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Review:

Apical ectodermal ridge regulates three principal axes of the developing limb*

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Abstract: Understanding limb development not only gives insights into the outgrowth and differentiation of the limb, but also has clinical relevance. Limb development begins with two paired limb buds (forelimb and hindlimb buds), which are initially undifferentiated mesenchymal cells tipped with a thickening of the ectoderm, termed the apical ectodermal ridge (AER). As a transitional embryonic structure, the AER undergoes four stages and contributes to multiple axes of limb development through the coordination of signalling centres, feedback loops, and other cell activities by secretory signalling and the activation of gene expression. Within the scope of proximodistal patterning, it is understood that while fibroblast growth factors (FGFs) function sequentially over time as primary components of the AER signalling process, there is still no consensus on models that would explain proximodistal patterning itself. In anteroposterior patterning, the AER has a dual-direction regulation by which it promotes the sonic hedgehog (*Shh*) gene expression in the zone of polarizing activity (ZPA) for proliferation, and inhibits *Shh* expression in the anterior mesenchyme. In dorsoventral patterning, the AER activates Engrailed-1 (*En1*) expression, and thus represses Wnt family member 7a (*Wnt7a*) expression in the ventral ectoderm by the expression of *Fgfs*, *Sp6/8*, and bone morphogenetic protein (*Bmp*) genes. The AER also plays a vital role in shaping the individual digits, since levels of *Fgf4/8* and *Bmps* expressed in the AER affect digit patterning by controlling apoptosis. In summary, the knowledge of crosstalk within AER among the three main axes is essential to understand limb growth and pattern formation, as the development of its areas proceeds simultaneously.

Key words: Apical ectodermal ridge (AER); Limb development; Fibroblast growth factor (FGF); Zone of polarizing activity (ZPA)

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1 Introduction

Studies on vertebrate limb development are of great significance for the understanding of pattern formation and morphogenesis in embryology (Casanova et al., 2011; Rodriguez-Leon et al., 2013). A good conception of limb development both includes the outgrowth and differentiation of the limb, and provides information of clinical relevance, such as reasons for congenital limb deficiencies, or clues about regeneration. Research to date has revealed that limb development begins with the formation of two paired limb buds (the forelimb and hindlimb buds), which are initially undifferentiated mesenchymal cells tipped with a thickening of the ectoderm, termed the apical ectodermal ridge (AER) (Fig. 1) (Irvine and Rauskolb, 2001; Towers and Tickle, 2009). It is believed that the AER is a signalling centre with a key

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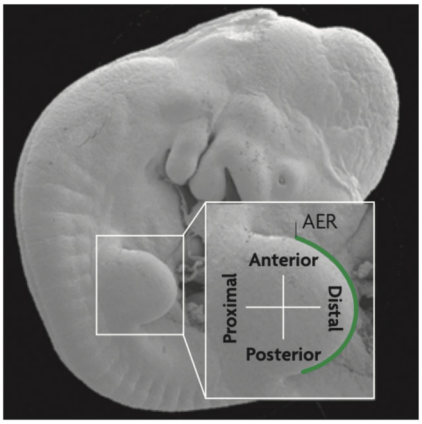

Fig. 1 Forelimb bud (at the level of the heart in mouse embryo)

The green layer indicates the apical ectodermal ridge. AER: apical ectodermal ridge. Reprinted from Zeller et al. (2009), Copyright 2009, with permission from Springer Nature

role in regulating limb growth and development (Casanova et al., 2011; Mallick, 2013).

The AER is a transitional embryonic structure that regulates gene expression involved with limb development in four stages through the secretion of signalling molecules (Mallick, 2013; Rodriguez-Leon et al., 2013). Thus, an understanding of AER development is essential for further research on limb growth. For the initiation of AER function, fibroblast growth factor 10 (FGF10) (encoded by *Fgf10* gene) expressed from mesenchymal cells binds the epithelial FGF receptor 2b (FGFR2b) with heparin sulfate to activate *Fgf8* in the AER precursor cells (Barrow et al., 2003; Itoh and Ohta, 2014). During maturation, Engrailed-1 (*En1*) is required to repress the expression of Wnt family member 7a (*Wnt7a*) to the dorsal ectoderm in order to form the dorsal–ventral boundary (Logan et al., 1997), while the upstream of *En1* is regulated by bone morphogenetic protein (BMP) (Pizette et al., 2001). Furthermore, Casanova et al. (2011) discovered that AT-rich interaction domain 3b (*Arid3b*) participated in AER maturation. The maintenance stage is highly relevant to limb outgrowth and pattern formation, where several signalling pathways function within the AER through a positive feedback loop termed sonic hedgehog (SHH)-Gremlin 1 (GREM1)-FGF regulatory loop (Bouldin et al., 2010; Rodriguez-Leon et al., 2013). Following the disconnection of the SHH-GREM1-FGF regulatory loop, the regression stage of the AER occurs for size regulation (Scherz et al., 2004).

Vertebral limb growth and development proceeds in three main axes (Fig. 2), with the AER acting throughout this process. Based on classical manipulation experiments (Saunders, 1948), this structure was used to be considered to control proximodistal patterning. However, the ridge also influences the regulation of anteroposterior patterning, dorsoventral patterning, and the shaping of the limbs (Choi et al., 2012; Delgado and Torres, 2017). This review aims to summarise the specific roles of the AER in three main axes and in shaping the developing limbs, by conducting a comprehensive search in the database sources up to 2019: MEDLINE, Embase, Scopus, Google Scholar, Cochrane Library, and Web of Science databases. The search terms used were “apical ectodermal ridge,” “developing limb/limb development,” and “limb bud” for searching valuable information in the title, keywords, and abstract. No restrictions were placed on the type of articles, study design, language, or publication year. The searching results were manually reviewed to exclude irrelevant articles.

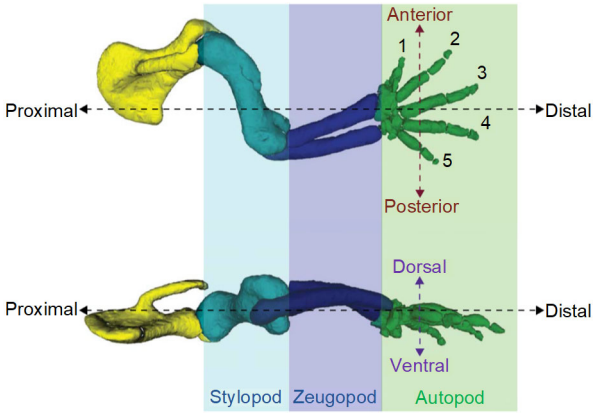

Fig. 2 Limb axis and elements

Proximodistal axis: proximal to distal (stylopod to autopod); Anteroposterior axis: anterior to posterior (thumb (digit 1) to little finger (digit 5)); Dorsoventral axis: dorsal to ventral (back of the hand to the palm). Reprinted from Duboc and Logan (2009), Copyright 2009, with permission from Elsevier

2 Role of AER in proximodistal patterning

The primary function of the AER is a regulation of proximodistal patterning. A truncation of the limb skeleton may occur in any stage of limb development when the AER is removed in experiments (Saunders, 1948; Summerbell et al., 1973). The removal of the

AER results in arrested limb development, with the achieved degree of development determined by the stage of development at which the AER is removed (Saunders, 1948; Tickle, 2003). These findings help us to better understand the mechanism of limb hypoplasia and truncation along the proximodistal axis in humans.

2.1 Current models for specifying positional information in proximodistal patterning

Several models have been proposed to explain proximodistal patterning, such as the progress zone (PZ) model (Summerbell et al., 1973), the early specification model (Dudley et al., 2002), and the two-signal gradient model (Tabin and Wolpert, 2007; Cooper et al., 2011). In the PZ model, positional information of the developing limb is from the PZ immediately beneath the AER (Summerbell et al., 1973; Wolpert, 2002). The PZ acts as a developmental clock suggesting that the length of time undifferentiated mesenchymal cells spend in the PZ specifies their positional value along the proximodistal axis (Wolpert, 2002; Tickle, 2003; Towers and Tickle, 2009). That is, positional values in the PZ model are produced when cells leave the zone (Tickle, 2003). However, the early specification model indicates that specific positional information is assigned upon the formation of the limb bud (Dudley et al., 2002; Tickle and Wolpert, 2002). The difference in positional values between these two models is illustrated in Fig. 3. As for the two-signal gradient model, it involves retinoic acid (RA) signalling as a proximal signal and FGFs as distal signals to regulate proximodistal patterning simultaneously (Mariani et al., 2008).

Therefore, the PZ model and early specification model contradict each other. Moreover, studies in recent years have supplied evidence for the two-signal gradient model (Roselló-Díez et al., 2014). In fact, there is still no consensus about models among researchers, but each of these models contributes to the understanding of limb development and roles of the AER, in particular, the signals expressed in the AER. Thus, substantial evidence is still required to propose or support a valid model that credibly explains the mechanism of proximodistal patterning.

2.2 Molecular and genetic bases of proximodistal patterning

FGF signalling is instructive in proximodistal patterning, and has been considered as the primary component of the AER signalling (Mallick, 2013). Evidence for the function of FGFs in proximodistal patterning was supplied by an FGF-soaked bead experiment in chick wing buds. During the experiment, the AER was replaced by an FGF-soaked bead compared with one no-treatment group and one ridge-removal group without the FGF bead. Results showed that the FGF-soaked bead rescued the outgrowth and proximodistal patterning even with the AER removed

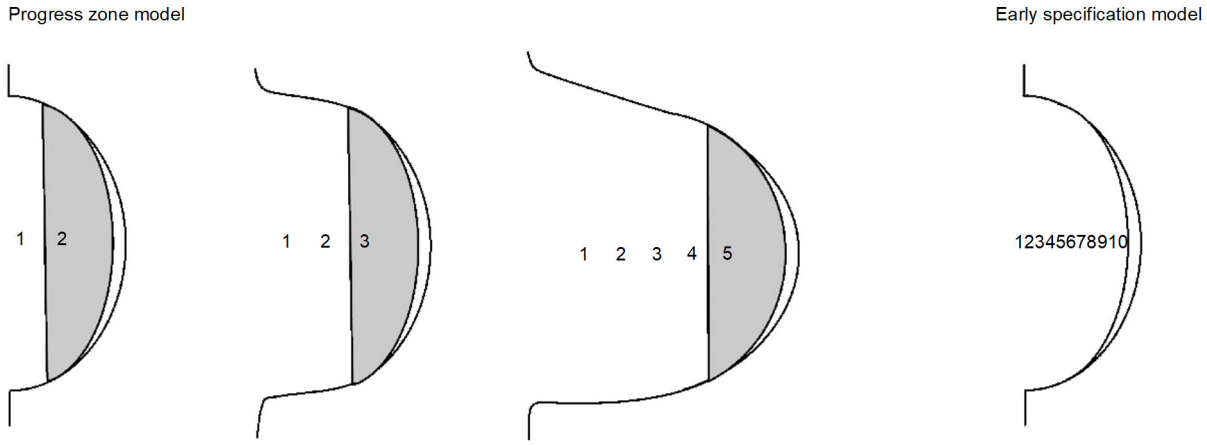

Fig. 3 Comparison between the progress zone model and the early specification model

Left: progress zone (PZ) model; positional values along the proximodistal axis. The shaded region is the "PZ." Right: early specification model; positional values are specified at the formation of the limb bud. Reprinted from Tickle (2003), Copyright 2003, with permission from Elsevier

(Niswander et al., 1993). Using mouse embryos, Moon and Capecchi (2000) tested the function of FGF signalling by the direct inactivation of *Fgf* genes (*Fgf8*) in the AER. As a result, bud outgrowth was reduced and truncation appeared. A similar study by Lewandoski et al. (2000) also supported these results. However, the inactivation of other *Fgf* genes does not affect limb development, except by the simultaneous inactivation of *Fgf4* and *Fgf8* (Martin, 1998; Moon et al., 2000; Sun et al., 2000; Towers and Tickle, 2009). The simultaneous absence of *Fgf4* and *Fgf8* in the AER at different stages of limb development eventually results in a lack of induction of limb development (Sun et al., 2002). Besides, both of these genes in the AER are involved in the maintenance of the undifferentiated zone adjacent to the AER.

The AER produces a large number of FGF proteins encoded by *Fgf4*, *Fgf8*, *Fgf9*, and *Fgf17* (in a mouse embryo), or *Fgf19* (in a chick embryo), which function at different stages of limb development (Fig. 4) (Tickle, 2003; Rodriguez-Leon et al., 2013). The gene *Fgf8* participates in regulating the outgrowth and development throughout proximodistal patterning, while other *Fgf* genes are activated at specific stages. The expression of *Fgf4* and *Fgf17* lasts until the beginning of digit patterning (Niswander and Martin, 1992), while the expression of *Fgf9* still persists in the later stage of digit patterning (Hajihosseini and Heath, 2002).

There are also other molecules and genes regulating proximodistal patterning, such as RA signalling (Yashiro et al., 2004), T-box transcription factor 5 (*Tbx5*) and *Tbx4* (Nishimoto et al., 2015), myeloid ecotropic viral integration site (*Meis*) (Mercader et al., 1999, 2000), Homeobox A11 (*Hoxa11*) and *Hoxa13* (Nelson et al., 1996; Mercader et al., 2009), and *Shh* (Laufer et al., 1994; Niswander et al., 1994). The disruption of RA signalling through the deletion of retinaldehyde dehydrogenase 2 (*Raldh2*) leads to forelimb abnormalities (Niederreither et al., 2002). Moreover, RA signalling controls the expression of *Tbx5* serving the forelimb and *Tbx4* serving the hindlimb with the expression of *Hox* genes and β-catenin, and later through an *Fgf10*-AER-*Fgf8* feed-forward mechanism to regulate limb growth and development (Fig. 5) (Nishimoto et al., 2015). Through the feed-forward loop, *Fgf10* is regulated by the RA both directly and indirectly through *Tbx* genes. The corresponding genes encode MEIS1/2, HOXA11, and HOXA13 proteins as proximodistal markers in the stylopod, zeugopod, and autopod, respectively (Fig. 6) (Roselló-Díez et al., 2014). The *Shh* gene, expressed in the zone of polarizing activity (ZPA), primarily regulates anteroposterior patterning. However, the expression of *Fgf4* requires SHH signalling through the SHH-FGH feedback loop to regulate proximodistal patterning (Fig. 7) (Pownall and Isaacs, 2010).

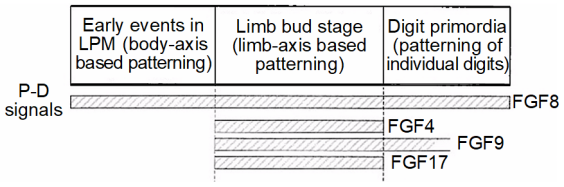

Fig. 4 FGF signalling of AER functions sequentially over time, not showing FGF19
P-D: proximal-distal; LPM: lateral plate mesoderm; AER: apical ectodermal ridge; FGF: fibroblast growth factor. Reprinted from Tickle (2003), Copyright 2003, with permission from Elsevier

2.3 Roles of FGF10 signalling in pre-AER and post-AER induction

The factor FGF10, as a paracrine FGF, plays different roles in pre- and post-AER phases along with limb development. During pre-AER induction,

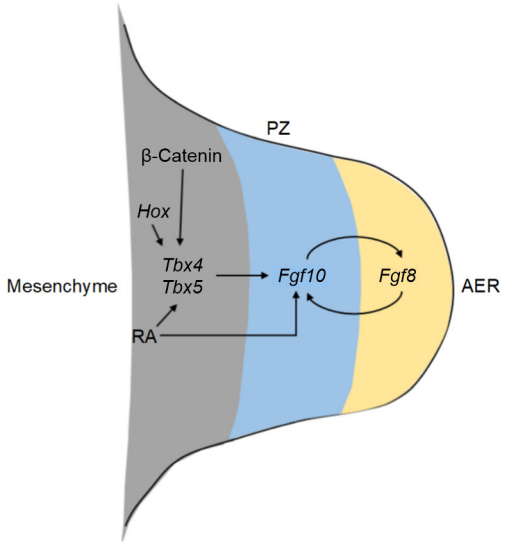

Fig. 5 Feed-forward mechanism
The factors retinoic acid (RA)/β-catenin/Homeobox (*Hox*) cooperatively activate T-box transcription factor 5 (*Tbx5*) in the forelimb and *Tbx4* in the hindlimb. Genes fibroblast growth factor 10 (*Fgf10*) and *Fgf8* are regulated by RA both directly, and indirectly with *Tbx*. PZ: progress zone; AER: apical ectodermal ridge

Fig. 6 Expression of *Meis*, *Hoxa11*, and *Hoxa13* in proximodistal patterning

PD: programmed death; *Meis*: myeloid ecotropic viral integration site; *Hoxa*: Homeobox A. Reprinted from Roselló-Díez et al. (2014), Copyright 2014, with permission from the Company of Biologists Ltd.

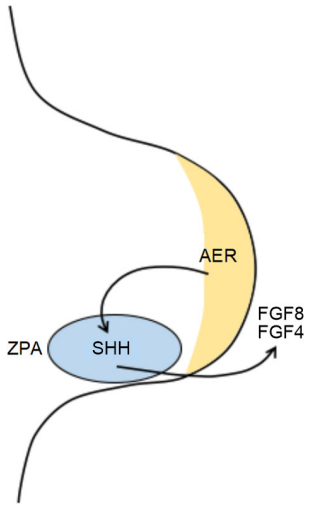

Fig. 7 SHH-FGF feedback loop

ZPA: zone of polarizing activity; SHH: sonic hedgehog; AER: apical ectodermal ridge; FGF: fibroblast growth factor

Fgf10 functions in the lateral plate mesoderm to initiate the formation of AER through *Fgf10*/AER-*Fgfr2b* signalling, and then establishes the *Fgf10*-AER-*Fgf8* feed-forward loop (Sekine et al., 1999; Danopoulos et al., 2013). Moreover, a new function of FGF10 found in a study on FGF10 null embryos is that FGF10 involves the regulation of epithelial to mesenchymal transition, thus allowing the formation of the progenitors of the limb (Gros and Tabin, 2014; Jin et al., 2019). It remains unclear, however, whether epithelial to mesenchymal transition is the direct consequence of *Fgf10* expression. During post-AER induction, *Fgf10* is responsible for maintaining the integrity of AER and FGFR2b signalling by promoting *Fgf8* and *Wnt3a* expression in the AER, which in turn maintain *Fgf10* expression in the PZ to initiate the amplification of the progenitors of the limb (Sekine et al., 1999; Mariani et al., 2008; Jin et al., 2019).

Collectively, the FGF family emanating from the AER regulates proximodistal patterning directly with different functions in corresponding stages of limb outgrowth and development. Meanwhile, newer "players" are being found that participate in the proximodistal patterning via specific feedback loops.

3 Role of AER in anteroposterior patterning

The main pathway where the AER participates in regulating anteroposterior patterning is the SHH-GREM1-FGF regulatory loop. As mentioned in the Introduction section, this feedback loop is essential for the maintenance stage of the AER. The gene *Shh* mediates anteroposterior patterning, and only exists in the posterior area of the limb bud, which is the so-called PA (Duboc and Logan, 2009). Within the SHH-GREM1-FGF regulatory loop (Fig. 8), the expression of *Fgf* genes, including *Fgf4*, *Fgf8*, *Fgf9*, and *Fgf17*, is required for the maintenance of SHH signalling. This loop is positively mediated by *Grem1* as an antagonist of *Bmp4*, which is responsible for a negative regulation of *Fgf* expression in the AER (Duboc and Logan, 2009).

Additionally, FGFs regulate E26 transformation-specific (ETS) variant 4 (*Etv4*) and *Etv5* of the ETS transcription factors, which are involved with restricting the expression of *Shh* in the anterior mesenchyme (Zhang et al., 2009). Authors of the same study found that the inactivation of *Fgf* genes in the AER or FGFRs leads to reduction of *Etv4* and *Etv5* expression. Furthermore, the ectopic expression of *Shh* gene occurs in the anterior mesenchyme and extra digits appear as *Etv4* and *Etv5* are inactivated (Fig. 9). These results are also supported by Lettice et al. (2012).

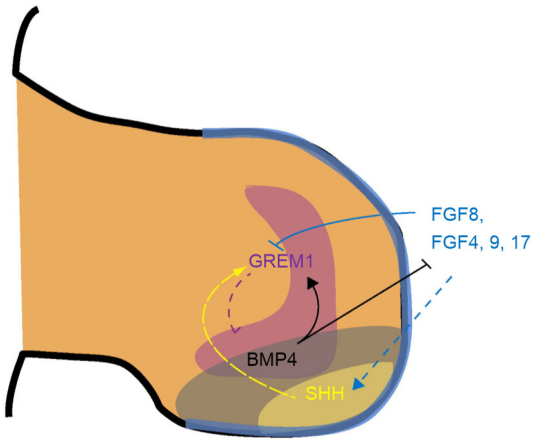

Fig. 8 SHH-GREM1-FGF regulatory loop
SHH: sonic hedgehog; GREM1: Gremlin 1; FGF: fibroblast growth factor; BMP: bone morphogenetic protein. Reprinted from Duboc and Logan (2009), Copyright 2009, with permission from Elsevier

Thus, the AER not only promotes *Shh* expression in ZPA for proliferation, but also inhibits *Shh* expression in the anterior mesenchyme. Anteroposterior patterning is regulated through the dual-direction regulatory loop established between signals from the ZPA (SHH) and AER (FGFs).

4 Role of AER in dorsoventral patterning

Dorsoventral patterning is regulated by the interaction between the AER and the ectoderm (dorsal and ventral regions). During normal limb development, the *Wnt7a* gene is expressed in the dorsal ectoderm, while *En1* expression is only detected in the ventral ectoderm (Delgado and Torres, 2017). Findings increasingly support that the *Fgf8*, *Sp6/8*, and *Bmp2/4/7* expressed in the AER have crucial roles in dorsoventral patterning (Fig. 10) (Fernandez-Teran and Ros, 2008; Haro et al., 2014). The genes *Fgfs*, *Sp6/8*, and *Bmps* activate the expression of *En1*, which in turn acts by restricting the expression of *Wnt7a* in the ventral ectoderm (Loomis et al., 1996). Conversely, *Wnt7a* expression in the dorsal ectoderm induces LIM homeodomain factor 1b (*Lmx1b*), and thus it triggers dorsal differentiation.

Furthermore, *Bmps*, *Wnt7a*, and *En1* give feedback to the AER through the SHH-FGF loop, that is, these factors are also crucial to the initiation, maturation, and maintenance of the AER as mentioned.

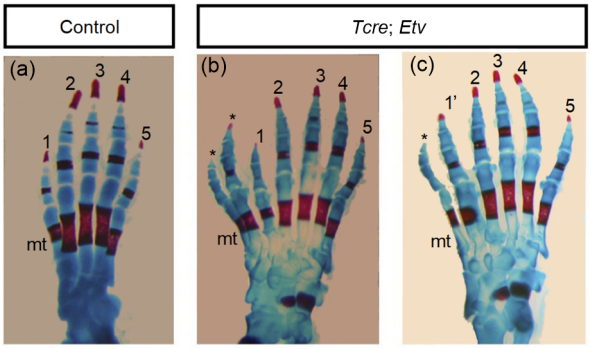

Fig. 9 Extra digits exhibited in the hindlimb
The asterisk indicates the extra digit. *Tcre*: transcervical resection of endometrium; *Etv*: E26 transformation-specific (ETS) variant; mt: metatarsal. Reprinted from Zhang et al. (2009), Copyright 2009, with permission from Elsevier

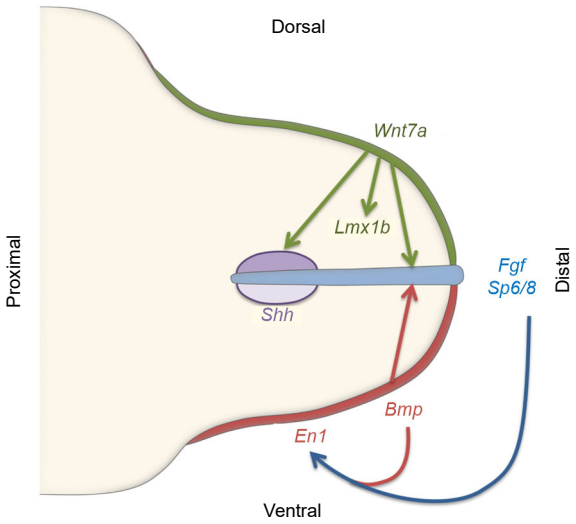

Fig. 10 Interaction between the AER and the ectoderm
AER: apical ectodermal ridge; *Shh*: sonic hedgehog; *Wnt7a*: Wnt family member 7a; *En1*: Engrailed-1; *Lmx1b*: LIM homeodomain factor 1b; *Bmp*: bone morphogenetic protein; *Fgf*: fibroblast growth factor. Reprinted from Delgado and Torres (2017), Copyright 2017, with permission from Elsevier

In this way, the location of the AER at the boundary between the ventral and dorsal ectoderms can be partly explained.

5 Role of AER in shaping the developing limb

Levels of the FGF4 and FGF8 proteins expressed in the AER affect the patterning of individual digits, and are regulated by *Bmps* in the AER (Fig. 11).

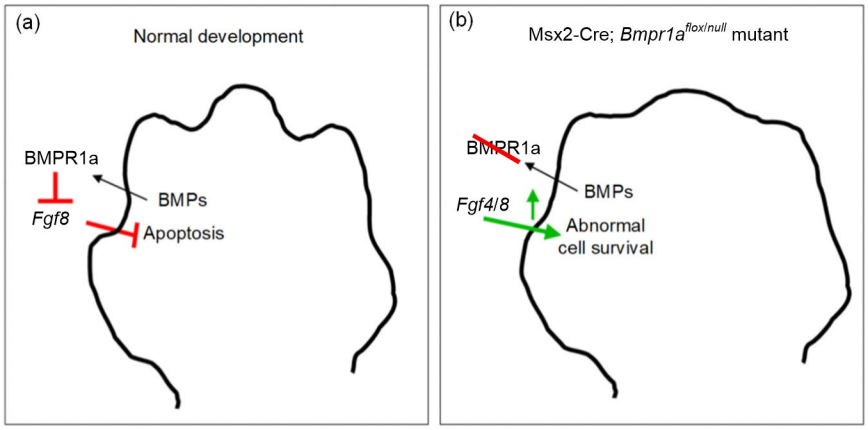

Fig. 11 Interaction of FGFs and BMPs in shaping individual digits (mouse embryo)

(a) During normal development, bone morphogenetic protein (BMP) receptor 1a (BMPR1a) downregulates fibroblast growth factor 8 (*Fgf8*) expression to activate apoptosis in the interdigit zone. (b) After removal of *Bmpr1a*, the expansion of *Fgf4/8* expression causes abnormal cell survival, and thus webbed digits appear. Reprinted from Pajni-Underwood et al. (2007), Copyright 2007, with permission from the Company of Biologists Ltd.

When the gene BMP receptor 1a (*Bmpr1a*) is inactivated, expression of *Fgf4* and *Fgf8* in the AER is upregulated, causing webbed digits and less cell death in the interdigit zone (Pajni-Underwood et al., 2007). This suggests that *Fgf4* and *Fgf8* can prevent apoptosis in the interdigital region. Additionally, the removal of *Bmp2/4* from the AER also results in increased expression of *Fgf4* and *Fgf8* with the same results. Furthermore, a study by Choi et al. (2012) including the removal of BMP ligands (*Bmp2/4/7*) from the AER reported polydactyly and webbed digits. Hence, *Fgf4/8* and *Bmps* from the AER are responsible for shaping developing digits.

6 Signalling interactions across different signalling centres during limb development

As explained previously, the AER, PZ, and ZPA, seen as three signalling centres besides the mesenchyme, regulate almost all phases of limb development simultaneously. Meanwhile, four AER compartments interact with each other by respective signalling pathways during the process. Jin et al. (2019) linked signalling to these four compartments with effects of *Fgf10* (Fig. 12), clearly showing pathways of RA/*Hox*/β-catenin signalling to *Tbx4*/*Tbx5*, *Fgf10*-AER-*Fgf8* feedback loop, SHH-GREM1-FGF regulatory loop, and repression of FGFs on *Shh* expression, as mentioned above. Additionally, *Fgf10* expression

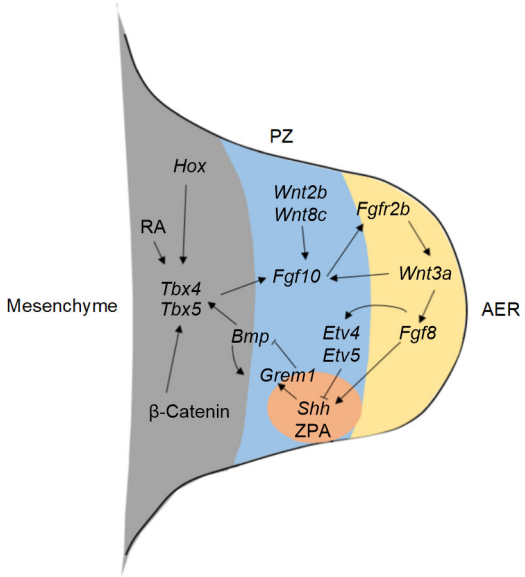

Fig. 12 Compositions of the limb bud and its signalling interactions

The limb bud consists of mesenchyme, progress zone (PZ), zone of polarizing activity (ZPA), and apical ectodermal ridge (AER) from proximal to distal. The closed loop regulating the outgrowth and development of the limb bud includes retinoic acid (RA)/homeobox (*Hox*)/β-catenin signalling, a fibroblast growth factor 10 (*Fgf10*)-AER-*Fgf8* feedback loop, and a sonic hedgehog (SHH)-Gremlin 1 (GREM1)-FGF regulatory loop. *Tbx*: T-box transcription factor; *Fgfr2b*: FGF receptor 2b; *Etv4*: E26 transformation-specific (ETS) variant 4; *Bmp*: bone morphogenetic protein

is stabilized by Wnt2b (forelimb) and Wnt8c (hindlimb), and *Fgf8* expression is stabilized by Wnt3a (Kawakami

et al., 2001). Furthermore, BMP signalling also upregulates *Tbx* expression, except for initiating *Grem1* expression (Rodriguez-Esteban et al., 1999; Sheeba and Logan, 2017). Reciprocal interactions form a basic loop to implement a regulation by signalling centres for the outgrowth and patterning of the limb.

7 Conclusions

The AER contributes to multiple axes of limb development through the coordination of signalling centres, feedback loops, and other cellular activities. The principal function of the AER is the regulation of proximodistal patterning. This is achieved directly through FGFs, while the specific model for explaining proximodistal patterning remains unclear. During anteroposterior patterning, the AER acts as a dual-direction regulator in which it promotes *Shh* expression in ZPA for proliferation, and inhibits *Shh* expression in the anterior mesenchyme. In dorsoventral patterning, the AER activates *En1* expression, and thus it suppresses *Wnt7a* expression in the ventral ectoderm by the expression of *Fgfs*, *Sp6*/*8*, and *Bmps*. It also plays a vital role in shaping the individual digits, as the levels of *Fgf4/8* and *Bmps* expressed in the AER affect digit patterning by controlling apoptosis. Consequently, it is important to understand the crosstalk among the three main axes of AER to accurately describe limb growth and pattern formation, as different processes occur simultaneously in limb development.

Contributors

Guo-hao LIN performed the literature research, wrote and edited the manuscript. Lan ZHANG provided expert comments, edited and revised the manuscript. Both authors have read and approved the final manuscript.

Compliance with ethics guidelines

Guo-hao LIN and Lan ZHANG declare that they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by either of the authors.

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中文概要

题　目：顶端外胚层嵴对肢体发育的立体调控

概　要：理解胚胎肢体发育有助于更加全面地了解肢体的发生和分化，同时对于相关基因疗法和肢体再生具有临床指导意义。本文综述了顶端外胚层嵴（AER）参与胚胎肢体发育调控的研究进展及机理，重点总结了 AER 对肢体发育三个轴向的立体调控，包括基因表达调控、信号通路及相关调控中心，并进一步讨论了各信号中心之间的交互作用及现有研究待解决问题，从而加深对肢体发育异常、肢端异常等疾病的理解。

关键词：顶端外胚层嵴（AER）；肢体发育；成纤维细胞生长因子（FGF）；极化活性区（APZ）