Interplay between the molecular signals that control vertebrate limb development

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ABSTRACT Vertebrate limbs display three obvious axes of asymmetry. These three axes are referred to as proximal-distal (Pr-D; shoulder to digit tips), anterior-posterior (A-P; thumb to little finger), and dorsal-ventral (D-V; back of hand to palm). At a molecular level, it is now possible to define the signals that control patterning of each of the three axes of the developing limb. These signals do not work in isolation though but rather their activity must be integrated such that the various limb elements are coordinately formed with relation to these three axes. This review will provide an overview of the intricate medley amongst the molecular signals that serve to establish and coordinate patterning information along the three primary axes of the limb.

KEY WORDS: *anterior-posterior, dorsal-ventral, FGF, limb patterning, proximal-distal, SHH, WNT*

Patterning along the three axes of the embryonic limb is regulated by three key organizing centers that produce the following primary signals. As reviewed by John Fallon in this journal issue, Pr-D growth is regulated by the apical ectodermal ridge (AER) which produces proteins of the Fibroblast Growth Factor (FGF) family that are secreted and act on the underlying limb mesenchyme. As described by Cheryll Tickle, A-P patterning is controlled by a population of cells in the posterior aspect of the limb mesenchyme that secrete the Sonic Hedgehog (SHH) protein. D-V patterning requires localization of the WNT7a signaling protein to the dorsal limb ectoderm via repression by the Engrailed-1 (En1) transcription factor which is localized to the ventral ectoderm (Loomis *et al.,* 1996; Parr and McMahon, 1995).

Integration of three-dimensional patterning occurs as a result of complex interplay amongst these three signaling centers. The organizing centers communicate with one another to position and refine the expression domains of these key signals. Through these interactions, growth and patterning are coordinated during limb development.

Coordination of the Signaling Centers

Ectodermal Signals (FGF and WNT7a) restrict the A-P (SHH) Organizer

One of the earliest gene markers of the presumptive AER is *Fgf8*. Experimental studies in the chick indicate that FGF signaling from the AER serves to induce the expression of *Shh* in the posterior-distal mesenchyme(see references in Martin, 1998). However, in mouse limbs lacking *Fgf8*, *Shh* is normally expressed

(Lewandowski *et al.,* 2000; Moon and Capecchi, 2000). At least three other *Fgf* genes are expressed in the AER (*Fgf4, Fgf9, Fgf17;* (Martin, 1998) and there may be functional redundancy of FGF signaling from the AER. FGF signals from the AER are also required to maintain *Shh* expression (Laufer *et al.,* 1994; Niswander *et al.,* 1994). Signals from the dorsal ectoderm also cooperate in the regulation of *Shh* expression. WNT7a signaling from the dorsal ectoderm is necessary for normal levels of *Shh* expression, thereby serving to restrict *Shh* to the dorsal mesenchyme (Parr and McMahon, 1995; Yang and Niswander, 1995). It is not known whether signaling from FGF8 and WNT7a to *Shh* is direct or indirect. Thus, ectodermal signals from the Pr-D organizer (FGF) and the D-V organizer (WNT7a) act to position the A-P organizer to the distal and dorsal limb mesenchyme. Later in this review will be described the genetic interactions that serve to restrict the position of the A-P organizer to the posterior aspect of the limb.

The A-P Organizer SHH controls Fgf4 Expression in the AER, via Regulation of BMP

In a reciprocal manner, SHH acts to limit the expression of another FGF family member, *Fgf4*, to the posterior aspect of the AER (Laufer *et al.,* 1994; Niswander *et al.,* 1994). However, the path from SHH to *Fgf4* is quite indirect. SHH acts via the Formin

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(limb deformity) gene product to positively regulate the expression of Gremlin, an antagonist of the Bone Morphogenetic Protein (BMP) signaling molecules. BMP in turn serves to repress expression of *Fgf4* in the AER (Capdevila *et al.,* 1999; Merino *et al.,* 1999; Pizette and Niswander, 1999; Zúñiga *et al.,* 1999). Thus, SHH activates an antagonist of a repressor of *Fgf4* expression to positively regulate AER signaling.

Relationship between D-V Patterning and AER Formation

Once the limb bud forms, D-V patterning is regulated by the limb ectoderm. This was first shown by experimental embryology studies in the chick where 180º rotation of the ectoderm relative to the mesenchyme resulted in a 180º inversion of the resulting mesenchymal structures (Geduspan and MacCabe, 1987; MacCabe *et al.,* 1974; Pautou, 1977). The key genes that regulate D-V patterning have now been identified by gene targeting in the mouse and by molecular experiments in the chick. D-V patterning is coordinated by the activity of EN1 in the ventral ectoderm which serves to restrict *Wnt7a* expression to the dorsal ectoderm (Logan *et al.,* 1997; Loomis *et al.,* 1996). WNT7a then activates the expression of the transcription factor *Lmx1b* in the dorsal mesenchyme and this is required for dorsal patterning (Chen *et al.,* 1998; Riddle *et al.,* 1995; Vogel *et al.,* 1995). Thus, these three genes are necessary for the establishment of D-V pattern.

The AER forms at the interface between dorsal and ventral limb ectoderm. Yet, the relationship between D-V patterning and AER induction is not absolute. Mouse embryos with loss of function mutations of En1 or Wnt7a (singly or in combination) display disrupted D-V patterning of the distal limb but AER induction is not affected (Cygan *et al.,* 1997; Loomis *et al.,* 1996; Loomis *et al.,* 1998; Parr and McMahon, 1995). En1 mutant mice have defects in AER morphogenesis (cells of the ventral AER do not compact towards the distal tip) yet, *Fgf8* expression is induced and Pr-D growth and patterning is relatively normal. This suggests that the interrelationship lies upstream of these D-V signals. This coordination appears to depend on BMP, and perhaps a different member of the Wnt ligand family.

Conditional gene targeting of the type I BMP receptor (BmpR-IA) in mice and molecular gain and loss of function studies in the chick demonstrate that BMP signaling is necessary and sufficient to regulate both D-V patterning and AER induction (Ahn *et al.,* 2001; Pizette *et al.,* 2001). BMP signaling within the ventral ectoderm positively controls *EN1* expression, hence serving to restrict *Wnt7a* to the dorsal ectoderm. Thus, BMP is upstream of EN1 in D-V patterning. BMP signaling is also involved in the induction of *Fgf8* expression in AER precursors. Moreover, BMP appears to act through a different set of transcription factors, members of the MSX family, to mediate AER induction (Pizette *et al.,* 2001). These studies indicate that D-V patterning and AER induction are coordinately regulated by BMP, and suggest that EN-1 and MSX function independently of one another downstream of BMPs to differentially mediate these two aspects of limb development.

Wnt signaling within the limb ectoderm is also implicated in AER formation. Molecular experiments in the chick limb suggest that Wnt3a signaling, through a β-catenin and Lef1-dependent pathway, is also necessary and sufficient to induce *Fgf8* expression (Kengaku *et al.,* 1998). Thus, Wnt and Bmp induce and most likely influence the position of the AER along the D-V interface.

The molecular interrelationship between the WNT and BMP pathways and how their activities converge during this process still remains to be determined.

Initiation of Limb Bud Formation: a Dance between WNT and FGF

Moving backwards in developmental time raises the questions of how budding of the limb is first initiated and what normally serves to restrict the positions of the limb buds along the rostral-caudal axis of the body. Molecular experiments in the chick suggest that an intricate dance between FGF and WNT signaling is involved in limb bud initiation (Kawakami *et al.,* 2001). A series of sequential signals are passed between WNT and FGF in a wave across the medial to lateral aspect of the body (somite, intermediate mesoderm, lateral plate mesoderm, ectoderm). In this dance the partners are exchanged while the overall melody remains the same.

In the presumptive forelimb region, Wnt2b becomes restricted along the rostral-caudal region to the intermediate and lateral plate mesoderm (Kawakami *et al.,* 2001). It is not yet known what genes are involved in defining the rostral-caudal domain of Wnt expression. Presumably axial patterning determinants are important, and these could include the Hox genes as mutation of Hoxb5 leads to a rostral shift of the forelimb field (Rancourt *et al.,* 1995).

Wnt2b, through a β-catenin-dependent pathway, appears to restrict the expression of *Fgf10* to the lateral plate mesenchyme of the limb field (Kawakami *et al.,* 2001). FGF10 is necessary for the induction of *Fgf8* in the AER. Limb formation fails in Fgf10-/- mice but interestingly, the initial budding of the limb appears normal (Min *et al.,* 1998; Sekine *et al.,* 1999). Further complexity in the dance between WNT and FGF is indicated by the results that FGF10 does not directly induce *Fgf8* but instead FGF10 acts to regulate another Wnt member, Wnt3a, in the ectoderm (Kawakami *et al.,* 2001). As outlined above, Wnt3a, perhaps in conjunction with BMP signaling, then serves to induce *Fgf8* expression.

There may then be a continuing dance between FGF10 and FGF8 as the limb continues to grow. Removal of the AER and replacement with FGF indicates that FGF signaling from the AER is needed to maintain *Fgf10* expression (Ohuchi *et al.,* 1997). It is not yet clear whether FGF10 in the mesenchyme is necessary after the AER has been established. Further roles for FGF10 could include the maintenance of FGF signaling in the AER or an independent role in the regulation of mesenchyme growth and patterning. It is also unclear whether WNT3a signaling plays a later role in maintenance of AER function. In contrast, the evidence suggests that BMP is not needed to maintain the AER and instead, after AER establishment, BMP negatively regulates the function of the AER by repressing *Fgf4* expression (Capdevila *et al.,* 1999; Merino *et al.,* 1999; Pizette and Niswander, 1999; Zúñiga *et al.,* 1999).

Mesenchymal Control of A-P Patterning: SHH-Dependent

As reviewed by Cheryll Tickle, SHH signaling is sufficient and necessary to regulate A-P patterning and growth of the intermediate (zeugopod) and distal (autopod) elements. Loss or gain of SHH signaling leads to a decrease or increase, respectively, of the number of elements along the A-P axis (Chiang *et al.,* 2001; Chiang *et al.,* 1996; Kraus *et al.,* 2001; Riddle *et al.,* 1993). For instance,

SHH protein can be applied to the anterior of the limb bud resulting in the formation of extra digits and these ectopic digits can adopt more posterior identity. Thus it is critical to tightly regulate the activity and the location of the SHH signal. One level of control lies within the SHH signal transduction pathway itself. There are a large number of modulators of SHH signaling, disruption of which leads to A-P patterning alterations. Many of these are negative regulators of the SHH signal transduction pathway (patched, Gli3, opb) (Eggenschwiler *et al.,* 2001; Hui and Joyner, 1993; Milenkovic *et al.,* 1999; Schimmang *et al.,* 1992). Moreover, pathway components such as Gli3 and patched serve to restrict *Shh* expression to the posterior of the limb bud as mice mutant for these genes are polydactylous and display an ectopic domain of *Shh* in the anterior of the limb bud (Masuya *et al.,* 1995; Milenkovic *et al.,* 1999). *opb* mutant limbs are also polydactylous (Günther *et al.,* 1994) and, although *Shh* is normally expressed, there is ectopic expression of the SHH target, patched, in the anterior of the limb (Eggenschwiler and Anderson, unpublished observations). In the chick *talpid* mutants, patched expression is expanded along the A-P axis of the distal limb bud while *Shh* is expressed in its normal domain. It is postulated that there is activation of the SHH signaling pathway in the absence of ligand leading to an increase in digit number and, in the *talpid* mutant limb, an apparent uniform distribution of positional identity (Caruccio *et al.,* 1999; Lewis *et al.,* 1999).

An additional level of refinement of SHH signaling appears to arise by restricting the signal in space and time. It has been proposed that there is a SHH autoregulatory loop in which SHH regulation of cell death in the posterior necrotic zone serves to modulate the domain and hence the level of SHH signaling (Sanz-Ezquerro and Tickle, 2000). SHH activity and/or range of signal is modulated by cholesterol modification, which occurs during processing to form the mature protein (Porter *et al.,* 1996). There also appears to be an intricate feedback and a relay system that provides temporal and spatial refinement. Experimental studies in the chick limb suggest the following model (Drossopoulou *et al.,* 2000). SHH first acts as a long range signal to prime the region for competence to form digits and to control digit number. SHH signaling is then limited by induction of, and binding to, its own receptor Patched, subsequently restricting SHH activity to a shorter range. SHH also acts to induce and maintain the expression of *Bmp2*. Subsequently, BMP acts on the primed cells to specify digit identity. Thus, A-P pattern is thought to be relayed from SHH to BMP.

It is clear that the AER and A-P organizer are tightly coupled (FGF induces and maintains *Shh;* SHH regulates *Fgf4*, and in *Shh-/-* limbs *Fgf8* and *Fgf4* expression is lost). However, SHH itself is not required for Pr-D patterning as in *Shh-/-* mouse limbs, elements representing all Pr-D levels are present (Chiang *et al.,* 2001; Kraus *et al.,* 2001).

Mesenchymal Control of A-P Patterning: SHH-Independent

It is not clear when A-P patterning is specified and whether this occurs at discrete intervals during Pr-D growth or continuously during limb development. Although SHH is necessary for normal limb development, there is a significant amount of A-P pre-pattern laid down prior to induction of *Shh* expression. Analysis of *Shh-/-* mutant mouse limbs indicates that A-P pattern of the proximal element, the stylopod (humerus/femur) is independent of SHH

(Chiang *et al.,* 2001; Kraus *et al.,* 2001). Moreover, there is asymmetric expression of genes, such as members of the *Hoxd* family in the mesenchyme and *Fgfs* in the AER, prior to, or in the absence of, SHH signaling (Chiang *et al.,* 2001; Grieshammer *et al.,* 1996; Kraus *et al.,* 2001; Noramly *et al.,* 1996; Ros *et al.,* 1996; Zúñiga and Zeller, 1999).

So what is this A-P pre-pattern and how is it established? The A-P pre-pattern appears to be generated at least in part through the localization of a set of transcription factors. Gli3 and Alx4, which act to repress the potential for polarizing activity, are expressed in the anterior of the limb field, whereas the basic helix-loop-helix gene product dHAND is expressed in the posterior of the limb field. These genes appear to act, prior to induction of *Shh* expression, to regulate the asymmetric expression of *Hoxd* members and other genes and to pattern the stylopod elements (Charité *et al.,* 2000; Fernandez-Teran *et al.,* 2000; Qu *et al.,* 1997; Takahashi *et al.,* 1998; Zúñiga and Zeller, 1999).

Mesenchymal Signals (Gli3, Alx, dHand) restrict the Position of the A-P Signaling Center

Gli3, Alx4, and dHand also act to position the domain of *Shh* expression to the posterior mesenchyme. The mouse mutants *extra toes (Gli3)* and *Strong's luxoid (Alx4)* were originally identified by their polydactylous (extra digit) phenotype. These mutant limbs display ectopic *Shh* expression in the anterior limb mesenchyme, indicating that they are required to restrict *Shh* expression to the posterior of the limb bud (Masuya *et al.,* 1995; Qu *et al.,* 1997; Takahashi *et al.,* 1998; Zúñiga and Zeller, 1999). Targeted mutagenesis and misexpression of dHand led to its identification as a positive regulator of *Shh* expression in posterior mesenchyme (Charité *et al.,* 2000; Fernandez-Teran *et al.,* 2000). Thus, genes that repress *Shh* are expressed anteriorly and genes that activate *Shh* are present posteriorly. Subsequently, it is thought that FGF8 signaling from the AER cooperates in the induction of *Shh* expression within the region of competence, the posterior mesenchyme (see Martin, 1998).

It is interesting to consider how SHH may influence skeletal patterning. One role of SHH signaling may be to regulate proliferation of the mesenchyme and/or the pattern of branching of the early skeletal condensations. It is intriguing that the proximal stylopod element, the patterning of which is SHH-independent, derives from an unbranched condensation. The transition to SHH-dependence appears to correlate with the transition to a branched condensation at the stylopod/zuegopod border. This model suggests a more direct link between the patterning signals and the emergence of the skeletal condensations.

Final Considerations

Lest one is left with the impression that all has been solved with regards to the fundamentals of limb development, it is important to raise the major unresolved question: how is the molecular interplay amongst these patterning signals interpreted such that limb elements of the proper shape and size are formed? There is a very large gap in our understanding of how the activity of *Shh, Fgf, Bmp,* and *Wnt* genes influences, for example, where the cartilage condensations will form, how the elements are sculpted, how the number of phalangeal elements are specified, and where

the tendon/muscle will insert. It is likely that some of these same sets of signaling molecules will be re-deployed to control these later aspects of limb development. It is already known that these families of signaling molecules are used multiple times during limb development. For example, BMP appears to regulate D-V patterning, AER formation, AER function, apoptosis and skeletal formation whereas WNT members regulate limb bud initiation, AER formation, D-V patterning and *Shh* expression. Thus, the roles change over time and depend on the cell receiving the signal. This highlights the importance of context-dependent responses and reveals the complexity of understanding the integration of these signals at a cellular level. There is much yet to be discovered in the ultimate quest for knowledge of how patterning relates to final limb form.

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