REVIEW

Patterning the limb before and after SHH signalling

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Abstract

The vertebrate limb is one of the most relevant experimental models for analysing cell–cell signalling during patterning of embryonic fields and organogenesis. Recently, the combination of molecular and genetic studies with experimental manipulation of developing limb buds has significantly advanced our understanding of the complex molecular interactions co-ordinating limb bud outgrowth and patterning. Some of these studies have shown that there is a need to revise some of the textbook views of vertebrate limb development. In this review, we discuss how signalling by the polarizing region is established and how limb bud morphogenesis is controlled by both long-range and signal relay mechanisms. We also discuss recent results showing that differential mesenchymal responsiveness to SHH signalling is established prior to its expression by the polarizing region.

Key words apical ectodermal ridge; ALX4; BMP; dHAND; GLI3; *Gremlin*; limb bud; SHH; ZPA.

Introduction: a history of the limb bud mesenchymal organizer called the polarizing region or ZPA

About half a century ago the seminal experiments by Saunders (1948) led to the discovery of two major signalling centres controlling vertebrate limb pattern formation. The first is a group of cells located in the posterior limb bud mesenchyme, the so-called polarizing region or zone of polarizing activity (ZPA, reviewed by Johnson & Tabin, 1997). The second signalling centre is a ridge of cells running along the distal margin of the limb bud ectoderm called the apical ectodermal ridge. When this is removed during the early development of a chicken limb bud, only proximal limb skeletal elements develop; removal at a later stage of development, on the other hand, ablates the more distal skeletal elements.

These studies showed that the apical ectodermal ridge is necessary to maintain limb bud morphogenesis, while apical ridge removal causes elimination of the underlying mesenchyme by apoptotic cell death (see review by Duboule, 2002). The polarizing region is a classical organizer as defined by Spemann & Mangold (2001; reprinted), which instructs limb bud mesenchymal cells with respect to their proliferation potential and fate. This was established by transplantation of polarizing region cells from the posterior mesenchyme to ectopic anterior locations, which induces complete mirror image duplications of the distal-most limb skeletal elements (digits, Saunders & Gasseling, 1968). In addition, it was shown that several other embryonic organizing centres such as the notochord and floor-plate also possess polarizing activity upon grafting to chicken limb buds. In trying to develop a molecular model of polarizing region signalling, Wolpert (1969) proposed that these cells produce a small diffusible molecule, termed morphogen. This morphogen would form a diffusion gradient across the limb bud, and the polarity and fate of digit precursor cells, for example, would be determined by their response to specific thresholds (Fig. 1A, also known as the 'French flag' model, see Wolpert, 1969). Wolpert's model provided a straightforward explanation for most, if not all, experimental manipulations of the polarizing region in chicken limb buds and the theoretical basis to search for this mysterious morphogen signal.

Tickle et al. (1982) achieved a new breakthrough by establishing that implantation of an inert bead soaked in retinoic acid into a chicken limb bud mimics transplants of polarizing region cells perfectly. Retinoic acid is indeed present in the limb bud mesenchyme (Thaller &
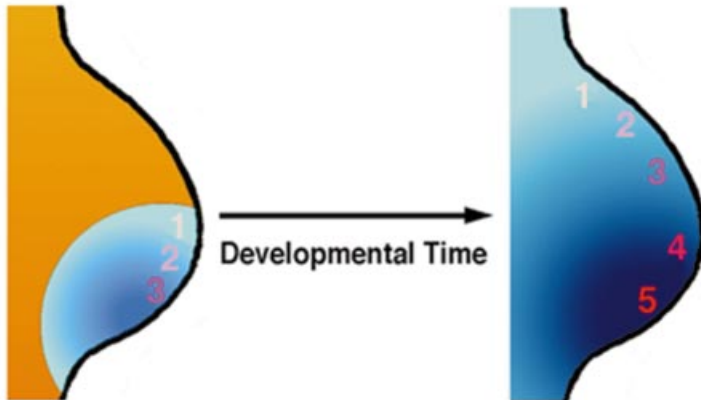
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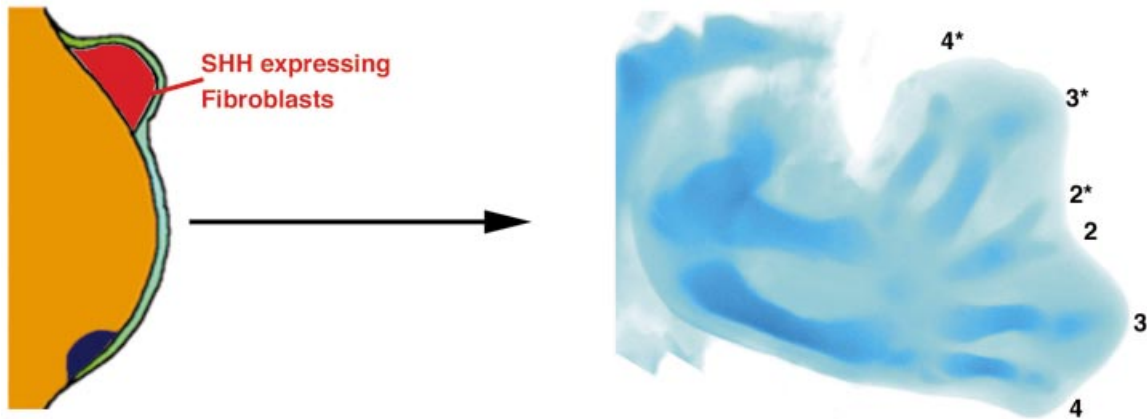
A Wolpert's Morphogen Hypothesis

Developmental Time

B *Shh* Expression

C SHH mediates Polarising Activity

SHH expressing Fibroblasts

D Loss of Distal A-P Patterning in *Shh* deficient Limbs

scapula

stylopod

zeugopod

autopod

Wt

Shh⁻ᐟ⁻
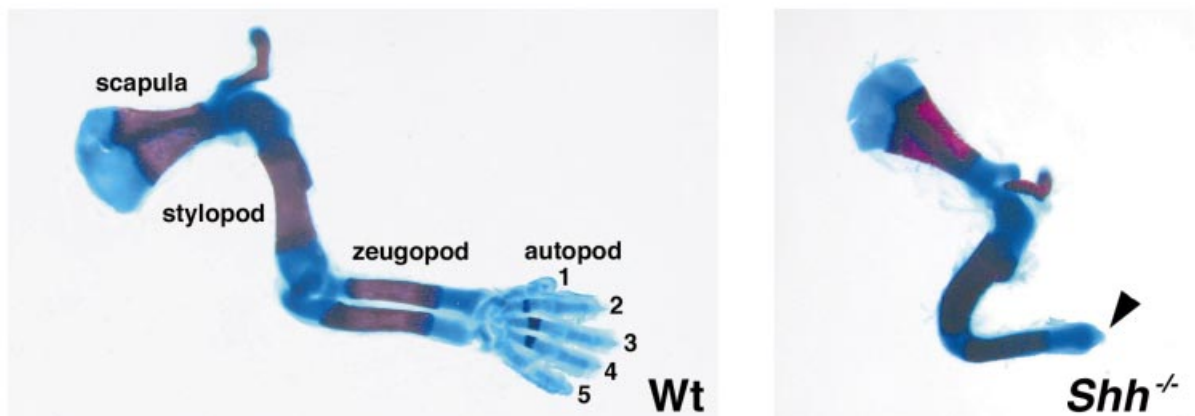
Eichele, 1987), but its specific production by polarizing region cells could not been shown (see also below). The continued search for Wolpert's morphogen led Tabin and colleagues (Riddle et al. 1993) to identify *Sonic hedgehog* (SHH), a homologue of the *Drosophila hedgehog* gene, as the signal expressed by the polarizing region in vertebrate limb buds (Fig. 1B). Furthermore, SHH expression in fibroblasts is sufficient to endow them with polarizing activity (as assessed by the induction of mirror image digit duplications; Fig. 1C). Inactivation of the *Shh* gene in the mouse results in a pleiotropic phenotype (for details see Chiang et al. 1996) including complete disruption of distal limb development and digit arch formation (Fig. 1D; Chiang et al. 2001; Kraus et al. 2001). Detailed genetic analysis provides evidence for long-range SHH signalling and threshold effects (see below), all in agreement with its proposed function as a morphogen. However, recent studies also showed that the SHH signal is relayed by activation of secondary signals in responding mesenchymal cells.

Furthermore, many regulator genes defined as SHH targets by experimental manipulation of chicken limb buds are in fact activated prior to and/or independent of SHH signalling (see below). In agreement, te Welscher et al. (2002a) provided evidence that the limb bud mesenchyme is not nascent at the time when it receives the SHH signal, but prepatterned by an upstream acting mechanism. These results show that SHH functions to maintain and modulate gene expression rather than acting as an inducer. Therefore, these recent insights are discussed in more detail in the following sections.

Current views on how the polarizing region is established

During the formation and patterning of the primary body axis, the positions of fore- and hindlimb fields are determined and polarized by molecular mechanisms that are still largely unknown. Hornbruch & Wolpert (1991), and more recently Tanaka et al. (2000), established that the potential to form limbs is initially spread throughout the flank. Prior to initiation of limb bud outgrowth, flank tissue possesses weak polarizing activity and the ability to activate *Shh* expression. During the initiation of limb bud outgrowth, polarizing activity is restricted to the posterior mesenchyme and up-regulated concurrent with activation of SHH signalling.

Molecular analysis of limbless-ness in snake embryos (Cohn & Tickle, 1999) and ectopic expression of *Hox* genes in mouse limb buds (Charité et al. 1994; Knezevic et al. 1997) indicates that the nested expression of *Hox* genes along the primary body axis may both position and polarize the limb field along its antero-posterior axis. In particular, anterior ectopic expression of the *Hoxb8* gene in forelimb buds results in establishment of an ectopic anterior SHH signalling centre and mirror image duplications of digits similar to polarizing region grafts (Charité et al. 1994).

However, loss-of-function analysis of this and many other *Hox* genes alone or in combination has to date not revealed any essential functions for the so-called *Hox* code in positioning and/or polarization of the limb field and polarizing region (see, e.g. van den Akker et al. 2001). In contrast, retinoic acid seems essential during these early stages as inactivation of RALDH2, an enzyme necessary for retinoic acid synthesis, completely disrupts limb bud formation and activation of SHH signalling (Niederreither et al. 1999).

Forelimb bud development of *Raldh2*-deficient mouse embryos can be rescued by administration of retinoic acid during limb field stages and early limb bud outgrowth (Niederreither et al. 2002). These studies establish that retinoic acid is critically required to induce SHH signalling during initiation of forelimb bud outgrowth. Niederreither et al. (2002) propose that retinoic acid and the bHLH transcription factor dHAND

Fig. 1 The polarizing region and SHH signalling. (A) Wolpert morphogen hypothesis (or French flag model): the posterior mesenchymal cells of the polarizing region produce a small diffusible molecule, which over time forms a gradient and patterns the mesenchyme according to threshold values. The hypothetical thresholds that pattern digits are indicated by numbers 1–5 according to the five digits of mouse and human limbs (1, most anterior digit – thumb; 5, most posterior digit – little finger). (B) *Shh* expression as revealed by whole mount *in situ* hybridization (purple colour) in a mouse forelimb bud of embryonic day 10.5. (C) Anterior grafts of fibroblasts expressing SHH (red in scheme) into an early chicken wing bud induces mirror-image duplications of the digit pattern. 2–4 indicate normal digits (2, most anterior; 4, most posterior), 2*–4* indicate duplicated digits. Note reversal of antero-posterior polarity in induced anterior ectopic digits. (D) Loss of distal limb structures and the digit arch in Shh$^{-/-}$ limb buds. Wt, Wild-type forelimb skeleton at embryonic day 15; stylopod, humerus; zeugopod, ulna and radius; autopod, metacarpals and digits. Shh$^{-/-}$, *Shh*-deficient mouse forelimb. Note: only one fused zeugopodal bone forms and the digit arch is completely absent, with the exception of a terminal phalange (arrowhead). In contrast, one complete digit forms in hind limbs (Chiang et al. 2001; Kraus et al. 2001). All limb buds are orientated with anterior to the top and posterior to the bottom.
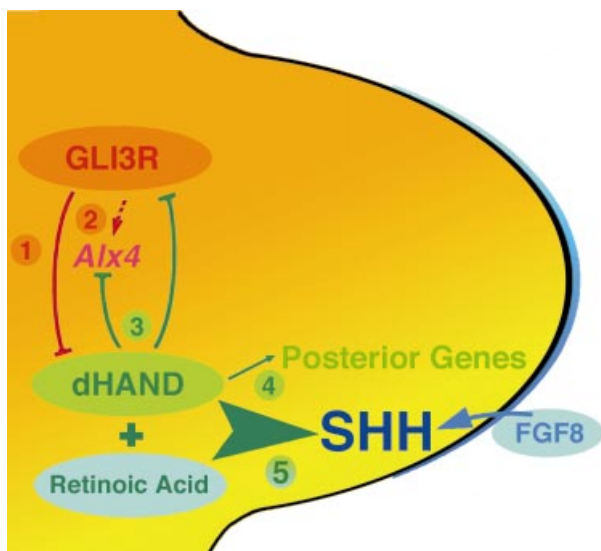
Fig. 2 Mutual antagonistic interaction of Gli3R and dHAND prepatterns the mesenchyme and controls establishment of the polarizing region. (1) GLI3R restricts *dHAND* expression to the posterior mesenchyme during initiation of limb bud outgrowth. (2) GLI3R acts upstream of other anterior genes such as ALX4 and is necessary for temporal up-regulation of *Alx4* expression. (3) dHAND in turn keeps *Gli3* and *Alx4* expression anteriorly restricted. (4) The genetic interaction of Gli3 with dHAND controls activation and spatial expression of posterior genes such as *5′Hoxd*, *Gremlin*, apical ectodermal ridge *Fgfs* (*Fgf4*, *–9*, *–17*). (5) Last but not least, dHAND interacts with retinoic acid (and FGF8 expressed by the apical ectodermal ridge) to establish SHH signalling and thereby the polarizing region in the posterior limb bud mesenchyme (scheme modified from te Welscher et al. 2002a).

together activate *Shh* expression in the posterior mesenchyme (Fig. 2). This proposal is based on the fact that inactivation of the *dHAND* gene in mice and zebrafish disrupts SHH activation and severely impairs paired appendage (limb/fin) bud development (Charite et al. 2000; Yelon et al. 2000). Ectopic expression of *dHAND* in limb buds in turn induces ectopic anterior *Shh* expression and digit polydactyly (Fernandez-Teran et al. 2000; McFadden et al. 2002). Interestingly, *dHAND* is initially expressed throughout the flank mesenchyme (reviewed by Cohn, 2000) as may be expected from the widespread competence to induce polarizing activity (Tanaka et al. 2000; see above).

During initiation of limb bud formation, *dHAND* expression is rapidly restricted to the posterior limb bud mesenchyme, which may be crucial to restrict polarizing activity to the posterior mesenchyme. Interestingly, expression of the *Gli3* transcriptional repressor (GLI3R, Wang et al. 2000) is activated in anterior mesenchyme concurrent with posterior restriction of *dHAND* (te

Welscher et al. 2002a). These authors showed that posterior restriction of *dHAND* is disrupted in *Gli3*-deficient mouse limb buds and further genetic analysis implicated the GLI3 repressor in positioning of the *Shh* expression domain (Zuniga & Zeller, 1999; see also below). In *Gli3*-deficient mouse limb buds, *dHAND* expression is maintained in the anterior mesenchyme and expression of 'posterior' genes is anteriorly expanded much prior to detecting ectopic anterior SHH signalling. This early loss of posterior restriction in *Gli3*-deficient limb buds (and not late ectopic anterior SHH signalling as previously assumed; Masuya et al. 1995; Buescher et al. 1997) is the most likely cause of the digit polydactyly with associated loss of identity (Zuniga & Zeller, 1999).

In *dHAND*-deficient mouse limb buds, expression of *Gli3* and another 'anterior' gene, *Alx4*, is conversely expanded posteriorly (te Welscher et al. 2002a). These studies establish that a mutual genetic antagonism between GLI3 and dHAND polarizes the nascent limb bud mesenchyme upstream of positioning and activating SHH signalling (Fig. 2).

This genetic interaction of GLI3 with dHAND uncovers essential components of the molecular mechanism, which positions the polarizing region at the posterior limb bud margin (Fig. 2). Previous work has also implicated FGF8 signalling from the apical ectodermal ridge in the activation of SHH signalling (Lewandoski et al. 2000). These results show that repression by anterior factors such as GLI3 in combination with retinoic acid and dHAND is crucial to polarize the limb bud and establish the polarizing region. In fact, the studies by te Welscher et al. (2002a) indicate that polarization of the early limb bud mesenchyme is triggered by GLI3-mediated anterior repression of *dHAND* expression (step 1, Fig. 2). Therefore, it will be important to identify the gene products regulating *Gli3* expression in the anterior limb field mesenchyme.

Many 'posterior' genes originally identified as transcriptional targets of SHH signalling are expressed considerably prior to *Shh* and their distribution is regulated by GLI3 repressor activity (Zuniga & Zeller, 1999; te Welscher et al. 2002a). In contrast to *Gli1* and *Patched* (*Ptc*), which are *bona vide* SHH targets, expression of mesenchymal SHH mediators such as, e.g. *5′Hoxd* genes, *Bmp2*, *Gremlin* and *Fgfs* in the posterior apical ectodermal ridge (see below), is activated independent of and prior to SHH signalling (Zuniga et al. 1999; Sun et al. 2000; Chiang et al. 2001; Panman et al.

unpublished results). In *Gli3*-deficient mouse limb buds, the expression of these genes is anteriorly expanded independent of ectopic SHH signalling (Zuniga & Zeller, 1999; te Welscher et al. 2002a,b). Therefore, it is likely that these genes are activated in response to retinoic acid and/or dHAND (see above) and excluded from anterior mesenchyme by GLI3-mediated repression. Taken together, these studies show that responsiveness to SHH signalling is established in the nascent mesenchyme by a prepatterning mechanism acting upstream of SHH signalling (Fig. 2). Rapid down-regulation of these genes in *Shh*-deficient limb buds (Zuniga et al. 1999; Sun et al. 2000; Chiang et al. 2001) shows that SHH signalling is essential to maintain and elaborate expression during progression of limb bud morphogenesis as discussed below.

Long- and short-range SHH signalling

Long-range

Analysis of SHH signalling in chicken limb buds provided clear evidence that SHH can act as a long-range morphogenetic signal and elicits a dose-dependent response as postulated by Wolpert's morphogen hypothesis (Yang et al. 1997). However, biochemical studies showed that the full-length SHH protein undergoes auto-proteolytic cleavage yielding a C-terminal peptide and the active N-terminal SHH peptide, which is covalently linked to cholesterol (Lee et al. 1994; Porter et al. 1995, 1996). Such cholesterol attachment results in retention of the active SHH peptide by plasma membranes, which poses an apparent paradox with respect to the proposed long-range signalling during limb bud development.

To shed light on this rather puzzling biochemical finding, Lewis et al. (2001) generated a mouse mutant able to express a modified SHH peptide that can no longer be cholesterol modified. Analysis of mouse embryos expressing this modified SHH peptide during limb bud development showed that formation of anterior digits is disrupted, while posterior digits are normal. Therefore, the cholesterol modification is essential for full-range activity of SHH signalling. Furthermore, analysis of target gene expression and protein distribution shows that the signalling range of the mutant, non-cholesterated SHH peptide, but not polarizing activity, is more restricted in comparison with the wild-type protein (Lewis et al. 2001).

Analysis of *Drosophila Hedgehog* (*Hh*) signalling showed that the *Dispatched* gene product is required to release cholesterol-bound *Hh* protein from cells to enable long-range signalling (Burke et al. 1999). In the absence of *Dispatched*, *Hh* protein accumulates in cells, suggesting that *Dispatched* is crucial for release and long-range signalling activity of *Hh* proteins (Burke et al. 1999). Therefore, it will be important to analyse genetically the potential requirement of vertebrate *Dispatched* homologues for long-range SHH signalling in mouse limb buds (Lewis et al. 2001).

Furthermore, Zeng et al. (2001) have shed light on a possible cellular mechanism that permits SHH to act in the long range. Experimental evidence reveals the existence of a multimeric, cholesterol-modified SHH peptide that is freely diffusible and seems to form a gradient across the limb bud. These authors propose that multiple cholesterol-modified SHH peptides accumulate in lipid rafts to form a soluble and freely diffusible complex. Alternatively, cholesterated SHH peptides may traffic across the limb bud mesenchyme through cytonemes, which are long actin-based cellular processes extended by mesenchymal cells in response to FGF signalling (Ramírez-Weber & Kornberg, 1999; see Fig. 3A). While it is clear that long-range SHH signalling is necessary during limb bud development, the underlying molecular and cellular mechanisms enabling such morphogenetic signalling remain in need of further investigation.

Short-range

Several mechanisms limit the range of Shh signalling during limb bud development to prevent ectopic activation of *Shh* target genes. The most direct of these mechanisms involves transcriptional up-regulation of the SHH receptor PTC in response to SHH signalling (Goodrich et al. 1996). In the absence of SHH signalling, PTC inhibits the *trans*-membrane protein *Smoothened* (SMO) and thereby signal transduction and transcriptional activation of *Shh* target genes. One of these transcriptional targets is *Ptc* itself, which results in up-regulation of PTC activity in cells receiving the SHH signal. Genetic analysis in the mouse shows that SHH targets are ectopically activated in *Ptc1*-deficient embryos (Goodrich et al. 1997). Reduction of PTC1 levels in mouse limb buds also results in ectopic anterior *Shh* expression and digit polydactyly (Milenkovic et al. 1999). In addition, PTC1 is also able to sequester the

A Cytonemes
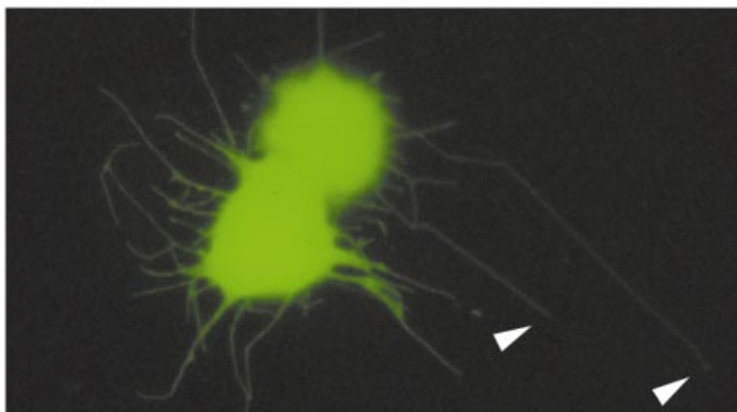
B SHH and BMP Pattern Digits
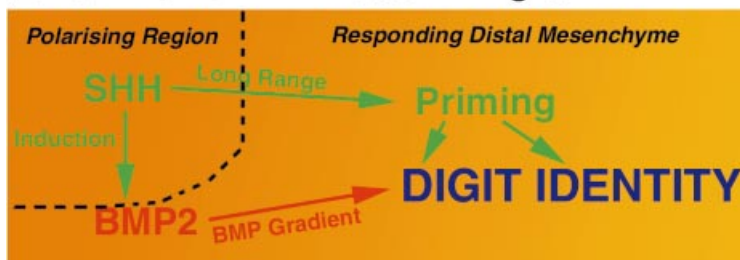
C SHH/FGF Feedback Loop

D SHH/GLI3R Interaction

Fig. 3 SHH signalling interactions in vertebrate limb buds. (A) Posterior mouse limb bud mesenchymal cells form long actin-based cellular processes, called cytonemes. GFP-expressing limb bud mesenchymal cells (green) were sandwiched with wild-type cells (non-fluorescent) and cultured for 1 h in the presence of 10 ng mL^{-1} FGF2 as described by Ramírez-Weber & Kornberg (1999). Arrowheads point to select cytonemes extending from two GPF-positive cells on a layer of non-fluorescent wild-type cells. (B) SHH and BMP interact to pattern digits. SHH signalling primes mesenchymal cells through long-range signalling and induces BMP2 in a fraction of posterior mesenchymal cells. Graded BMP signalling provides primed mesenchymal cells with positional identity (modified from Drossopoulou et al. 2000). (C) Establishment and maintenance of the SHH/FGF feedback loop. SHH up-regulates and maintains expression of the secreted BMP antagonist *Gremlin* in a *Formin*-dependent manner in posterior distal mesenchyme. Gremlin-mediated BMP antagonism enables expression of *Fgfs* such as *Fgf4*, *Fgf9* and *Fgf17* in the posterior apical ectodermal ridge. FGF signalling by the apical ridge in turn maintains and propagates SHH signalling by the polarizing region in the posterior–distal limb bud mesenchyme (modified from Zuniga et al. 1999). (D) Antagonistic interaction of SHH with GLI3. SHH signalling by the posterior polarizing region inhibits processing of the full-length GLI3 protein into an active repressor (GLI3R). Graded SHH signalling results in establishment of a GLI3R gradient with its highpoint in the anterior mesenchyme. GLI3R activity in turn participates in keeping *Shh* expression posteriorly restricted (modified from Wang et al. 2000).

cholesterol-free SHH peptide, as reduction of PTC1 levels in mouse limbs expressing only the non-cholesterated SHH peptide also expands the SHH signalling range anteriorly (Lewis et al. 2001).

In vertebrates, the SHH signalling range is further restrained by the Hedgehog-interacting protein, HIP (Chuang & McMahon, 1999). *Hip* expression is activated in limb bud mesenchymal cells in response to SHH signalling. The HIP protein generates an additional negative feedback loop by sequestering the ligand and thereby limiting the *Hedgehog* activity range (Chuang & McMahon, 1999).

Signal relay

A fraction of SHH responsive limb bud mesenchymal cells activate the secondary signal BMP2 (Fig. 3B, Drossopoulou et al. 2000). BMP2 is a vertebrate homologue of *Dpp*,

which is activated in *Drosophila* wing discs in response to *Hh* signalling (Basler & Struhl, 1994). However, BMP2 is not able to mimic all morphogenetic functions of SHH during vertebrate limb development. Ectopic expression of BMP2 in chicken limb buds only duplicates anterior-most digits, in contrast to the complete digit duplications induced by ectopic SHH signalling (Duprez et al. 1996). Although BMP2 does not have the full morphogenetic potential of SHH, an important role for BMP2 in determining digit identities has been proposed.

Experimental manipulation of chicken limb buds led Drossopoulou et al. (2000) to propose that mesenchymal cells are primed by long-range SHH signalling and that induction of *Bmp2* expression by SHH signalling results in graded BMP activity, which together with the priming effect of SHH specifies digit identity (Fig. 3B). Even during advanced limb development, when the digit primordia have long been determined, changes in interdigital BMP levels cause transformations of digit identities (Dahn & Fallon, 2000). This suggests that positional information is initially set by long-range SHH in combination with graded BMP signalling. However, identities of particular digits may only be specified much later under the influence of BMP signalling from the interdigital mesenchyme, just prior to the regression of this tissue by apoptosis.

SHH not only signals to the limb bud mesenchyme, but maintains and propagates its own expression through establishment of a positive feedback loop between polarizing region and apical ectodermal ridge (Fig. 3C; SHH/FGF feedback loop, reviewed by Capdevila & Izpisua Belmonte, 2001). Rather than acting long-range, the SHH signal is relayed to the apical ectodermal ridge through *Formin*-dependent activation of the BMP antagonist *Gremlin* in responding mesenchymal cells (Fig. 3C). *Gremlin*-mediated BMP antagonism enables expression of several *Fgfs* (*Fgf4, –9, –17*) in the posterior apical ectodermal ridge (Zuniga et al. 1999; L. Panman et al. unpublished). FGF signalling by the posterior apical ridge in turn maintains and propagates SHH signalling distally through the SHH/FGF4 feedback loop (Niswander et al. 1993; Laufer et al. 1994), thereby co-ordinating outgrowth with patterning.

The mouse *limb deformity* (*ld*) mutation disrupts the functioning of both *Formin* and *Gremlin*, which prevents establishment of the SHH/FGF feedback loop (Haramis et al. 1995; Zuniga et al. 1999). The *limbs* of *ld* mutant mice exhibit fusions of ulna and radius and digit syndactyly (reviewed by Zeller et al. 1999), indicat-

ing that antero-posterior patterning and to some extent outgrowth are affected.

Shh-deficient mouse limbs display a much more dramatic limb phenotype as only one zeugopod bone forms and the digit arch is absent (Chiang et al. 2001; Kraus et al. 2001; see also Fig. 1D). The phenotypic differences between *ld* and *Shh* mutant limbs indicate that SHH patterns the mesenchyme (Fig. 3B) largely independent of the SHH/FGF feedback loop (Fig. 3C).

GLI proteins and SHH signal transduction

In the *Drosophila* imaginal discs, nuclear response to *Hh* signalling is mediated by the *Cubitus interruptus* (*Ci*) transcription factor. In the absence of *Hh* signalling, the *Ci* protein is processed to generate a repressor of *Hh* target genes (Aza-Blanc et al. 1997). *Hh* signalling inhibits PKA-mediated *Ci* cleavage and the full-length *Ci* protein in turn acts as a transcriptional activator in the presence of *Hh* signalling (Methot & Basler, 1999). Several vertebrate *Ci* homologues, called GLI proteins, have been identified. In particular, *Gli1* is a direct target of SHH signalling (Lee et al. 1997) and has been suggested to mediate response to SHH signalling (Ruiz i Altaba, 1998). However, the lack of a phenotype in *Gli1*-deficient mice indicates that GLI1 alone is not sufficient to mediate nuclear response to SHH signal reception (Park et al. 2000). The neural tube phenotype of *Gli2*-deficient mouse embryos suggests that GLI2 mediates aspects of SHH signalling during ventral neural tube patterning (Ding et al. 1998; Matize et al. 1998). However, neither *Gli2*-deficient nor *Gli1*; *Gli2* double homozygous mouse embryos display striking limb phenotypes (Park et al. 2000), suggesting that another GLI protein regulates the expression of SHH target genes during limb bud morphogenesis. As discussed below, the GLI3 protein is a good candidate for regulating expression of genes in response to SHH signalling.

In the absence of SHH signalling, the GLI3 protein is constitutively processed to a repressor similar to *Ci* in *Drosophila* (Wang et al. 2000). SHH signalling inhibits GLI3 processing and experimental evidence indicates that SHH signalling in the limb bud generates an antero-posterior GLI3 protein repressor gradient with highest GLI3 repressor levels in the anterior mesenchyme (Fig. 3D; Wang et al. 2000). The balance between SHH-mediated activation and GLI3-mediated

repression of target genes might provide limb bud mesenchymal cells with the necessary positional cues to enable antero-posterior patterning. Indeed, analysis of *Shh*$^{-/-}$; *Gli3*$^{-/-}$ double mutant mouse embryos has revealed that antagonistic interactions between SHH and GLI3 control pattern formation. Despite the fact that the direct SHH transcriptional targets such as *Gli1* are not activated in double mutant embryos (Litingtung & Chiang, 2000; te Welscher et al. 2002b), expression of many 'posterior' genes (e.g. *5′Hoxd*, *Hoxa13*, *Gremlin*) and distal limb patterning are successively restored and anteriorly expanded by removing one or both *Gli3* alleles in the context of a *Shh*-deficient limb bud (te Welscher et al. 2002b). These results show that SHH normally functions to overcome GLI3-mediated repression and down-regulation of posterior genes to enable distal progression of limb morphogenesis. As limbs of *Gli3*$^{-/-}$, *Shh*$^{-/-}$ double mutant embryos are polydactylous and indistinguishable from *Gli3* single mutant limbs, these results confirm that GLI3 acts initially upstream of SHH. Subsequent balancing of graded SHH (and BMP, Fig. 3B; Drossopoulou et al. 2000) signalling with GLI3 repressor activity (Fig. 3D) enables co-ordinated progression of limb bud outgrowth and patterning to determine numbers and identity of digits.

In summary, SHH signalling prevents GLI3R formation in the posterior–distal mesenchyme (Fig. 3D) and thereby enables maintenance and propagation of the expression of posterior–distal genes (5′Hoxd, *Hoxa13*, *Gremlin*, etc.). Their expression is induced and positively regulated by factors acting upstream of SHH such as dHAND, most likely in combination with cellular response to retinoic acid (Fig. 2). Further research will be needed to shed light on the molecular cascades by which SHH inhibits processing of GLI3 to its active repressor form (GLI3R) and on the interactions by which GLI3R keeps SHH posteriorly restricted.

Acknowledgements

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