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Synovial Joint Formation during Mouse Limb Skeletogenesis: Roles of Indian Hedgehog Signaling

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Abstract

Indian hedgehog (*Ihh*) has been previously found to regulate synovial joint formation. To analyze mechanisms, we carried out morphological, molecular and cell fate map analyses of interzone and joint development in wild type and *Ihh*^{-/-} mouse embryo long bones. We found that *Ihh*^{-/-} cartilaginous digit anlagen remained fused and lacked interzones or mature joints, whereas wrist skeletal elements were not fused but their joints were morphologically abnormal. E14.5 and E17.5 wild type digit and ankle anlagen expressed hedgehog target genes including *Gli1* and *Gli2* and interzone-associated genes including *Gdf5*, *Erg* and *tenascin-C*, but expression of all these genes was barely detectable in mutant joints. For cell fate map analysis of joint progenitor cells, we mated *Gdf5-Cre*^{+/-}/*Rosa R26R*^{+/-} double transgenic mice with heterozygous *Ihh*^{+/-} mice and monitored reporter β -galactosidase activity and gene expression in triple-transgenic progeny. In control *Gdf5-Cre*^{+/-}/*R26R*^{+/-}/*Ihh*^{+/-} limbs, reporter-positive cells were present in developing interzones, articulating layers and synovial lining tissue and absent from underlying growth plates. In mutant *Gdf5-Cre*^{+/-}/*R26R*^{+/-}/*Ihh*^{-/-} specimens, reporter-positive cells were present also. However, the cells were mostly located around the prospective and uninterrupted digit joint sites and, interestingly, still expressed *Erg*, *tenascin-C* and *Gdf5*. Topographical analysis revealed that interzone and associated cells were not uniformly distributed, but were much more numerous ventrally. A similar topographical bias was seen for cavitation process and capsule primordia formation. In sum, *Ihh* is a critical and possibly direct regulator of joint development. In its absence, distribution and function of *Gdf5*-expressing interzone-associated cells are abnormal, but their patterning at prospective joint sites still occurs. The joint-forming functions of the cells appear to normally involve a previously unsuspected asymmetric distribution along the ventral-to-dorsal plane of the developing joint.

Keywords

synovial joint patterning; limb skeletogenesis; Indian hedgehog; interzone; joint progenitor cells; hedgehog signaling; Gli proteins; transcription factor Erg

INTRODUCTION

The biology and developmental biology of synovial joints continue to be the focus of intense research activity owing to the fact that joints are essential for skeletal function and quality of life and are susceptible to malfunction during natural aging and in congenital or acquired conditions including osteoarthritis. Classic embryological studies showed several years ago that the onset of limb skeletogenesis involves formation of an uninterrupted Y-shaped mesenchymal condensation made of a seemingly uniform population of cells.¹ The proximal arm of the Y-shaped condensation corresponds to the future femur or humerus and the two arms of the Y will give rise to tibia/fibula or radius/ulna, but the condensation initially displays no overt morphological sign of knee or elbow joint. Additional uninterrupted mesenchymal condensations appear in the autopod, are termed digital rays² and represent the primordia of tarsal/carpal and phalangeal elements. The first overt evidence of joint formation is the emergence of the interzone at each future joint location.³⁻⁵ The interzone consists of closely-associated and flat-shaped mesenchymal cells and provides a clear demarcation between adjacent cartilaginous elements. The interzone is widely believed to be essential for joint formation,³ but its specific roles are not fully clear.⁶ To address this and related issues, genetic cell fate mapping studies were recently carried out in which *Rosa R26R* reporter mice⁷ were mated with mice expressing Cre-recombinase in incipient joints under the control of growth and differentiation factor-5 (*Gdf5*) regulatory sequences.⁸ The data indicated that *Gdf5*-expressing and interzone-associated progenitor cells are likely to give rise to several joint tissues, including articular cartilage and synovial lining.

Indian hedgehog (*Ihh*) is widely recognized as a critical regulator of long bone development and growth. *Ihh* is expressed in the pre-hypertrophic zone of growth plate⁹⁻¹¹ and regulates a number of processes. We found that *Ihh* promotes osteogenic cell differentiation, leading us to first propose that one key role for *Ihh* is to induce intramembranous bone collar formation around the developing diaphysis.^{10, 12} Other studies indicated that *Ihh* regulates the rates of chondrocyte proliferation and maturation in concert with peri-articular derived parathyroid hormone-related protein (PTHrP).^{11, 13} Studies on *Ihh*^{-/-} mouse embryos provided further and more conclusive support for such multiple *Ihh* roles in long bone development.^{14, 15} Strikingly, these studies led to the realization that *Ihh* also regulates synovial joint formation.¹⁴ The autopod region of *Ihh*^{-/-} mouse embryos was found to contain uninterrupted cartilaginous digital rays lacking obvious joints, when in fact joints were forming in the digits of wild type littermates. More moderate defects involving partial joint fusion or other defects were observed in more proximal mutant joints such as elbow and knee. Despite the remarkable nature of these observations and their potentially fundamental implications, it remains largely obscure how *Ihh* regulates joint formation and what specific roles it actually has. The present study was carried out to tackle these interesting and fundamental issues, using a combination of gene expression analyses and genetic cell fate map approaches in wild type and *Ihh*^{-/-} mouse embryos.

RESULTS

Anatomical and gene expression studies

Whole mount inspection of mouse embryo limbs was used to further analyze whether development of interphalangeal joints is more affected by lack of hedgehog signaling than that of more proximal limb joints. Staining with alcian blue did show that the cartilaginous anlagen of E18.5 *Ihh*^{-/-} mouse embryo toes were uninterrupted, lacked overt interzones and stained uniformly with alcian blue (Fig. 1B). Instead, skeletal elements in neighboring mutant ankle were distinct and separated from each other, as revealed by presence of alcian blue-negative mesenchymal tissue (Fig. 1B, arrows). Standard joint formation processes were well under

way in wild type littermate toes and ankle that displayed obvious alcian blue-negative joint-associated tissues (Fig. 1A).

To examine hedgehog signaling, we determined the gene expression patterns of hedgehog target and effector genes by in situ hybridization. In early E14.5 wild type joints, interzones were apparent between adjacent cartilaginous anlagen and displayed clear levels of *Gli1* and *Gli3* transcripts (Fig. 2A, C, E, arrows). *Gli2* expression was not as prominent (Fig. 2D), expression of hedgehog receptor *Patched-1* demarcated the cartilaginous anlagen (Fig. 2B) and expression of hedgehog signaling receptor *Smoothened* was widespread (Fig. 2F). At E17.5 when the cartilaginous anlagen become physically separated, expression of *Gli1*, *Gli3* and *Patched-1* was still appreciable and seemed to be more prominent on the concave side of the developing joints (Fig. 2G, H, I, K, arrows). In E17.5 *Ihh*^{-/-} limbs, however, *Patched-1*, *Gli1* and *Gli2* transcripts were essentially undetectable (Fig. 2M, N-P); interestingly, *Gli3* and *Smoothened* transcripts were present, but largely occupied the mesenchymal tissues flanking the prospective and uninterrupted joint site (Fig. 2M, R, arrowheads).

To corroborate these latter findings, we examined expression of *Gdf5* and also *Erg* and *tenascin-C*. *Erg* is an *ets* transcription factor family member that we found to be associated with joint and articular cartilage formation and function,¹⁶⁻²⁰ and *tenascin-C* is a pericellular matrix protein abundant in developing and adult articular cartilage.^{16, 17, 21} In both E15.5 and E17.5 wild type digits, *Gdf5*, *Erg* and *tenascin-C* were all expressed in the interzones and associated articular layers (Fig. 3A-F). However, in *Ihh*^{-/-} digits, expression of the three genes was essentially restricted to the mesenchymal tissues flanking the prospective and uninterrupted joint sites (Fig. 3G-M, arrowheads), thus resembling the gene expression pattern of *Gli3* and *Smoothened*.

As pointed out in Fig. 1B, mesenchymal tissue separates the various skeletal elements in *Ihh*^{-/-} ankles and wrists. To clarify its nature, we processed control and mutant specimens for in situ hybridization. The tissue present in control E15.5 wrists was conspicuous and well-organized (Fig. 4F) and strongly expressed *Gdf5* and *Patched-1* (Fig. 4G-H). Instead, the tissue present in the *Ihh*^{-/-} wrists was thin and inconspicuous (Fig. 4M) and expressed barely detectable levels of *Patched-1* and *Gdf5* (Fig. 4N-O).

Genetic cell fate map analysis

Next, we carried out genetic cell fate map analysis of joint progenitor cells in wild type and *Ihh*^{-/-} littermates by mating *Gdf5-Cre*^{+/-}/*Rosa R26R*^{+/-} double transgenic mice with heterozygous *Ihh*^{+/-} mice and analyzing their triple-transgenic progenies. Resulting embryonic limbs were processed for detection of reporter β -galactosidase activity by whole mount staining and histochemistry.²² As shown in Fig. 4A, reporter activity was clearly prominent and restricted to the developing interphalangeal and wrist joints in control *Gdf5-Cre*^{+/-}/*R26R*^{+/-}/*Ihh*^{+/-} heterozygous embryos. Identical patterns were seen in *Ihh* wild type (*Gdf5-Cre*^{+/-}/*R26R*^{+/-}/*Ihh*^{+/+}) mice (not shown). Histochemical analysis showed that reporter-positive cells were present in interzone, associated articulating layers and developing lining tissue but were absent in underlying shaft and growth plate cartilage (Fig. 4B) in agreement with previous findings.⁸ A similar distribution of reporter-positive cells were seen in the wrists (Fig. 4F). When we examined mutant *Gdf5-Cre*^{+/-}/*R26R*^{+/-}/*Ihh*^{-/-} specimens by whole mount, we were surprised to observe a band of reporter-positive cells located at the prospective location of interphalangeal joints (Fig. 4I, arrow), a finding seemingly to indicate that digit joints were actually forming in the *Ihh*^{-/-} embryos. However, histochemical analysis revealed that the reporter positive cells were mostly located outside and around the prospective joint site and were largely absent from the uninterrupted cartilaginous tissue (Fig. 4J, arrow). A moderate number of reporter-positive cells characterized the mutant wrist joints (Fig. 4M).

To more clearly understand the topography of distribution of reporter-positive cells, E15.5 control and mutant triple-transgenic autopods were cross-sectioned that the dorso-ventral distribution of the cells could be appreciated and analyzed. Interestingly, we found that the reporter-positive cells were not distributed uniformly along such dorso-ventral plane, but appeared to be more numerous and concentrated in the ventral half in control joints (Fig. 4C-E, arrowheads). The ventral side displayed also conspicuous bilateral masses of mesenchymal cells (Fig. 4E, double arrowhead) and, interestingly, the cavitation process was well underway ventrally but not dorsally (Fig. 4E, arrow). The primordia of extensor (*et*) and flexor (*ft*) tendons were clearly visible at their characteristic dorsal and ventral locations (Fig. 4C-E). When we examined *Ihh*^{-/-} prospective digit joint sites, we found that the reporter-positive cells also appeared to be slightly more numerous on the ventral aspect of the uninterrupted site (Fig. 4K-L). However, there was no overt sign of cavitation or presence of ventral-lateral mesenchymal masses (Fig. 4K-L, arrow and double arrowhead, respectively).

DISCUSSION

Since it was discovered about 10 years ago and found to be expressed by pre-hypertrophic chondrocytes in the growth plate, *Ihh* has received a great deal of well-deserved attention. It can safely be said that *Ihh* is a true master regulator of limb skeletogenesis. Its expression in the pre-hypertrophic zone places it in a developmentally strategic position. We theorized long ago that the pre-hypertrophic zone is critical for the overall orchestration, coordination and temporo-spatial unfolding of the chondrocyte maturation program and long bone anlage development.²³ *Ihh* likely represents a key element in such pre-hypertrophic chondrocytes' function, and it is now well established and recognized that *Ihh* regulates central processes and functions in developing long bones that include intramembranous bone collar formation, chondrocyte proliferation and maturation rates, expression of PTHrP, and endochondral ossification.^{24, 25}

The data in our present study now provide further insights into the fact that *Ihh* is also important for synovial joint formation and that joint initiation and morphogenesis are disrupted in *Ihh*^{-/-} limbs. We find that wild type interzones and early joints express *Gli1* and *Gli3*, and *Patched-1* expression characterizes the contiguous cartilaginous tissue in line with previous studies.²⁶⁻²⁸ Given that these genes are mediators of hedgehog signaling, the findings indicate that hedgehog signaling is locally involved in joint formation. Indeed, *Gli1* and *Patched-1* are not expressed at appreciable levels in mutant *Ihh*^{-/-} autopods, and this is associated with, and possibly causally linked to, absence or defects in joint formation. It remains to be clarified whether *Ihh* action in joint formation is direct or indirect, the former implying that *Ihh* itself diffuses and reaches the joint-forming sites. *Ihh*, and hedgehog proteins in general, are known to be able to travel from their site of synthesis to far-away targets.^{29, 30} This task is rather demanding and difficult since hedgehog proteins contain C-terminal and N-terminal-bound cholesterol and palmitoylate tails and tend to stay close to their synthetic site.³¹ One diffusion and traveling strategy characterizing hedgehog proteins is the possible formation of micelles by which the hydrophobic tails would be buried on the inside and the more hydrophilic protein moiety would face the extracellular fluids.³² An additional strategy is likely provided by the fact that hedgehog proteins contain a heparin-binding domain, and interaction with heparan sulfate proteoglycans modulates their diffusion.³³ Previous work from our group and others has shown that growth plate chondrocytes express a number of heparan sulfate proteoglycans. Syndecan-3 and -4 are particularly evident in the proliferative zone^{34, 35} and syndecan-2 characterizes the perichondrium.³⁶ Given their binding characteristics and location, these macromolecules could attract *Ihh* and regulate its diffusion along specific spatial directions. Indeed, we and others have shown that *Ihh* diffuses away from its site of synthesis and is present in the proliferative zone and surrounding perichondrial tissue^{29, 30} and that defects in heparan sulfate proteoglycan synthesis or expression are accompanied by aberrant distribution of *Ihh*.

^{37, 38} It is thus possible that mechanisms such as these could allow *Ihh* to influence joint development.

Our cell fate tracking data using *Gdf5-Cre/Rosa R26R* mice now reveal that reporter-positive cells are present at prospective joint sites in mutant *Ihh*^{-/-} digits and are distributed outside of the uninterrupted cartilaginous tissue. Gene expression analysis shows that the cells express joint marker genes including *Erg*, *tenascin-C* and *Gdf5*. Thus, these cells exhibit interzone-like characteristics and may represent interzone precursors or full-fledged interzone cells, implying that the cells become developmentally determined and patterned even in the absence of overt joint formation. Why then would the cells be largely located around the periphery of the prospective mutant joint site? One possibility is that in the absence of *Ihh* signaling, the cells may not be able to participate in interzone formation because chondrocytes occupying the site do not undergo de-differentiation and maintain the skeletal anlage uninterrupted. If so, this would suggest that interzone formation may normally involve participation of peri-joint precursor cells migrating into the joint site under the auspices of hedgehog signaling, a possibility also raised in previous studies.^{39, 40}

Whatever the explanation, it is noteworthy that our data provide novel and unexpected insights into interzone and joint organization and functioning. We show that reporter-positive cells are not uniformly distributed across the dorso-ventral axis of developing control digit joints and display a gradient-like distribution, with more numerous cells present in the ventral half. Our data also show that the cavitation process appears to follow a similar ventral-to-dorsal pattern and is evident in the ventral side already at E15.5 but unappreciable on the dorsal side. Interestingly, there appears to be some ventral crowding of reporter-positive cells around prospective (uninterrupted) *Ihh*^{-/-} digit joint sites, suggesting that such distribution is independent of hedgehog signaling and overt joint formation. Several previous studies have focused on the important question of how the dorsal-ventral axis of limb development and symmetry is regulated, and factors such as *Wnt7a* and *Lmx1* proteins are critical players.⁴¹⁻⁴³ Thus, it is possible that interzone precursors and associated cells are patterned by similar mechanisms and attain the differential distribution revealed by our data. Why should the cells be differentially distributed along the ventral-to-dorsal axis? One obvious possibility is that this asymmetric distribution is linked to, and needed for, digit joint formation. The digits can only bend in one direction (toward the ventral side) and thus their joints must develop accordingly to permit and direct such uni-directional movement. Presence of more numerous reporter-positive cells in the ventral half, presence of conspicuous ventro-lateral cell masses, and the ventral-to-dorsal direction of the cavitation process may all be needed for appropriate formation, organization and functioning of digit joints.

As previously realized and reiterated here, developing limb joints are differentially sensitive to the absence of *Ihh*. Interphalangeal joints fail to form in *Ihh*^{-/-} embryos and the intervening cartilaginous tissue remains uninterrupted. Instead, the mutant wrist and ankle sites contain cartilaginous elements separated by mesenchymal tissue (Fig. 1) and, as shown previously, elbow and knee sites contain separated or partially separated cartilaginous elements.¹⁴ It has been known for a while that though limb synovial joints are comparable biomechanical instruments, their development and organization are responsive to, and directed by, somewhat distinct mechanisms. For instance, major defects are seen in interphalangeal joints in mice lacking *Gdf5*, whereas the more proximal joints are minimally affected.⁴⁴ Proximal joints are significantly affected only in double *Gdf5/Gdf6*-null mice,⁴⁵ strongly indicating that *Gdf5* and *Gdf6* have redundant function in development of proximal joints, whereas development of interphalangeal joints largely relies on *Gdf5*. It is possible then that the different limb joints have distinct compensatory mechanisms or redundant pathways that would render them differentially sensitive to *Ihh* deficiency. Clearly, much more work is needed to sort out this important issue. In any case, it is interesting to note that even though they are not fully ablated

by *Ihh* absence, the developing mutant wrist (and ankle) joints are profoundly mis-shaped and disorganized. The reciprocal concave-convex contours that characterize normal wrist elements and sculpt the two sides into functional and inter-locking instruments are absent in the mutants and both sides display a convex shape; in addition, the intervening mesenchymal tissue is poorly organized and thin (see Fig. 4M). Thus, *Ihh* signaling appears to be essential for joint morphogenesis, and this suggestion is reiterated by our observation that the opposing sides of wild type digit joints display small but appreciable differences in gene expression of hedgehog target and receptor genes (see Fig. 2G-L).

A major future task is to clarify how articular cartilage, ligaments, synovial lining and other differentiated joint tissues form during joint development and to what extent they do or do not derive from interzone cells. Based on matrilin-1 gene expression patterns and genetic tracking, it has been proposed recently that articular chondrocytes do not derive from interzone cells but are descendants of the original mesenchymal cell condensations.⁴⁶ Interzone cells would instead give rise to the other joint tissues such as synovial lining. Our *Gdf5-Cre/Rosa R26R*-based cell fate studies do not allow us to confirm or refute such possibility, given that both interzone cells and most-epiphyseal early chondrocytes express *Gdf5* and would become reporter-positive. In other studies, we have shown that *Erg* is likely to have an important role in establishing the stable permanent status of articular chondrocytes.²⁰ Important roles are also found to be played by TGF β signaling mechanisms.^{47, 48} These and related studies emphasize the fact that joint formation is very complex and requires a multitude of mechanisms regulating: joint site patterning and determination; interzone formation and joint morphogenesis; and formation of differentiated joint tissues. This multitude of mechanisms and processes needs to be coordinated and orchestrated spatio-temporally such that appropriate joints form at each specific location along the longitudinal axis of the limb. The challenge now is to figure out exactly how this feat is accomplished and whether disturbances in these fundamental mechanisms underlie congenital joint defects.

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FIGURE 1. Anatomical examination of E18.5 wild type and *Ihh*^{-/-} mouse embryo hindlimbs
 Specimens were stained with alcian blue to distinguish cartilaginous from non-cartilaginous structures. (A) Wild type specimen in which the alcian blue-positive phalangeal and ankle skeletal elements are clearly interrupted at prescribed locations by alcian blue-negative joints. (B) *Ihh*^{-/-} specimen in which the digit primordia are uniformly stained and uninterrupted and lack obvious joints. Arrows point to alcian blue-negative tissue surrounding the mutant ankle elements.

FIGURE 2. Gene expression patterns of hedgehog target and effector genes

(A-F) E14.5 wild type forelimb digit early joints in which nascent interzone cells clearly display *Gli1* and *Gli3* transcripts (arrow). *Patched-1* expression characterizes contiguous cartilaginous tissue (B), *Gli2* expression is low (D), and *Smoothened* (*Smo*) expression is widespread (F). (G-L) E17.5 wild type forelimb digit joints in which the cartilaginous elements are now well separated from each other. Note that transcripts for *Patched-1*, *Gli1* and *Gli3* seem to be slightly more abundant on the convex side of the developing joint (H, I, K, arrow). (M-R) E17.5 *Ihh*^{-/-} forelimb specimen from littermates in which joints are absent and prospective joint sites are uninterrupted. Note that *Patched-1*, *Gli2* and *Gli2* transcripts are essentially undetectable (M-P), while *Gli3* and *Smoothened* transcripts are present but flank the prospective joint site (Q-R, arrowhead).

FIGURE 3. Gene expression patterns of joint marker genes
(A–F) E15.5 and E17.5 wild type forelimb digit joints in which interzone and associated cells strongly express *Gdf5*, *Erg* and *tenascin-C (Tn)*. (G–M) E15.5 and E17.5 *Ihh*^{−/−} forelimb digits in which transcripts for *Gdf5*, *Erg* and *tenascin-C (Tn)* are present in tissue flanking the prospective joint sites (arrowheads).

FIGURE 4. Anatomical and histochemical analyses of *Gdf5-Cre/ROSA R26R* control and *Ihh*^{-/-} autopods

(A-E) Control E15.5 autopods showing obvious presence of reporter activity (blue color) at prescribed interphalangeal joint locations (A). Histochemical analysis (B) shows that reporter-positive cells represent interzone cells, adjacent most-epiphyseal chondrocytes and joint lining cells, but the cells are absent in underlying growth plate and shaft. Joint cross sections (C-E) corresponding to location I and II in panel (A) reveal that the ventral half of the joints contain more numerous reporter-positive cells (arrowhead), large ventro-lateral mesenchymal cell masses (double arrowhead) and ongoing cavitation process (arrow). (F-H) Sections of control E15.5 wrists reveal peri-cartilaginous distribution of reporter-positive cells and well-contoured and reciprocally-shaped developing joints (F) that express *Patched-1* and *Gdf5* (G-H). (I-L) E15.5 *Ihh*^{-/-} autopods showing surprising presence of reporter activity at prospective joint site (arrow in I), but histochemical analysis reveal that the reporter-positive cells are located around the uninterrupted site (arrow in J). Cross sections corresponding to location III in panel (I) show some crowding of reporter-positive cells in the ventral aspect of the uninterrupted joint site (arrowhead in L) and absence of mesenchymal masses (double arrowhead in L) and cavitation (arrow in L). (M-O) Sections of E15.5 *Ihh*^{-/-} wrists showing that there are few reporter-positive cells and that the prospective joints are completely mis-shaped and lack

reciprocal concave-convex contours (M) and express barely detectable levels of *Patched-1* and *Gdf5* (N-O).