Interplay between the molecular signals that control vertebrate limb development

LEE NISWANDER*
Molecular Biology Program and Howard Hughes Medical Institute, Memorial Sloan-Kettering Cancer Center, New York, USA

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

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

Abbreviations used in this paper: AER, apical ectodermal ridge; A-P, anterior-posterior; BMP, bone morphogenetic protein; D-V, dorsal-ventral; En1, engrailed 1; fgg, fibroblast growth factor; Pr-D, proximal-distal; Shh, sonic hedgehog.

*Address correspondence to: Dr. Lee Niswander. Molecular Biology Program and Howard Hughes Medical Institute, Memorial Sloan-Kettering Cancer Center, New York, NY 10021, USA. Fax: +1-212-717-3623. e-mail: l-niswander@ski.mskcc.org

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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 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 polydactyous 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 polydactyous (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 intrinsic 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 polydactyous (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/zeugopod 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, 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.

References


