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Role of *Epiprofin*, a zinc-finger transcription factor, in limb development

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ABSTRACT

The formation and maintenance of the apical ectodermal ridge (AER) is critical for the outgrowth and patterning of the vertebrate limb. In the present work, we have investigated the role of *Epiprofin* (*Epfn*/*Sp6*), a member of the SP/KLF transcription factor family that is expressed in the limb ectoderm and the AER, during limb development. *Epfn* mutant mice have a defective autopod that shows mesoaxial syndactyly in the forelimb and synostosis (bony fusion) in the hindlimb and partial bidorsal digital tips. *Epfn* mutants also show a defect in the maturation of the AER that appears flat and broad, with a double ridge phenotype. By genetic analysis, we also show that *Epfn* is controlled by WNT/b-CATENIN signaling in the limb ectoderm. Since the less severe phenotypes of the conditional removal of *b-catenin* in the limb ectoderm strongly resemble the limb phenotype of *Epfn* mutants, we propose that EPFN very likely functions as a modulator of WNT signaling in the limb ectoderm.

Introduction

The apical ectodermal ridge (AER), a specialized thickened epithelium at the distal edge of the developing limb bud, is a major signaling center for limb development. The AER, through the production of several members of the fibroblast growth factor (FGFs) family, controls appropriate gene expression, survival, and proliferation of the subjacent mesoderm (Niswander, 2003; Tickle, 2003; Saunders, 1948; Dudley et al., 2002, Rowe et al., 1982; Mariani et al., 2008). In the chick, several FGFs have been shown capable of substituting for AER function (Niswander et al., 1993; Fallon et al., 1994; Martin, 1998). In the mouse, the genetic removal of a significant amount of FGF signaling from the AER results in the absence of limbs (Sun et al., 2002; Boulet et al., 2004; Mariani et al., 2008).

The formation of the AER is a complex process that includes the induction of the AER precursor cells. In the mouse, these are initially located in the ventral ectoderm (Kimmel et al., 2000), but are later positioned and compacted at the dorso-ventral tip of the limb bud to form the linear and thickened mature AER (Loomis et al., 1998; Kimmel et al., 2000; Fernández-Terán and Ros, 2008).

The establishment of the AER is directed by complex interactions between the FGF, WNT/b-CATENIN, and BMP signaling pathways, which operate within the ectoderm as well as between the ectoderm and mesoderm components of the early limb bud. Furthermore, this process is linked to the initiation of the limb bud and to the establishment of dorso-ventral (DV) patterning. It is currently accepted that an ectodermal active WNT/b-CATENIN pathway is required for both AER induction and maintenance in the chick and the mouse (Soshnikova et al., 2003; Barrow et al., 2003; Kawakami et al., 2001). BMP signaling is also essential for induction of the AER, probably through action upstream of WNT signaling (Ahn et al., 2001; Soshnikova et al., 2003; Barrow et al., 2003; Pizette et al., 2001). Paradoxically, once the AER has been induced, further BMP signaling becomes detrimental to AER maintenance and is involved in AER regression (Pizette et al., 2001; Zuñiga et al., 1999; Fernández-Terán and Ros, 2008).

Despite this intensive study, the genetic mechanisms that regulate AER formation and maintenance are not yet fully understood. In particular, very few transcription factors have been identified for

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these processes. In this context, the importance of SP8, one member of the SP family of transcription factors, has been indicated for AER maintenance and maturation (Bell et al., 2003a,b; Treichel et al., 2003; Kawakami et al., 2004). The SP protein subfamily is characterized by a highly conserved carboxy-terminal DNA binding domain composed of three Cys2His2 zinc finger motifs and a buttonhead box located N-terminal to the zinc finger domain. The amino-terminal region is more variable and contains transcriptional activation or repression domains (reviewed in Suske et al., 2005; Kaczynski et al., 2003; Kadonaga et al., 1987; Wimmer et al., 1993). In addition to *Sp8*, other *Sp* family members, including *Sp5*, *Sp9* and *Epiprofin* (*Epfn*/*Sp6*), are also expressed in the limb bud (Harrison et al., 2000; Nakamura et al., 2004; Bell et al., 2003a,b; Treichel et al., 2001; Kawakami et al., 2004). The present study was aimed at characterizing the function of *Epfn*/*Sp6* in limb development.

We show that the limbs formed in the absence of *Epfn* exhibit an altered digital pattern that is characterized by mesoaxial syndactyly, including synostosis in the hindlimb and a partial dorsalization of the digital tips. These limbs develop with a defect in the maturation of the AER, which adopts a double ridge phenotype. By genetic analysis, we also show that *Epfn* is downstream of WNT/b-CATENIN while it does not require FGF signaling for expression. All our results fit with the *Epfn* phenotype resulting from a mild deficit of WNT signaling in the limb ectoderm that affects BMP signaling but not FGF signaling.

Materials and methods

Mutant mice

The generation of *Epiprofin* (*Epfn*/*Sp6*) mutant mice is described in Nakamura et al., 2008. *Brn4Cre;DN-b-catenin*, *Brn4Cre;b-catenin*$^{flox/flox}$ and *Axin2-LacZ* reporter mice, which express *lacZ* under the control of the endogenous *Axin2* promoter (Soshnikova et al., 2003), were also used in this study. All mice were maintained on a mixed genetic background and genotyped based on previously published reports. *Msx2Cre;Fgf4;Fgf8* mutant embryos (Sun et al., 2002) were kindly provided by Gail Martin and Francesca Mariani and *Fgf10* mutant embryos (Sekine et al., 1999) by Thomas Shimmang.

Skeletal preparations, in situ hybridization and measurements of the limb

After removing skin and visceras, mouse embryos were fixed in 95% ethanol. Alizarin Red and Alcian blue skeletal staining was performed according to standard protocols, cleared by KOH treatment and stored in glycerol. *In situ* hybridization (ISH) was performed in whole-mount and in sections following standard procedures. At least two specimens for each gene and stage were analyzed. The length of the AER was measured on *Fgf8* hybridized *Epfn* mutant and wild type limbs of equivalent stage using the measure tool from Image J software. The wild type value was considered as 100.

Cell death and cell proliferation assays

Detection of cell death was performed in sections of paraffin-embedded tissue using terminal deoxynucleotidyl transferase mediated dUTP nick-end labelling (TUNEL) with the Apoptag Fluorescein Direct *In Situ* Apoptosis Detection Kit (Intergen) following the manufacturer's instructions. Analysis of cell death was also performed in whole limb buds using LysoTracker (Molecular Probes L-7528, Invitrogen). The limb buds were incubated with 5 μl/ml LysoTracker solution in HBSS at 37 °C for 30 minutes before been fixed in 4% PFA and analyzed.

Detection of cell proliferation in sections was performed by immunohistochemical assay using the anti phosphorylated histone H3 antibody (rabbit polyclonal Phospho H3 from Upstate Biotechnology, USA) diluted at 1/100.

Results

Pattern of expression of Epiprofin in the developing limb

Epfn is expressed in proliferating dental epithelium during early tooth development, in the matrix of hair follicles, and in the AER of the limb (Nakamura et al., 2004, 2008; Hertveldt et al., 2008). Since we were interested in studying the function of *Epfn* during limb development, we first analyzed in detail its spatial and temporal pattern of expression, by *in situ* hybridization.

At E9, prior to the emergence of the forelimb bud, *Epfn* was strongly expressed in the prospective limb ectoderm, at both the fore and hind limb levels (Fig. 1A). The expression was similar in the dorsal and ventral ectoderm, as shown in the section at forelimb level in Fig. 1B (the level of the section is indicated in Fig. 1A). After the initial limb budding at E9.5, *Epfn* expression continued throughout the entire limb ectoderm, but with a higher level of expression in the ventral ectoderm (Fig. 1C). This could be clearly observed in transverse sections (Fig. 1D; the level of the section is indicated in Fig. 1C). From E10 on, *Epfn* expression progressively declined, first from the dorsal and then from the ventral ectoderm. By E11.5, its expression was mainly confined to the AER (Figs. 1E, F). At later stages, *Epfn* expression was observed to extend from the AER into the dorsal and ventral ectoderm, predominantly at the tip of the digits, while its expression gradually faded over the regressing interdigits (E12.5, Fig. 1G). At E15.5, *Epfn* expression continued to persist over the tip of the digits (Fig. 1H). Notably, *Epfn* expression was always restricted to the limb ectoderm, as confirmed through the analysis of sections of hybridized embryos (Figs. 1B, D and not shown).

At intermediate stages of limb development, in particular, the pattern of expression of *Epfn* was very similar to that of *Fgf8* (Figs. 1E, F; Crossley and Martin, 1995), although the domain of expression of *Epfn* was wider than that of *Fgf8* and extended further into the dorsal and ventral ectoderm. In light of this similarity in expression pattern between *Fgf8* and *Epfn* (Figs. 1A–F; Crossley and Martin, 1995), and their very early activation in the presumptive limb ectoderm, we investigated which gene was activated first. To precisely compare relative activation times, we performed *in situ* hybridization to *Epfn* and to *Fgf8* in consecutive transverse tissue sections (6 μm apart) at the forelimb level of E9 embryos. *Epfn* expression was activated earlier than *Fgf8* in the limb ectoderm (Figs. 1I, J). Its expression also continued at the tip of the digits, well after *Fgf8* expression had ceased (Fig. 1H; Crossley and Martin, 1995), indicating that neither the activation nor the maintenance of *Epfn* expression in the limb ectoderm required *Fgf8* expression.

Overall, the pattern and dynamics of *Epfn* expression in the limb ectoderm suggests its possible involvement in the establishment and/or maintenance of the AER.

Limb defects in the Epiprofin$^{-/-}$ *mice*

To investigate the biological role of *Epfn* during limb development, we analyzed the limb phenotype of mice that had a targeted deletion of the *Epfn* gene (Nakamura et al., 2008).

At birth, the limbs of homozygous *Epfn* mutants showed a normal external morphology, apart from some abnormalities of the digits (Fig. 2). In the forelimbs, these abnormalities consisted of soft-tissue syndactyly of digits 2 and 3, and occasionally digit 4 (arrowhead in Fig. 2B; control in Fig. 2A). In the hindlimbs, these abnormalities were characterized by oligodactyly of four digits due to the fusion of digits 3 and 4 although there were cases in which