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Coordination of limb development by crosstalk among axial patterning pathways

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ABSTRACT

Vertebrate limb development relies on the activity of signaling centers that promote growth and control patterning along three orthogonal axes of the limb bud. The apical ectodermal ridge, at the distal rim of the limb bud ectoderm, produces WNT and FGF signals, which promote limb bud growth and progressive distalization. The zone of polarizing activity, a discrete postero-distal mesenchymal domain, produces SHH, which stimulates growth and organizes patterning along the antero-posterior axis. The dorsal and ventral ectoderms produce, respectively, WNT7A and BMPs, which induce dorso-ventral limb fates. Interestingly, these signaling centers and the mechanisms they instruct interact with each other to coordinate events along the three axes. We review here the main interactions described between the three axial systems of the developing limb and discuss their relevance to proper limb growth and patterning.

1. Introduction

Limb bud patterning is organized along three orthogonal axes. Each of these axes develops under the control of a specific signaling center: the proximo-distal (P-D) axis, extending from the body trunk to the tip of the digits, is controlled by the apical ectodermal ridge (AER); the antero-posterior (A-P) axis, running from the thumb to the little finger, is regulated by the zone of polarizing activity (ZPA); and the dorso-ventral (D-V) axis, from the dorsum to the palm of the hand, is patterned by non-AER ectoderm. Two of these three fundamental signaling centers —AER and ZPA— and their basic functions were discovered by John Saunders long before molecular genetics impacted developmental biology (Saunders and Gasseling, 1968; Saunders, 1948) and today remain as essential paradigms for the understanding of limb patterning. Furthermore, Saunders made very important early observations on the role of dorsal ectoderm in D-V limb bud patterning (MacCabe et al., 1974).

The AER is an ectodermal thickening at the most distal region of the limb bud with important signaling roles, mainly contributed by fibroblast growth factor (FGF) and WNT signals (reviewed in (Fernandez-Teran and Ros, 2008)). Several FGF family members of and WNT3a are expressed from the AER and signal to the underlying mesoderm, promoting P-D limb axis extension and patterning (Mariani et al., 2008; Barrow et al., 2003).

The ZPA is a group of mesodermal cells localized at the posterior region of the limb bud that were identified and characterized by John Saunders. When the ZPA is grafted anteriorly, a mirror image duplication of the posterior digits occurs. The ZPA is thus responsible for the polarization of the digital plate (Saunders and Gasseling, 1968) and promotes A-P growth. These early experiments by Saunders inspired the morphogen gradient model for limb A-P patterning (Tickle, 1981; Wolpert, 1969; Summerbell, 1979) and set the stage for the advanced molecular and cellular characterization of this developmental paradigm. The signaling molecule responsible for ZPA activity is sonic hedgehog (*Shh*), which is required for, and can replace, ZPA activity (Martí et al., 1995; Riddle et al., 1993).

Dorso-ventral limb patterning is governed by ectodermal signals. In the ventral ectoderm, BMP signaling activates *En1*, which restricts *Wnt7a* to the dorsal ectoderm (Pizette and Niswander, 1999; Ahn et al., 2001). WNT7A signaling from the dorsal ectoderm then acts on the mesoderm to activate the LIM-homeodomain factor Lmx1b and establish the D-V pattern (Chen and Johnson, 2002; Riddle et al., 1995; Chen et al., 1998).

Initially, mechanisms operating along the three main limb bud axes were studied independently; however, accumulating evidence shows that patterning and signaling involves interactions among the three axes. Indeed, the pattern of A-P polarity varies greatly according to the P-D limb segment: the anatomy and gene expression patterns of the AER are modulated along the A-P axis, and the position of the AER is established at the interface of the dorsal and ventral ectoderm, indicating crosstalk between axes for patterning and establishment of signaling centers. The complicated pattern of the mature limb thus can

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only be achieved by a continuous interaction among the mechanisms that control the three spatial axes.

In this review we summarize the main interactions described between axial patterning systems at different stages of limb development.

2. P-D/A-P interactions

As mentioned above, P-D patterning is regulated by FGFs and WNTs expressed from the AER, whereas A-P patterning is controlled by SHH produced by the ZPA. In this section we focus on the involvement of these molecules in cross-regulation between axes. The need for crosstalk between these axes is evident from the variations in A-P pattern in different proximo-distal limb segments. In the canonical tetrapod limb, the proximal segment—the stylopod—contains a single A-P element (humerus or femur), the intermediate segment—the zeugopod—contains two A-P elements (radius or tibia and ulna or fibula), and the distal-most element—the autopod—contains five A-P elements (tarsals or carpals and digits). P-D and A-P patterning therefore need to be modulated coordinately in order to generate the correct A-P elements in each specific P-D segment. Furthermore, the timing of patterning also requires coordination, because limb development takes place in a P-D sequence and therefore the different A-P patterns need to be established at different developmental times.

The best example of P-D and A-P interaction is the well-established ZPA-SHH/AER-FGF positive feedback-loop (Fig. 1), which involves the signaling molecules Gremlin1 (GREM1) and bone morphogenetic proteins (BMPs) (Benazet et al., 2009; Laufer et al., 1994; Nissim et al., 2006; Niswander et al., 1994; Scherz et al., 2004; Zuniga et al., 1999; Verheyden and Sun, 2008). During the period of activity of the AER and ZPA, BMPs are expressed in the mesenchyme beneath the AER, and their activity represses AER-FGFs. ZPA maintenance is promoted by AER-FGF activation of Shh expression, while AER maintenance is promoted by Shh activation of *Gremlin*, which represses BMPs (Fig. 1). In addition, AER-FGFs prevent overactivation of the positive feedback loop by promoting *Gremlin* downregulation.

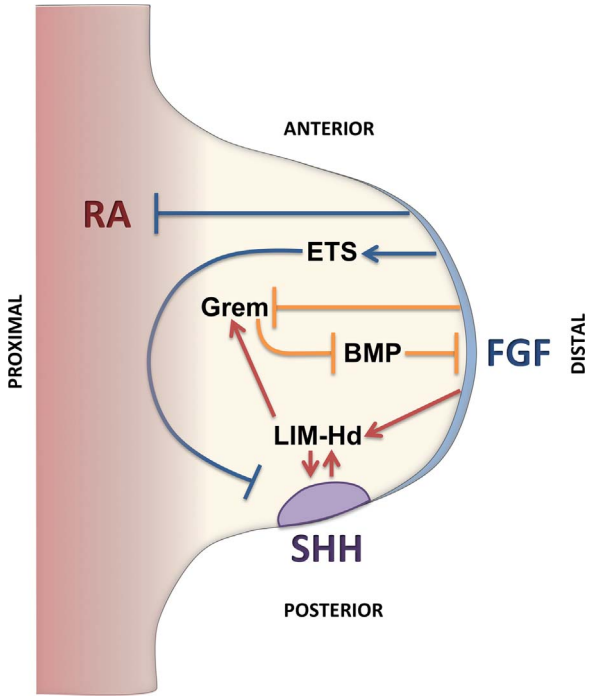

Fig. 1. The FGF-SHH positive feedback loop maintains AER and ZPA activity. SHH/GREM1/FGF feedback loop (yellow lines), showing the integration of LIM-Hx transcription factors (red lines) and the negative regulation of *Shh* by FGF targets (blue lines). In addition, AER-FGF signals maintain the restriction of RA to the proximal limb.

The involvement of LIM-homeodomain transcription factors, which will be further discussed below, is essential for the regulatory connections between AER and ZPA (Tzchori et al., 2009) (Fig. 1). This regulatory loop is essential for limb development, and its premature disruption results in apoptosis of progenitors at the mesenchymal core and loss of AP identity and digits (Zuniga et al., 1999; Michos et al., 2004; Khokha et al., 2003; Panman et al., 2006). The SHH/GREM1/FGF feedback loop thus allows distal progression/patterning of the limb bud in coordination with A-P growth/patterning. At later stages, the loop coordinates cessation of ZPA and AER activities, regulating the simultaneous termination of limb growth associated with the patterning phase. During normal limb development, growth creates a gap between the *Grem1* and *Shh* domains, due to expansion of former ZPA cells, which are refractory to SHH signaling. When this gap is sufficiently large, SHH cannot efficiently promote *Grem1*, and the feedback loop is naturally disrupted (Scherz et al., 2004). The natural disruption of the loop is also a consequence of the progressive increase in FGF signaling that normally occurs as the limb grows. This increase in FGFs inhibits *Grem1* in the distal region, contributing to high BMP levels and loop termination (Benazet et al., 2009; Scherz et al., 2004; Verheyden and Sun, 2008).

An additional point of control between the AER and ZPA was demonstrated by the negative regulation of *Shh* by FGF targets. The ETS transcription factors *Etv4* and *Etv5* are transcriptional activators that are downstream targets of FGF/MAPK signaling and are involved in many embryonic processes (Brent and Tabin, 2004; Firnberg and Neubuser, 2002; Raible and Brand, 2001; Roehl and Nusslein-Volhard, 2001). Accordingly, in many tissues, conditional inactivation of these factors results in phenotypes similar to those observed in *Fgf* mutants. In contrast, mutant phenotypes in the limb do not show the expected P-D alterations, instead showing anterior expansion or ectopic *Shh* expression, leading to pre-axial polydactyly (Mao et al., 2009; Zhang et al., 2009). In one study, these phenotypes were also accompanied by shortening of the P-D skeletal elements (Mao et al., 2009). It was further shown that members of the ETS family directly bind to the ZRS—the main *Shh* limb enhancer—to restrict the *Shh* spatial expression pattern to the posterior region (Lettice et al., 2012). This cross-regulation represents another instance of A-P and P-D axis interaction through which the system ensures that the FGF-SHH positive loop does not extend to anterior limb bud regions (Fig. 1).

There are other relevant processes in which P-D and A-P axis communicate with each other. A fascinating issue is how SHH signaling is not only antero-posteriorly biased but also regulated during P-D patterning. Genetic tracing of cells expressing *Gli1*—a direct transcriptional target of Hedgehog signaling—showed that in the stylopod only posterior muscle and skin responded to SHH signaling; in contrast, the zeugopod and autopod contained descendants of SHH-responding cells contributing to all skeletal elements except the most anterior ones—radius/tibia and digit 1 (Ahn and Joyner, 2004). These observations indicate that SHH signaling distribution varies along the P-D axis. The main cause of this differential SHH signaling distribution is the regulation of the *Shh* mRNA expression domain, and more specifically the timing of *Shh* activation during P-D limb outgrowth. In fact, *Shh* expression starts when the limb bud is already well established. In his first seminal contribution to understanding limb development, John Saunders established a fundamental feature of limb P-D development: its temporal progression from proximal to distal (Saunders, 1948). This study gave an extraordinary boost to limb research and promoted the elaboration and discussion of P-D patterning models for several decades (reviewed in (Tabin and Wolpert, 2007; Delgado and Torres, 2016)). Due to this temporal sequence of P-D development, *Shh* activation takes place when the stylopod is already specified, so that the stylopod skeletal element contains no cells that ever expressed *Shh* (Harfe et al., 2004). In addition, cells that form the stylopod skeletal element never activate the Shh pathway (Ahn and Joyner, 2004), either because Shh does not diffuse proximally or because they become