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ABSTRACT

Primary cilia regulate several developmental processes and mediate hedgehog signaling. To study their roles in cranial base development, we created conditional mouse mutants deficient in *Polaris*, a critical primary cilium component, in cartilage. Mutant post-natal cranial bases were deformed, and their synchondrosis growth plates were disorganized. Expression of *Indian hedgehog*, *Patched-1*, *collagen X*, and *MMP-13* was reduced and accompanied by decreases in endochondral bone. Interestingly, there was excessive intramembranous ossification along the perichondrium, accompanied by excessive *Patched-1* expression, suggesting that *Ihh* distribution was wider and responsible for such excessive response. Indeed, expression of heparan sulfate proteoglycans (HS-PGs), normally involved in restricting hedgehog distribution, was barely detectable in mutant synchondroses. Analyses of the data provides further evidence for the essential roles of primary cilia and hedgehog signaling in cranial base development and chondrocyte maturation, and point to a close interdependence between cilia and HS-PGs to delimit targets of hedgehog action in synchondroses.

KEY WORDS: *Polaris*, primary cilium, cranial base synchondrosis, hedgehog signaling, heparan sulfate proteoglycans.

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Roles of the Primary Cilium Component *Polaris* in Synchondrosis Development

INTRODUCTION

Primary cilia are solitary, immotile, microtubule-based organelles that project from the surfaces of most cells, including chondrocytes (Scherft and Daems, 1967). Cilia are characterized by a system of motor and adaptor proteins that endows them with intraflagellar transport (IFT) and also sustains organelle assembly and growth (Singla and Reiter, 2006). Mutations in the structural and functional components of primary cilia can cause defects in major developmental processes, including limb patterning (Huangfu *et al.*, 2003; Zhang *et al.*, 2003). The findings reflect the fact that primary cilia and their IFT system have turned out to mediate biological signals and action elicited by hedgehog (Hh) proteins (Haycraft *et al.*, 2005; Huangfu and Anderson, 2005). Hh proteins act by binding to the cell-surface receptor Patched1 (Ptch1), and signals are transmitted by the receptor Smoothened via zinc-finger transcription factors Glis. Following hedgehog interaction with Patched-1, Smoothened is activated and translocates to the primary cilium, resulting in production of Gli activator forms that travel to the nucleus and induce expression of hedgehog target genes (Christensen and Ott, 2007). Loss of IFT function deranges Hh signaling at the level of Gli processing and causes developmental defects (Haycraft *et al.*, 2005; Huangfu and Anderson, 2005).

The cranial base and its synchondroses function as growth centers for the neurocranium. At early stages of embryogenesis, a cranial base primordium forms, consisting of three pairs of mesenchymal condensations that differentiate into cartilage and fuse to form the cranial base template (Thorogood, 1988). Primary ossification centers then emerge within this uninterrupted structure at specific times and locations, and the cartilaginous structures remaining between the ossification centers represent the synchondroses (Kjaer, 1990). Each synchondrosis is composed of mirror-image growth plates that share a central resting zone and contain zones of proliferating, pre-hypertrophic, and hypertrophic chondrocytes, followed by endochondral bone and marrow and flanked by intramembranous bone.

We showed previously that *Indian hedgehog* (*Ihh*) is expressed by pre-hypertrophic chondrocytes in synchondrosis growth plates, and *Ihh* itself is present not only in the pre-hypertrophic zone, but also in the preceding proliferating zone and the flanking perichondrium, where it regulates chondrocyte proliferation and intramembranous ossification. In *Ihh*-null embryos, however, synchondrosis chondrocytes underwent precocious maturation, and both intramembranous and endochondral ossification processes were deranged (Young *et al.*, 2006). In mouse mutants deficient in *Kif3a*, a component of kinesin-II motor protein complex required for cilium function, we found synchondrosis growth plate malfunction characterized by defective chondrocyte proliferation and maturation (Koyama *et al.*, 2007).

Figure 1. Cranial base organization, synchondrosis development, and ossification patterns are defective in *Polaris*-deficient mice. Skulls from P14 control (**A,B**) and *Polaris*-deficient (**F,G**) mice were analyzed by μ CT and are shown from a 'bird's eye' view. Boxed areas in **A** and **G** are shown at higher magnification in **B** and **H**, respectively. Intrasphenoidal (*is*), speno-occipital (*so*), intra-occipital (*io*) synchondroses, and basi-sphenoidal (*bs*) and basi-occipital (*bo*) bones are indicated in controls (**B**). *Polaris*-deficient cranial bases (**F**, double arrow) were shorter by 10-15% along the anteroposterior axis than those of control littermates (**A**, double arrow). Parasagittal H&E-stained sections from control (**C**) and *Polaris*-deficient (**H**) speno-occipital (*so*) synchondrosis; note that the cellular arrangement in the mutant synchondrosis (**H**) was significantly altered compared with controls (**C**). (**D, I**) Sphenio-occipital synchondrosis serial sections were processed for *in situ* hybridization analysis of bone markers (*Osterix*) in primary spongiosa and perichondrial bone. (**E, J**) Synchondrosis growth-plate sections were processed for immunodetection of acetylated tubulin. Scale bars: 0.5 cm in **A** for **A, F, G**; 0.2 cm in **B** for **B, G**; 150 μ m in **C** for **C-D, H-I**; and 15 μ m in **E** for **E and J**.

Analysis of the above data, though quite clear, left several questions unanswered. For example, *Kif3a* has roles not only in cilia but also in the cytoplasm, and thus some of the cranial base defects in *Kif3a*-mutant mice could theoretically be due to such additional *Kif3a* functions. To address this issue, we created cartilage-specific conditional mutants deficient in *Polaris*, a highly specific primary cilium structural component. These studies provided us with the opportunity to address mechanisms by which primary cilia participate in the control of *Ihh*'s physical and functional topography (both altered in the *Kif3a*-mutants above). Specifically, we focused on heparan sulfate proteoglycans (HS-PGs), which have been shown to regulate *Hh* distribution and short- and long-range action in lower organisms, including *Drosophila* (Takei et al., 2004) and are strongly suspected to have

similar roles in higher organisms (Kozziel et al., 2004; Koyama et al., 2007).

MATERIALS & METHODS

Generation of Conditional *Polaris*-deficient Mice

We created conditional mutant mice by mating *Polaris* floxed mice with *Col2a1-Cre* mice (Haycraft et al., 2007; Song et al., 2007). The genotyping of transgenic mice is described in the Appendix. The *Kif3a* conditional allele has been previously described (Lin et al., 2003), and both *Polaris*^{*fl/fl*} and *Kif3a*^{*fl/fl*} mouse lines were maintained on mixed backgrounds. Animal protocols were approved by the IACUC.

Micro-computed Tomography

Post-natal day 14 (P14) skulls from *Polaris*-deficient mice and control littermates were fixed in buffered 4% paraformaldehyde and were subjected to micro-computed tomography (μ CT) in a CT40 SCANCO Medical System (Southeastern, PA, USA), scanned at 45 kV of energy, 12 μ m scanning thickness, and medium resolution. Three-dimensional reconstructions were generated with the following parameters: filter width sigma = 0.8, support level = 1.0, and threshold = 173.

Immunohistochemistry and Gene Expression Analysis

For immunofluorescence staining, 10- μ m-thick frozen sections were incubated with 1:200 dilution of anti-acetylated α -tubulin monoclonal antibody (Clone 6-11B-1; Sigma, St. Louis, MO, USA), followed by biotinylated secondary antibodies and Cy3-conjugated streptavidin (Vector Laboratories, Burlingame, CA, USA) to yield signal. Sections were counterstained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) (Invitrogen, Carlsbad, CA, USA) to reveal nuclei. The control was slides without the primary antibody, followed by incubation with secondary antibodies and Cy3-conjugated streptavidin. Serial para-sagittal sections from *Polaris*-deficient and *Kif3a*-deficient mice and control littermates were placed on the same slides. Mouse cDNA clones used were described previously (Shibukawa et al., 2007) and in the Appendix.

RESULTS

Cranial Base and Synchondrosis Defects in Conditional *Polaris*-deficient Mice

To create mice deficient in *Polaris* in cartilage, we mated floxed *Polaris* mice with *Col2a1-Cre* mice, and skulls from resulting *Polaris*^{*fl/fl*};*Col2a1-Cre* mice (heretofore termed *Polaris*-deficient) and *Polaris*^{*fl/wt*};*Col2a1-Cre* and *Polaris*^{*fl/fl*} mice (heretofore termed controls) were processed for micro-computed tomography (μ CT). Control cranial bases displayed a typical elongated morphology along the anteroposterior axis, and their intrasphenoidal (*is*), speno-occipital (*so*), and intra-occipital (*io*) synchondroses were well-defined (Figs. 1A, 1B). In contrast, *Polaris*-deficient cranial bases (Fig. 1F, double arrow) from littermates were shorter by 10-15% along the anteroposterior axis and also wider laterally,

resulting in an overall round neurocranium (Fig. 1F). Mutant spondyloses displayed different levels of abnormalities, with complete fusion in intra-occipital spondyloses (Figs. 1F, 1G, arrows; see also Appendix Fig. 1) and partial fusion in speno-occipital and intersphenoidal spondyloses (Figs. 1F, 1G, arrowheads). The process of endochondral bone formation was severely altered and was associated with the virtual absence of primary spongiosa (Figs. 1H, 1I, arrow) compared with controls (Figs. 1C, 1D, arrow). However, there was excessive intramembranous ossification along the perichondrial border (Fig. 1H, arrowhead), and the basi-sphenoidal and basi-occipital bones exhibited imperfections and even holes (Fig. 1G). *Osterix* and *collagen 1* transcripts were detected in control osteoblasts in primary spongiosa and perichondrial intramembranous bone (Fig. 1D and Appendix Fig. 2, arrows and arrowheads, respectively), but were nearly absent in mutant primary spongiosa (Fig. 1I and Appendix Fig. 2, arrow) and were ectopically expressed along the entire perichondrial tissue (Fig. 1I and Appendix Fig. 2, arrowhead).

To verify that *Col2a1-Cre* had effectively acted on the floxed *Polaris* gene and led to a reduction in primary cilia, we processed control and mutant sections for immunohistochemistry, using antibodies against primary cilium-associated acetylated α -tubulin. Positively stained primary cilia were readily detectable on controls, but not *Polaris*-deficient chondrocytes (Figs. 1E, 1J, arrowheads).

Growth Plate Organization and Gene Expression Patterns

Closer analysis showed that control spondylosis growth plates were rather compact and contained narrow, but still appreciable, chondrocyte zones (Fig. 2A). Resting chondrocytes were ellipsoidal and formed pairs near the perichondrium (Fig. 2A, top inset). Proliferative chondrocytes were flat, and pre-hypertrophic and hypertrophic chondrocytes were round and enlarged. Transcripts of *Sox9*, *aggrecan*, and *type II collagen* were abundant in proliferating and pre-hypertrophic chondrocytes (Figs. 2A, 2B and Appendix Fig. 2), and *Collagen X* and *MMP13* transcripts were prominent in hypertrophic chondrocytes and post-hypertrophic chondrocytes near primary spongiosa, respectively (Figs. 2C, 2D).

In *Polaris*-deficient spondyloses, the resting zone was larger and disorganized, and displayed chondrocyte pairs and clusters scattered throughout the tissue (Fig. 2E, inset). The other zones were ill-defined, and the chondro-bone/marrow border was deformed (Fig. 2E). *Sox-9* and *aggrecan* were low and scattered (Appendix Fig. 2), strong *collagen II* expression was maintained, but was topographically deranged (Fig. 2F), and *collagen X* and *MMP-13* transcripts were quite low (Figs. 2G, 2H), implying that chondrocyte maturation and hypertrophy were severely delayed.

Chondrocyte Proliferation, Hedgehog Signaling, and Heparan Sulfate Proteoglycans

Primary cilia mediate hedgehog signaling (Haycraft *et al.*, 2005; Huangfu and Anderson, 2005), and *Ihh* in the growth plate regulates proliferation and ossification (Koyama *et al.*, 1996b; Lanske *et al.*, 1996; Nakamura *et al.*, 1997; St-Jacques *et al.*,

Figure 2. Growth plate organization was abnormal in *Polaris*-deficient spondyloses. (A,E) Parasagittal sections from P14 control and *Polaris*-deficient speno-occipital spondyloses were stained with H&E, and boxed areas in A and E are shown at higher magnification in insets. Note the formation of chondrocyte clusters in mutant spondyloses, while cell doublets are present at the periphery along the chondro-perichondrial border (A, top inset) and individual cells seen at the central zone in controls (A, bottom inset). Serial sections were processed for *in situ* hybridization analysis of: type II collagen (*Col II*) (B,F); type X collagen (*Col X*) (C,G); and matrix metalloproteinase 13 (*MMP13*) (D,H). Scale bars: 35 μ m in A for insets in A,E; 150 μ m in E for A-H.

1999). Thus, we investigated whether and how deficiencies in *Polaris* and primary cilia would affect these and related parameters in spondyloses. *Ihh* was expressed by pre-hypertrophic chondrocytes (Figs. 3A, 3B), while *Ptch-1* and the hedgehog target/mediator factor *PTHrP* were expressed in preceding resting and proliferative zones (Fig. 3C and Appendix Fig. 2). Proliferating chondrocytes were identified by the expression of *histone H4C* (Fig. 3D); proliferating cells were also visible in bone marrow. In *Polaris*-deficient spondylosis, *Ihh* was low, but still appreciable (Figs. 3G, 3H, arrowhead), and *Ptch-1*, *PTHrP*, and *H4C* transcripts were barely detectable (Figs. 3I, 3J and Appendix Fig. 2). Interestingly, *Ptch-1* was ectopically expressed all along the perichondrial tissue (Fig. 3I, arrow), where excessive intramembranous bone formation was taking place (see Figs. 1G-1I above).

Given that HS-PGs control short- and far-range diffusion of hedgehog proteins and are required for hedgehog signaling (Koyama *et al.*, 1996a; Bernfield *et al.*, 1999; Gritli-Linde *et al.*, 2001; Yin *et al.*, 2002), we hypothesized that HS-PG gene expression might be altered in mutant spondyloses. To test this, we first analyzed the expression of *Syndecan-3* and *Perlecan*. We selected these HS-PGs based on our previous work on *Syndecan-3* (Shimo *et al.*, 2004) and the severe growth plate phenotype and deranged *Ihh* gene expression previously described in *Perlecan*-null mice (Arikawa-Hirasawa *et al.*, 1999). Interestingly, *Syndecan-3* and *Perlecan* were co-expressed in proliferating chondrocytes (Figs. 3E, 3F) that are the target of

Figure 3. *Polaris*-deficient synchondroses display alterations in cellular organization and expression for Hedgehog-related and HS-PG genes. Serial sections of control (**A-F**) and *Polaris*-deficient (**G-L**) P14 spheno-occipital synchondroses were processed for safranin O-fast green (**A,G**) staining. *In situ* hybridization analysis shows that, in controls, *Ihh* was expressed in the pre-hypertrophic zone (**B**), while *Ptch1* (**C**), *Syndecan-3* (**E**), and *Perlecan* (**F**) were all prominent in proliferating *H4C*-positive (**D**) chondrocytes. In *Polaris*-deficient synchondroses, residual *Ihh* expression (**H**, arrowhead) was accompanied by a marked reduction in the expression of *Ptch-1* (**I**), *H4C* (**J**), *Syndecan-3* (**K**), and *Perlecan* (**L**) in chondrocytes, while there was ectopic *Ptch-1* expression all along the perichondrium (**I**, arrow). Scale bars: 150 μ m in **A** for **A-L**.

Ihh action reflected by strong *Ptch-1* expression (Fig. 3C). Strikingly, both *Syndecan-3* and *Perlecan* were minimally expressed in *Polaris*-deficient synchondroses (Figs. 3K, 3L).

For completeness, we directly compared *Polaris*-deficient synchondroses with *Kif3a*-deficient synchondroses (Appendix Fig. 3, Appendix Table). In general, *Kif3a*-deficient synchondroses were much more severely disorganized. There were only remnants of the growth plate, subdivided into peculiarly shaped regions of *collagen II*-expressing chondrocytes and surrounded in every spatial direction by bone-like and *Osteopontin*-expressing

Figure 4. Schematic of phenotypic consequences in *Polaris*-deficient synchondroses. (**A**) In controls, growth plate zonal organization and gene expression patterns were well-defined and typical. The strong expression of *Ihh* in the pre-hypertrophic zone is accompanied by equally strong and distinct expression of *Patched-1* (*Ptch1*), *Syndecan-3* (*Synd3*), and *Perlecan* in the proliferative zone. As discussed in the text, such restricted HS-PG expression would be instrumental in directing and delimiting *Ihh* action in the proliferative zone. (**B**) In *Polaris*-deficient synchondroses, the growth plates are entirely disorganized, lack typical zones, and display a seemingly enlarged and widespread resting zone made of chondrocyte doublets or clusters. The latter are present only at the chondro-perichondral border in controls (see **A**). The growth plate disorganization in mutants is accompanied by reduced *Ihh* expression and very low expression of *Patched-1* and HS-PGs. The latter may have caused wider distribution of residual *Ihh*, ectopic action on perichondrial tissue, and excessive intramembranous ossification. rz/pz/phz/hz, resting/proliferating/pre-hypertrophic/hypertrophic growth plate zones.

cells. The remnant cartilaginous tissues were composed of unusual chondrocyte pairs and clusters, with a significantly expanded inter-territorial matrix, that were post-mitotic. *Syndecan-3* and *Perlecan* expression was also reduced. For more insight into the nature of synchondrosis defects, see the wild-type and mutant specimens schematically depicted in Fig. 4.

DISCUSSION

This study provides clear evidence that conditional *Polaris* deficiency and the accompanying loss of primary cilia in chondrocytes are incompatible with normal neurocranial and bone development and normal synchondrosis function. Also, *Polaris* deficiency severely affects the synchondrosis growth plates that lack distinct growth-plate zones and characteristic gene expression patterns and fail to sustain a seamless transition from hypertrophic cartilage into endochondral bone and marrow. At the same time, mutant growth-plate chondrocytes appear to be unable to respond to residual *Ihh* expression, as indicated by the near-absence of *Patched-1* and *PTHrP* gene expression; this finding reaffirms the essential nature of primary cilia in hedgehog responsiveness. The

data greatly extend those from previous studies on conditional *Kif3a* mutants from this and other groups (Haycraft *et al.*, 2007; Koyama *et al.*, 2007; Song *et al.*, 2007). Our direct side-by-side comparison of *Polaris*- and *Kif3a*-deficient synchronoses at identical post-natal ages shows that the *Kif3a* phenotype is more severe than the *Polaris* phenotype. There are several possible explanations for such differences, including possible gene function redundancies or differential susceptibility of floxed genes to Cre action. A more plausible explanation is that the more severe phenotype of *Kif3a* mutant mice reflects this protein's involvement in multiple cellular mechanisms such as mitosis, in addition to its role in primary cilia (Cole *et al.*, 1998). *Kif3a* deficiency could thus have led to broader cellular disturbances.

While the *Polaris*-deficient growth-plate chondrocytes are unable to mature properly and secure a normal endochondral bone formation process, the perichondrial tissues flanking the synchronoses exhibit excessive intramembranous ossification. A clue to explain this phenomenon is our observation that perichondrial tissue adjacent to the *Polaris*-deficient growth plates exhibits excessive and ectopic *Patched-1* gene expression. Because *Patched-1* is a direct target of hedgehog signaling, its excessive expression must reflect an equally excessive presence and broad distribution of Ihh. It is thus plausible that residual amounts of Ihh produced by mutant chondrocytes may have diffused and migrated more broadly than normal, and may have reached the entire length of the perichondrium, triggering an equal length-wise response and ossification. As pointed out above, work in *Drosophila* originally revealed that HS-PGs are necessary for Hh movement away from its site of synthesis, and also for establishing the outer limits of its diffusion and action (Takei *et al.*, 2004). Our own work and that of another group suggested that HS-PGs would have equivalent roles in Ihh diffusion and action in chick and mouse skeletal elements (Koziel *et al.*, 2004; Shimo *et al.*, 2004). The data we present here add another element to the story and point to the possible roles of Syndecan-3 and Perlecan. By being strongly and specifically expressed in the proliferative zone, these HS-PGs could be well-positioned to attract Ihh away from the prehypertrophic zone where it is made, and could help Ihh to migrate toward, and act on, the proliferative zone; they would also establish its outer diffusion limit. The significant down-regulation of both HS-PGs in *Polaris*-deficient growth plates could thus have led to, or even caused, wider and abnormal Ihh diffusion and ectopic action.

An interesting outcome of the present study relates to the synchronosis resting zone. As analysis of our data now reveals, the distribution and configuration of resting chondrocytes in control synchronoses are specific, with cell doublets present at the very periphery along the chondro-perichondrial border and individual cells seen through the bulk of the zone. The cellular configuration and distribution are altered in both *Polaris*- and *Kif3a*-deficient synchronoses, with the appearance of numerous doublets or clusters of round non-proliferating chondrocytes occupying much of the very enlarged resting zone. A similar accumulation of immature chondrocytes was observed in our previous study on Wnt/ β -catenin-deficient cranial base synchronoses, whereas a sharp reduction and near absence of immature chondrocytes (and concomitant widespread maturation) were seen in the *Ihh*-deficient and constitutive active Wnt/ β -catenin pathway (Nagayama *et al.*,

2008). In long-bone growth plates, pacing of maturation rates is regulated by the Ihh-PTHrP loop (Lanske *et al.*, 1996; Tamamura *et al.*, 2005; Maeda *et al.*, 2007). Clearly, then, negative and positive regulators control the resting immature status of chondrocytes and their ingression and progression through maturation. Given recent evidence that cilia may regulate Wnt signaling in addition to Hh signaling (Cano *et al.*, 2004; Ross *et al.*, 2005), analysis of our *Polaris* and *Kif3a* data indicates that chondrocytes cannot obey either positive or negative regulatory cues in the absence of primary cilia. It is tempting to speculate also that chondrocyte clustering could have consequences on its own and could have contributed to the accumulation of immature cells. If so, primary cilia would also be needed by chondrocytes to establish physiologic cell arrangements and configurations, and such parameters could in turn influence the transition from resting to proliferating/maturing chondrocytes.

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