous template in the absence of TGFβ signaling provides another indication that bone eminences are derived from a separate pool of progenitors, because the specification of one cell population requires TGFβ signaling whereas the other is refractory to it.

To establish further the role of TGFβ signaling in bone eminence formation, we examined *Prx1-Tgf-βRII* mutants at E18.5 by CT analysis. As can be seen in Fig. 8C-E, various eminences were completely lost in the mutants. These results clearly indicate the extensive effect of TGFβ signaling on eminence formation and, consequently, on attachment unit development.

DISCUSSION

Several studies have provided a histological and, to some extent, molecular description of the mature tendon-to-bone insertion site (Benjamin et al., 2002; Biewener et al., 1996; Thomopoulos et al., 2011; Thomopoulos et al., 2010). Nevertheless, the fundamental question of how an attachment unit is formed during musculoskeletal assembly has been largely neglected. In order to reveal the mechanisms by which the AU is established, we studied the development of its bony side, the bone eminence.

In this work, we reveal a unique pool of progenitors that form bone eminences. We show that two distinct pools of progenitor cells form the cartilaginous template of the long bone in a modular fashion. *Sox9*-positive progenitors form the primary, cylindrical structure of the cartilaginous anlage, whereas a previously unknown second pool of *Sox9*- and *Scx*-positive progenitors gives rise to bone eminences. Moreover, we show that these two pools are regulated separately, as TGFβ signaling is necessary for specification of bone eminence progenitors, but not of progenitors of the primary cartilage. Subsequently, the differentiation of the *Sox9*- and *Scx*-positive eminence progenitors to chondrocytes involves BMP signaling (Fig. 9).

Our finding that eminence-forming cells are not descendants of primary cartilage chondrocytes, but rather originate from a distinct pool of *Sox9*- and *Scx*-positive progenitors, is the first indication that bones form in a modular fashion. The early events that lead to the formation of this distinct pool of progenitors and the exact origin of the *Sox9*- and *Scx*-positive cells are still not understood.

Although later in development *Scx* expression is restricted to forming tendons (Schweitzer et al., 2001), previous works reported early and extensive expression of *Scx* in the limb bud mesenchyme (Asou et al., 2002; Cserjesi et al., 1995). Thus, it is possible that some of the limb mesenchymal cells that express both *Sox9* and *Scx* are the first progenitors of the bone eminence.

Intriguingly, the development of long bones progressively from two pools of progenitor cells bears a resemblance to the developmental modularity of the mammalian heart. During

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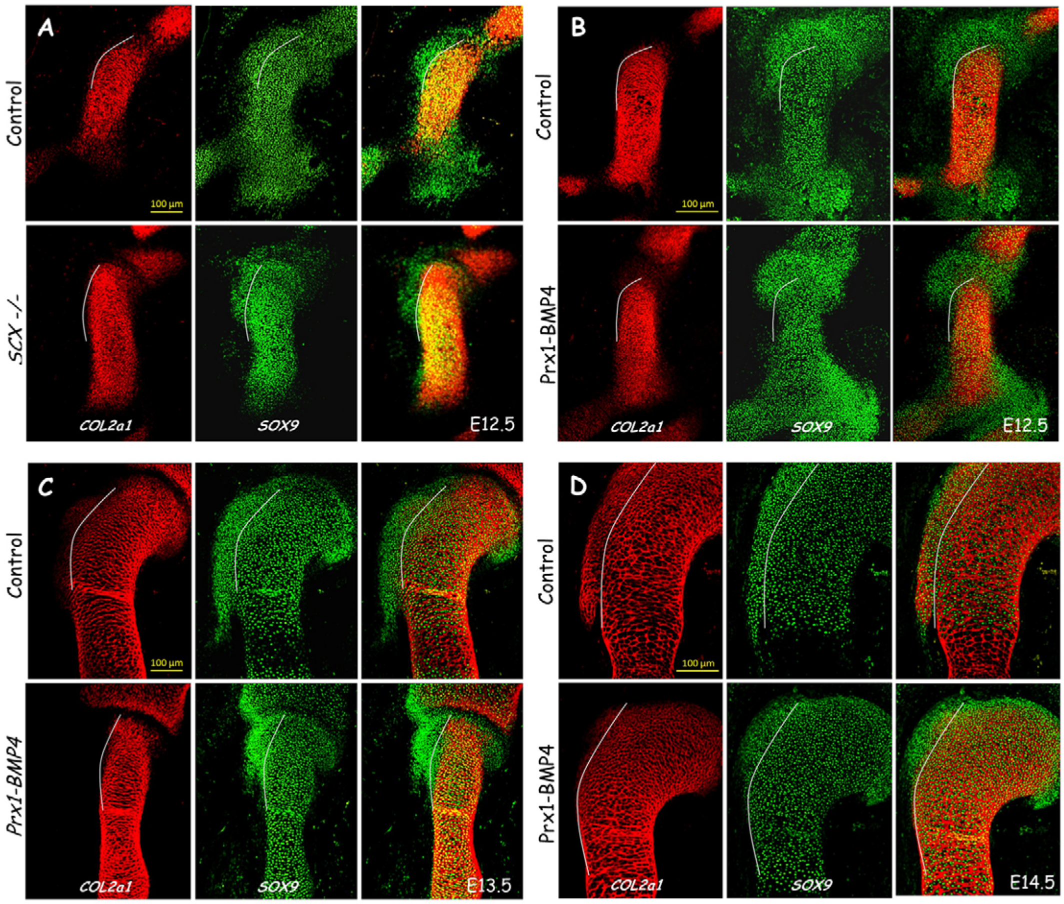

Fig. 7. SCX/BMP4 signaling regulates differentiation of eminence progenitors. (**A-D**) Immunofluorescence staining of humeral sections using anti-collagen II (COL2A1) and anti-SOX9 antibodies indicates the presence of eminence progenitors. (A) Sections from E12.5 control and *Scx*$^{-/-}$ mutants. (B-D) Sections from E12.5-E14.5 control and *Prx1-Bmp4* mutants. White lines mark the progenitor pool from which the deltoid tuberosity and the great tuberosity develop.

development, the heart is formed by cells from two distinct pools, known as the first and second heart fields. Initially, the heart tube is formed by cells of the first field. Then, cells that originate in the second heart field contribute to its elongation and to the formation of the outflow tract (Kelly et al., 2001; Mjaatvedt et al., 2001; Waldo et al., 2001). This similarity highlights the use of modularity in organogenesis in order to allow morphological and functional complexity.

Our finding that bone development is a modular process provides new perspectives on various aspects of musculoskeletal function and assembly. Functionally, different parts of the same bone are subjected to different mechanical loading conditions. For example, bone eminences are loaded in tension, whereas the joint surfaces experience compression. The finding that bones form modularly by two pools of progenitors may provide a mechanistic explanation for the ability of different anatomical regions of the bone to cope with different mechanical conditions. Developmentally, modularity can facilitate the assembly of the musculoskeletal system by coordinating interactions between the bone and the attaching tendon without interference with the construction of the entire bone. This notion underscores the evolutionary advantage of a modular strategy of bone morphogenesis, which may be easily manipulated by adding, removing or altering modules instead of by reshaping the whole structure.

Our discovery of a second pool of progenitor cells that form bone eminences suggests that a different regulatory mechanism controls specification and differentiation of these progenitors. Indeed, by blocking the expression of *Tgfbr2* in limb mesenchyme we demonstrate the central role of TGFβ signaling in regulating eminence progenitors, as this pathway is necessary exclusively for their specification. Previously, we showed that tendon cells are involved in the initiation of bone eminence formation (Blitz et al., 2009). The observation that *Sox9*- and *Scx*-positive eminence progenitors are already specified by E11.5 demonstrates that bone eminence development initiates at E11 rather than at E14, as we previously suggested (Blitz et al., 2009). Because TGFβ signaling is necessary for tendon formation (Pryce et al., 2009), one might argue that the absence of eminence progenitors was secondary to the loss of tendons. However, in *Prx1-Tgf-βRII* mutants, abnormal *Scx* expression and tendon development were only manifested at E12.5 (Pryce et al., 2009). Therefore, it is unlikely that arrested tendon formation was the reason for the loss of eminence progenitors in the absence of TGFβ signaling.

Finally, it is tempting to speculate that by controlling both tendon and bone eminence formation, TGFβ signaling is a key regulator of tendon-bone attachment unit. Indeed, it has been suggested that TGFβ coordinates cartilage and tendon differentiation during limb development (Lorda-Diez et al., 2009).

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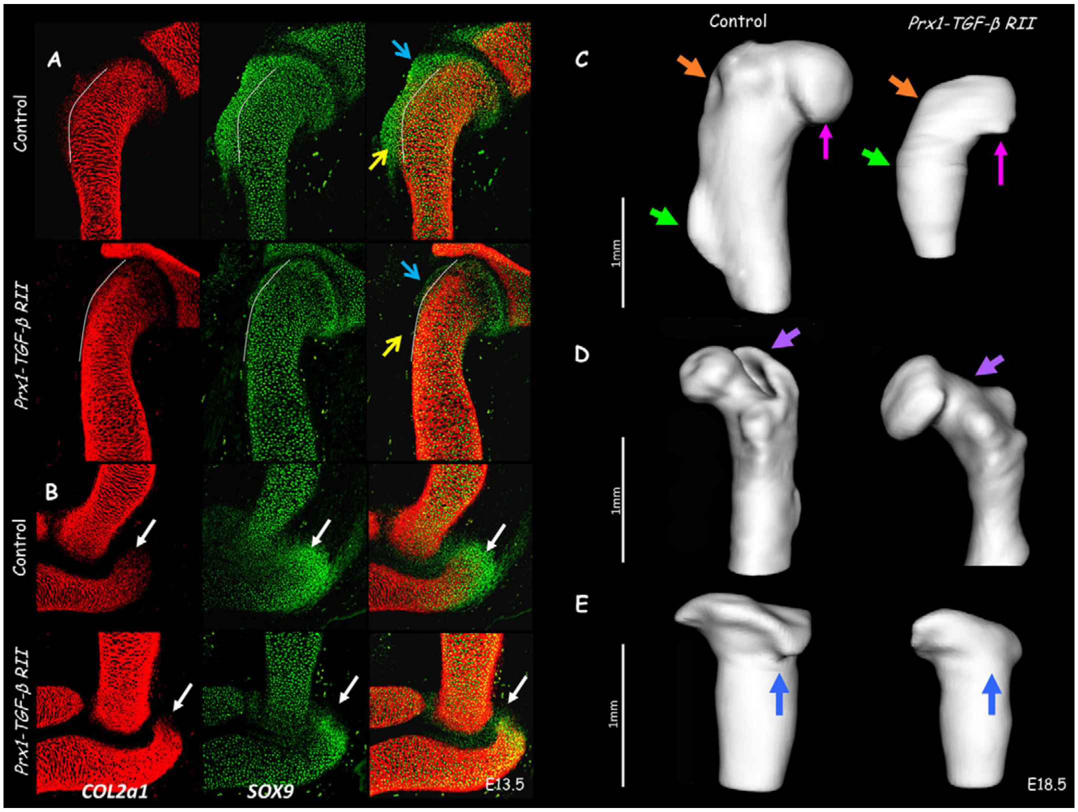

Fig. 8. TGFβ signaling regulates specification of bone eminence progenitors. (**A**,**B**) Immunofluorescence staining of humerus (A) and ulna (B) sections from E13.5 control embryos and *Prx1-Tgf-βRII* mutants, using anti-collagen II (COL2A1) and anti-SOX9 antibodies. White lines mark the field from which the deltoid tuberosity and the great tuberosity develop; blue arrows indicate the great tuberosity and yellow arrows indicate deltoid tuberosity; white arrows indicate the olecranon field. (**C-E**) Reconstructed CT images of forelimb and hindlimb bones from wild-type and *Prx1-Tgf-βRII* embryos at E18.5 show the morphology of bone eminences in the wild type and their absence in the mutants. (C) Deltoid tuberosity (green arrows), great tuberosity (orange arrows) and lesser tuberosity (pink arrows) of the humerus. (D) Greater trochanter of the femur (purple arrows). (E) Medial condyle of the tibia (blue arrows).

The mechanism that underlies the role of TGFβ signaling in specification of eminence progenitors is still unclear, especially in light of the broad expression of *Tgfbr2* and TGFβ ligands in the developing limb (Pryce et al., 2009). Nonetheless, our finding may help to resolve the ambiguity surrounding the role of this pathway in skeletogenesis. Previous *in vitro* studies suggested that TGFβs play a crucial role both in the induction of chondroprogenitors and in their differentiation to chondrocytes (Carrington and Reddi, 1990; Chimal-Monroy and Díaz de León, 1997; Kulyk et al., 1989; Leonard et al., 1991; Merino et al., 1998). However, ablation of TGFβ signaling in limb mesenchyme did not appear to affect these processes (Seo and Serra, 2007; Spagnoli et al., 2007). Our findings may imply that this pathway does regulate chondrogenesis, but its influence is limited to the secondary pools of progenitors that establish bone eminences.

Previously, we showed that the SCX/BMP4 pathway induces eminence formation (Blitz et al., 2009); however, we lacked understanding of the exact contribution of this pathway to the process. Here, we provide the missing information by showing that this pathway regulates differentiation of eminence progenitors. Another aspect of the involvement of SCX/BMP4 signaling is the possibility that its serves as a non-autonomous signal by which tendons regulate bone eminence development.

Our finding that eminence progenitors are *Scx* positive raises a new hypothesis that *Bmp4* expression might be regulated by *Scx*-positive cells within the AU, thus ruling out a non-autonomous role for tendons. Although our lineage experiment cannot provide a clear verdict in this matter, our expression analysis may offer an important clue. As mentioned, between E11.5 and E12.5 there is an overlap in the expression domains of *Sox9* and *Scx* in the forming AU; yet, by E13.5 clear spatial separation between the domains is visible, as *Scx* expression is restricted to the tendon. At that exact stage, SCX drives *Bmp4* expression in cells at the tip of the tendon (Blitz et al., 2009) and *Sox9*-positive cells undergo differentiation to chondrocytes. This suggests that in the forming tendon, SCX drives *Bmp4* expression to regulate non-autonomously the differentiation of *Sox9*-positive cells to *Sox9*- and *Col2a1*-positive chondrocytes, which form the bone eminence.

Finally, as TGFβ signaling was previously shown to regulate *Scx* expression (Murchison et al., 2007; Pryce et al., 2009), it is possible that TGFβ and BMP4 signaling together form a hierarchical mechanism that coordinates specification and differentiation of bone eminence progenitors.

In this study, we demonstrate that long bones develop modularly from two separate groups of progenitor cells. The first gives rise to the primary structure of the bone, whereas a previously unknown second pool establishes bone eminences. Expression of both *Sox9* and *Scx* by eminence progenitors provides a mechanistic basis for this modularity. The molecular mechanism that underlies the modular morphogenetic process of

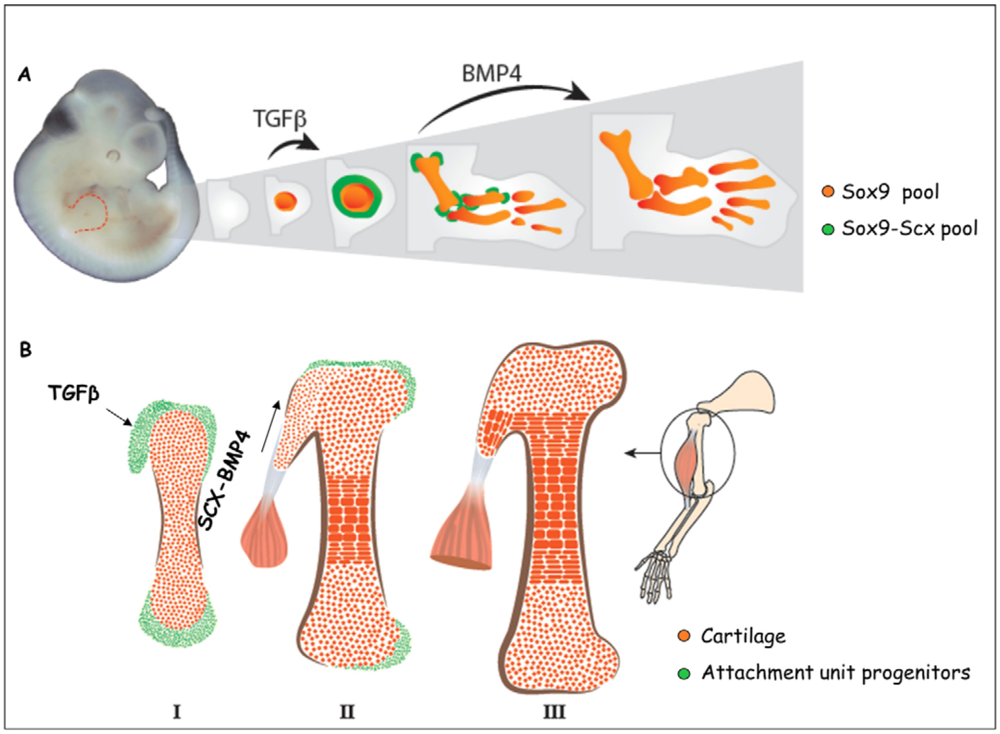

Fig. 9. A modular model for bone eminence development. (**A**,**B**) The formation of bone eminences is illustrated in the context of limb development (A) and of musculoskeletal development (B). Two distinct pools of progenitor cells form the cartilaginous template of the long bone in a modular fashion. The first pool of *Sox9*-positive progenitors forms the primary, cylindrical structure of the cartilaginous anlage. Then, a previously unknown second pool of *Sox9*- and *Scx*-positive progenitors forms the module of the bone eminence. TGFβ signaling controls the specification of eminence progenitors, whereas SCX/BMP4 signaling mediates their differentiation.

bone eminence formation involves TGFβ as well as BMP4 signaling. These findings shed new light on two central developmental processes, namely bone morphogenesis and musculoskeletal assembly

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Competing interests statement
The authors declare no competing financial interests.

Author contributions
E.B. conceived the study and jointly designed it with E.Z., conducted the experiments, analyzed and interpreted the data, and wrote the manuscript. A.S. conducted soft tissue micro-CT scans. H.A. provided the *Sox9-CreER* mice. E.Z. supervised the project, analyzed and interpreted the data, and wrote the manuscript.

Supplementary material
Supplementary material available online at
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