Hox11 genes establish synovial joint organization and phylogenetic characteristics in developing mouse zeugopod skeletal elements

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SUMMARY

Hox11 genes are essential for zeugopod skeletal element development but their roles in synovial joint formation remain largely unknown. Here, we show that the elbow and knee joints of mouse embryos lacking all Hox11 paralogous genes are specifically remodeled and reorganized. The proximal ends of developing mutant ulna and radius elements became morphologically similar and formed an anatomically distinct elbow joint. The mutant ulna lacked the olecranon that normally attaches to the triceps brachii muscle tendon and connects the humerus to the ulna. In its place, an ulnar patella-like element developed that expressed lubricin on its ventral side facing the joint and was connected to the triceps muscle tendon. In mutant knees, both tibia and fibula fully articulated with an enlarged femoral epiphyseal end that accommodated both elements, and the neo-tripartite knee joint was enclosed in a single synovial cavity and displayed an additional anterior ligament. The mutant joints also exhibited a different organization of the superficial zone of articular cartilage that normally exerts an anti-friction function. In conclusion, Hox11 genes co-regulate and coordinate the development of zeugopod skeletal elements and adjacent elbow and knee joints, and dictate joint identity, morphogenesis and anatomical and functional organization. Notably, the ulnar patella and tripartite knee joints in the mouse mutants actually characterize several lower vertebrates, including certain reptiles and amphibians. The re-emergence of such anatomical structures suggests that their genetic blueprint is still present in the mouse genome but is normally modified to the needs of the mammalian joint-formation program by distinct Hox11 function.

KEY WORDS: Hox genes, Synovial joint formation, Elbow and knee joints, Limb skeletogenesis, Mouse

INTRODUCTION

Synovial joints display distinct morphologies and organizations but the mechanisms by which they acquire such anatomical and functional characteristics are largely unknown (Archer et al., 2003; Pacifici et al., 2005; Pitsillides and Ashhurst, 2008). In the embryonic limb, joint formation initiates with the appearance of the so-called interzone that comprises flat and tightly packed mesenchymal cells and demarcates the boundary between adjacent cartilaginous skeletal anlage (Holder, 1977; Mitrovic, 1978). The interzone is required for joint formation (Holder, 1977), but its specific roles have long remained unclear. To tackle this key issue, we carried out genetic cell tagging and tracking studies using Gf3-Cre mice (Rountree et al., 2004) mated with RosaR26R mice (Soriano, 1999). We found that the lacZ-positive interzone cells gave rise to most if not all joint tissues, including articular cartilage layers, synovial lining and intra-joint ligaments, signifying that the interzone cells represent a specialized cohort of progenitor cells exclusively devoted to joint formation (Koyama et al., 2007a; Koyama et al., 2008). Other important studies showed that the Wnt/β-catenin signaling pathway maintains the function of interzone cells, cooperates with growth-plate-derived indian hedgehog and is essential for joint formation (Guo et al., 2004; Mak et al., 2006; Spater et al., 2006). Additional studies indicated that TGFβ signaling and TGFβ-versican interplays are needed for initiation of joint formation (Choocheep et al., 2010; Spagnoli et al., 2007) and that mechanical stimulation is required as well (Kahn et al., 2009; Pitsillides, 2006; Pitsillides et al., 1995). Despite these important advances on multiple fronts, other fundamental aspects of joint formation remain unclear and in particular, how joints acquire their diverse morphologies and organization.

Hox genes encode conserved homeodomain-containing nuclear proteins that regulate body plan, axis formation and organogenesis. Mammals have 39 Hox genes residing in four separate linkage clusters, termed A, B, C and D, that are subdivided into 13 paralogous groups each composed of 2-4 members. Hox9 through Hox13 group genes are specifically expressed during limb development, where they exert fundamental roles in patterning and growth of skeletal elements (Zakany and Duboule, 2007). In particularly striking examples, double-mutant mice lacking Hoxa11 and Hoxd11 were found to exhibit a significant retardation in radius and ulna development (Boulet and Capecchi, 2003; Davis et al., 1995), and triple mutants lacking all three Hox11 paralogous genes (Hoxa11, Hoxc11 and Hoxd11) displayed similar alterations in tibia and fibula development, whereas neighboring elements were largely normal (Wellik and Capecchi, 2003). Although seminal and pathbreaking in many respects, these and other studies did not examine whether or how Hox genes regulate synovial joint formation and what specific aspects of this process might be under Hox jurisdiction (Kmita et al., 2005; Yokouchi et al., 1991). The present study was carried out to tackle this key issue.

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MATERIALS AND METHODS

Mouse mutants and skeletal analysis

Mouse embryo mutants were generated by in vitro fertilization followed by transfer to foster mothers (Wellik and Capecchi, 2003), and all animal protocols were approved by the IACUC. Double Alizarin Red-Alcian Blue skeletal staining was performed according to established methods.

In situ hybridization and imaging

In situ hybridization was carried out as described (Koyama et al., 2007b). Paraffin sections pretreated with 10 μg/ml proteinase K for 10 minutes were post-fixed in 4% paraformaldehyde, washed with PBS containing 2 mg/ml glycine and treated with 0.25% acetic anhydride in triethanolamine buffer. Sections were hybridized with antisense 35S-labeled riboprobes (approximately 1×10⁶ DPM/section) at 50°C for 16 hours, coated with Kodak NTB-3 emulsion diluted 1:1 with water and exposed for 10-14 days. Slides were developed with Kodak D-19 at 20°C and stained with Hematoxylin. Probes used were described previously (Koyama et al., 2002) (see Fig. S1D in the supplementary material).

RESULTS AND DISCUSSION

Triple mouse embryo mutants lacking all Hox11 genes and designated as Hox11aaccdd displayed short limbs and increased spinal curvature (see Fig. 1A in the supplementary material) and, interestingly, their limbs failed to undergo normal rotation (Coates et al., 2002) (see Fig. 1D in the supplementary material) compared with wild-type littermates (see Fig. S1A,B in the supplementary material). Alcian Blue and Alizarin Red staining showed that, as expected, embryonic day (E) 18.5 wild-type zeugopod elements exhibited typical morphologies and joint organization (Fig. 1A,D). The ulna (ul) was slightly wider than the radius (ra), and the normal tripartite elbow joint comprised direct articulation among the proximal portion of ulna containing a well-formed olecranon (ole) attached to the triceps brachii muscle tendon (Fig. 1C, asterisk), the proximal portion of radius and the distal portion of humerus (hu) (Fig. 1B,C). Theibia (ti) was far larger than the fibula (fi) (Fig. 1D); the normal bipartite knee joint involved direct articulation of the proximal tibia with distal femur (Fig. 1E) and was enclosed in a synovial cavity (Fig. 1F, opposing arrowheads), and the proximal portion of fibula articulated with the lateral condyle of the tibia (Fig. 1E, arrowhead).

Such stereotypic features were completely altered in E18.5 triple-Hox11-null embryos (4/4). Mutant zeugopod elements were hypomorphic and entirely cartilaginous (Fig. 1G-J, arrows), had lost their morphologic distinction in terms of length and width (Coates et al., 2002; Sears et al., 2007) and displayed elbow and knee joints with novel characteristics (Fig. 1H,K). In mutant elbows, the proximal articulating portions of ulna and radius resembled one another and the ulna lacked the olecranon (Fig. 1H) that, in addition to serving as the attachment site for the triceps brachii muscle tendon, is crucial for elbow extension and forward movement of forelimb and is absent or poorly developed in lower amniotes (Barnett and Lewis, 1958; Drapeau, 2004; Jenkins, 1973). A triceps brachii muscle tendon was still present (Fig. 1I, asterisk) but, strikingly, was connected to an ectopic skeletal element that closely resembled the ulnar patellar characteristic of elbow joints of certain reptiles, amphibians and birds (Barnett and Lewis, 1958; Haines, 1969; Maisano, 2002; Vickaryous and Olson, 2007) (Fig. 1I, single arrowhead). The ectopic patellar was attached to an apparently functional patellar tendon that reached the ulna metaphysis and resembled the tendon present in normal knees (Fig. 1I, double arrowheads). The ectopic patella could be observed as a
distinct and sesamoid-like anatomical structure in whole-mount skeletal preparations (Fig. 2E, arrowhead) and displayed gene expression patterns typical of a functional articular skeletal element, including lubricin expression on its ventral side facing the joint (Fig. 2G, arrowhead) and abundant collagen IIB expression throughout its core (Fig. 2H). The changes in mutant knees were as encompassing. The proximal portions of both tibia and fibula fully articulated with an enlarged distal end of the femur that was able to accommodate both elements, thus resulting in a neo-tripartite joint (Fig. 1K). Indeed, the joint was enclosed in a single synovial cavity (Fig. 1L, opposing arrowheads) and exhibited an anterior ligament bridging femur to fibula (see Fig. S2E in the supplementary material, arrow). These anatomical features are also characteristic of knee joints in certain lower vertebrates (Dye, 1987; Haines, 1942).

Next, we asked whether the absence of the olecranon in mutant ulna was due to involution over developmental time or absence of embryonic specification. Thus, we first closely analyzed olecranon development in wild-type embryos (n=30). At E12.0, the cartilaginous and collagen-IIB-expressing humerus was still directly connected to the ulnar and radial anlage and there was no overt sign of elbow joint formation (Fig. 3A,G, arrowheads). Approximately 12 hours later, the mesenchymal collagen-IIB-
negative interzone had formed (Fig. 3B,H, arrowheads) and an olecranon primordium was already appreciable as a direct outgrowth and extension of the proximal ulnar epiphysis (Fig. 3B,H, curved arrow). By E13.6, the olecranon appeared well-consolidated and circled the distal humeral epiphysis (Fig. 3C,I, curved arrow). Thus, we examined the crucial E12.5 stage in companion triple-\textit{Hox11}-null embryos (5/5) and found that a collagen-IIB-negative interzone was forming but there was no obvious sign of olecranon formation (Fig. 3E,K, arrowhead). The collagen-IIB-negative interzone was forming but there was no obvious sign of olecranon formation (Fig. 3E,K, arrowhead). The collagen-IIB-negative interzone had formed (Fig. 3B,H, arrowheads) and an olecranon primordium was already appreciable as a direct outgrowth and extension of the proximal ulnar epiphysis (Fig. 3B,H, curved arrow).

To analyze the roles of individual \textit{Hox11} paralogous genes, we compared the elbow and knee joints in five-allele \textit{Hox11} mutants (4-5 for each genotype) that carry a wild-type copy (designated as \textit{Hox11aacdd}, \textit{Hox11aaccdd} and \textit{Hox11accdDd}, respectively). Interestingly, elbow organization and ulnar olecranon development were largely restored in E18.5 \textit{Hox11aacdd} and \textit{Hox11aaccdd} mutants, but not \textit{Hox11aacdd} mutants (see Fig. S2B-C in the supplementary material) and knee organization was largely restored in \textit{Hox11aaccdd} and \textit{Hox11aacccdd} mutants, but not \textit{Hox11aaccdDd} mutants (see Fig. S2E-G in the supplementary material), suggesting that \textit{Hox11} gene function is not wholly redundant but includes forelimb- and hindlimb-specific joint formation roles.

Finally, we examined the superficial zone of articular tissue that establishes joint boundary and is essential for joint function and movement (Hunziker et al., 2007). In wild-type embryos, the superficial zone exhibited normal characteristics that included: flat and spindle-shaped cells oriented along the main axis of the articular perimeter; and low Safranin-O staining that reflects a physiologically low proteoglycan content compared with flanking epiphyseal cartilaginous tissue (Fig. 4A,B). In triple-\textit{Hox11}-null mutants, the superficial layer was ill-defined. Its cells were round and their matrix stained as strongly with Safranin-O as the adjacent epiphyseal cartilaginous tissue (Fig. 4D,E). Changes in epiphyseal definition were further demonstrated by the relatively low expression of \textit{Ucma} (Fig. 4F), a matrix gene product that is normally expressed very strongly in developing epiphyses (Tagariello et al., 2008) (Fig. 4C). We previously observed similar superficial zone changes in \textit{Hox11}-null wrist and ankle joints (Koyama et al., 2010). In addition, some of the joints were partially fused and partial joint fusions were previously reported for more global \textit{Hox} cluster deletions (Kmita et al., 2005). Thus, \textit{Hox11} genes are required for superficial zone formation in mammalian zeugopod elements, and other family members expressed in zeugopods, including \textit{Hox10} genes, cannot compensate for their function. Whether the changes in the superficial zone mirror native characteristics in lower vertebrate joints remains unclear.

Our data demonstrate that \textit{Hox11} genes co-regulate and coordinate zeugopod skeletal element development and the formation of adjacent synovial joints. In the absence of \textit{Hox11} function, the developing elbow and knee joints display novel anatomical characteristics and organization that include an ulnar patella in place of the olecranon and a single tripartite knee joint shared equally and fully by distal femur and proximal tibial and fibular ends. An essential feature of joints is that the opposing sides of each joint are reciprocally sculpted and interlocking and create functional biomechanical devices each perfectly suited for site-specific skeletal movement (Pacifici et al., 2005). Our data suggest that the opposing sides of mutant elbow and knee joints appear to undergo coordinated remodeling and produce adequate reciprocal morphologies most clearly seen in the enlarged distal femoral end that is able accommodate both tibial and fibular proximal ends. This suggests that joints are programmed to develop as single functional units and that the normal range of \textit{Hox11} action does not stop at the zeugopod-stylopod anatomical joint boundary.

Loss-of-function mutations in \textit{Hox} genes often lead to homeotic transformations in which certain anatomical structures lose their identity and assume the identity of more anterior structures (McIntyre et al., 2007). A case in point is the anterior homeotic transformation in \textit{Hox10} triple mutants in which ectopic ribs form in the lumbar and sacral regions of their spine (Welik and Capecchi, 1991).
A similar process could have occurred in our *Hox11* triple mutants such that the posterior ulna and fibula primordia would have undergone anterior homoeotic transformation into the radius and tibia, respectively, resulting in morphologically similar structures. However, this interpretation does not account for the presence in the *Hox11* triple-mutant joints of anatomical structures and organization typical of other and more ancestral species, including an ulnar patella and a tripartite knee joint (Fig. 5). What could these striking but typical of other and more ancestral species, including an ulnar patella, mean to be comprehensive and is used here to provide a phylogenetic representation and interpretation of the results. Note that the above scheme is not meant to be comprehensive and is used here to provide a phylogenetic representation and interpretation of the results. hu, humerus; ra, radius; ul, ulna; fe, femur; fi, fibula; ti, tibia; ole, olecranon; up, ulnar patella.

**Fig. 5. Schematic of possible phylogenetic links between wild-type and mutant joints.** This schematic depicts the general organization and morphologies of elbow and knee joints in representative animal groups and species (Barnett and Lewis, 1958; Drapeau, 2004; Dye, 1987; Fujiwara et al., 2010; Haines, 1969; Soren and Waugh, 1994) compared with the joints in triple-*Hox11*-null mouse mutants. Most mammals have a conspicuous olecranon that encircles the distal end of the humerus, whereas the indicated groups of amphibians, reptiles and avians have a markedly smaller or even absent olecranon but have an ulnar patella (up, red). Thus, the presence of an ulnar patella and the absence of a clear olecranon in the *Hox11* mutants suggest that their elbow joints have acquired characteristics of more-ancestral vertebrate groups. Absence of the olecranon and presence of an ulnar patella in certain species led to the suggestion that evolution of the olecranon might have involved fusion of the ulnar patella (a sesamoid) to the proximal end of the ulna (Barnett and Lewis, 1958), but this remains unclear (Haines, 1969). With regard to hind limbs, the tripartite knee joint in which fibula and tibia fully articulate with the femur within a single synovial cavity is characteristic of many reptiles and certain amphibians. Thus, the mutant mouse knee joint shares characteristics with those species. Note that the above scheme is not meant to be comprehensive and is used here to provide a phylogenetic representation and interpretation of the results. hu, humerus; ra, radius; ul, ulna; fe, femur; fi, fibula; ti, tibia; ole, olecranon; up, ulnar patella.

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**Competing interests statement**

The authors declare no competing financial interests.

**Supplementary material**

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**References**


