

# Novel roles of *Fgfr2* in AER differentiation and positioning of the dorsoventral limb interface

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## Summary

The epithelial *b* variant of *Fgfr2* is active in the entire surface ectoderm of the early embryo, and later in the limb ectoderm and AER, where it is required for limb outgrowth. As limb buds do not form in the absence of *Fgfr2*, we used chimera analysis to investigate the mechanism of action of this receptor in limb development. ES cells homozygous for a loss-of-function mutation of *Fgfr2* that carry a  $\beta$ -galactosidase reporter were aggregated with normal pre-implantation embryos. Chimeras with a high proportion of mutant cells did not form limbs, whereas those with a moderate proportion formed limb buds with a lobular structure and a discontinuous AER. Where present, the AER did not contain mutant cells, although mutant cells did localize to the adjacent surface ectoderm and limb mesenchyme. In the underlying mesenchyme of AER-free areas, cell proliferation was reduced, and transcription of *Shh* and *Msx1* was diminished. *En1* expression in the ventral

ectoderm was discontinuous and exhibited ectopic dorsal localization, whereas *Wnt7a* expression was diminished in the dorsal ectoderm but remained confined to that site. *En1* and *Wnt7a* were not expressed in non-chimeric *Fgfr2*-null mutant embryos, revealing that they are downstream of *Fgfr2*. In late gestation chimeras, defects presented in all three limb segments as bone duplications, bone loss or ectopic outgrowths. We suggest that *Fgfr2* is required for AER differentiation, as well as for *En1* and *Wnt7a* expression. This receptor also mediates signals from the limb mesenchyme to the limb ectoderm throughout limb development, affecting the position and morphogenesis of precursor cells in the dorsal and ventral limb ectoderm, and AER.

Key words: FGF signaling, Chimeras, Limb outgrowth, AER differentiation, Dorsoventral compartments

## Introduction

Vertebrate limbs develop along a lateral axial line that constitutes the dorsoventral boundary of the trunk ectoderm and is established before limb outgrowth (Altabef et al., 1997; Michaud et al., 1997). During early limb development three signaling centers are set up (for a review, see Niswander, 2003). They direct growth and patterning along the proximodistal, anteroposterior and dorsoventral axes. The proximodistal signaling center, the apical ectodermal ridge (AER), is a specialized epithelial thickening at the distal tip of the limb bud. Its surgical removal abrogates limb outgrowth, which can be rescued by externally added FGF (Niswander et al., 1993). Cells in the posterior limb bud mesenchyme, in the zone of polarizing activity (ZPA), are regulated by SHH and BMP2, which are crucial for the anteroposterior limb pattern (Drossopoulou et al., 2000; Riddle et al., 1993). The distal dorsoventral limb pattern is established in the dorsal limb ectoderm, by *Wnt7a* dorsally and *En1* ventrally (Parr and McMahon, 1995; Loomis et al., 1996).

Fibroblast growth factors and their receptors have multiple roles in limb development. They form epithelial-mesenchymal interaction loops, and are involved in both the proximodistal and anteroposterior limb pattern (for reviews, see Martin, 1998; Niswander, 2003). *Fgf4*, *Fgf8*, *Fgf9* and *Fgf17* are

expressed in the AER. Recent genetic evidence for the role of *Fgf4* and *Fgf8* in mesenchymal proliferation and limb outgrowth suggests that the proximodistal limb pattern is established during the early stages of limb development (Sun et al., 2002). AER-derived FGF signals also affect anteroposterior specification by maintaining SHH signaling in the ZPA (Niswander et al., 1994). Although the mesenchymal FGF receptor that processes AER-derived FGF signals has yet to be identified, *Fgfr1c*, which is transcribed in the progress zone (PZ) mesenchyme and is responsible for the shape and adhesion of its cells (Saxton et al., 2000), is a likely candidate for this interaction loop.

A second interaction loop, which connects the PZ mesenchyme to the AER, includes *Fgf10* in the mesenchyme and *Fgfr2b* in the AER (Ohuchi et al., 1997). Targeted disruption of *Fgf10* eliminated AER differentiation, *Fgf8* expression and limb outgrowth (Min et al., 1998; Sekine et al., 1999). Targeted mutations of *Fgfr2* (Arman et al., 1999; Xu et al., 1998), or *Fgfr2b* (De Moerlooze et al., 2000), exhibited phenotypes very similar to those of *Fgf10*, suggesting that they function together as a receptor-ligand pair. This ligand-receptor interaction, which leads from the mesenchyme to the epithelium, complements the AER to mesenchyme loop, and the two together contribute to AER formation, dorsoventral patterning, and proximodistal limb outgrowth and patterning.

Despite a considerable body of evidence and a coherent scheme of gene interactions, the molecular and cellular mechanisms controlled by the FGF-FGFR system are poorly understood. Studying chimeric mouse embryos may help this analysis. Chimeras grown from mutant and wild-type cells can rescue the early lethality of certain mutations. They can reveal structures to which the activity of a receptor is directed, and if the chimera displays a mutant phenotype, interaction between its components may indicate whether the activity of a gene is intracellular, i.e. cell-autonomous, or extracellular, i.e. non-cell-autonomous.

We are interested in the mechanism of action of *Fgfr2*. Loss of *Fgfr2* causes amelia, the complete absence of all four limbs (Xu et al., 1998; Arman et al., 1999; De Moerlooze et al., 2000); therefore its null mutations are not suited for detailed study. We hoped that chimera analysis would help to overcome this difficulty. Here, we report that *Fgfr2* contributes to ectodermal cell movement towards and into the limb, to AER formation, and to certain aspects of dorsoventral ectodermal polarity.

## Materials and methods

### Generation of homozygous *Fgfr2*<sup>Δ2/Δ2</sup> ES cells for chimeric analysis

In the *Fgfr2*<sup>Δ2/Δ2</sup> mutation used in this study the transmembrane region (exon 10) was deleted and a pMCNeo-cassette was inserted into the *EcoRV* site of exon 9, by using the 'Osdupdel' vector (a gift of Oliver Smithies, Chapel Hill, NC). The targeting construct and homozygous mutant ES cells, described previously (Arman et al., 1999), were generated by selection at high G418 concentrations (Mortensen et al., 1992). For chimera experiments, a *Pgk1-lacZ* cassette was introduced into *Fgfr2*<sup>Δ2/Δ2</sup> and, as a control, into wild-type (R1) ES cells. Although this cassette was inserted independently of the mutant construct, the two clones used here exhibited similar localization in chimeras and reflected the published limb phenotypes of *Fgfr2* and *Fgfr2b* (Xu et al., 1998; Arman et al., 1999; De Moerlooze et al., 2000), indicating that β-galactosidase expression faithfully detects most, probably all, mutant cells. The two *lacZ*-positive control ES clones used were employed in multiple chimera experiments, and consistently showed widely distributed colonization in the embryo between E6.5 and 11.5.

### Generation and analysis of chimeric embryos

Small clumps (6–8 cells) of homozygous mutant *Fgfr2*<sup>Δ2/Δ2</sup> or control ES cells, expressing *lacZ*, were either aggregated with E2.5 CD1 mouse morulae (Nagy et al., 1993), or were microinjected into blastocysts. Chimeric embryos were transplanted into pseudopregnant CD1 female mice. Midgestation embryos were isolated 6.5 to 8 days after embryo transfer, corresponding to E9.0–E10.5; perinatal embryos were collected between day 14.5 and 16 after transfer, which corresponds to E16.5 and E18.5. Colonization by mutant-derived cells was estimated by whole-mount β-galactosidase staining, which when necessary was followed by Bouin's fixation, embedding in paraffin wax, and Haematoxylin and Eosin staining. Calcified bone and cartilage was detected by Alizarin and Alcian Blue staining, respectively (Kaufman, 1992).

### BrdU and TUNEL assay

Pregnant female mice were injected with 10 mg/ml BrdU (100 μg/g body weight). Embryos stained for β-galactosidase were post-fixed in Bouin's fixative and embedded in paraffin wax. Tissue sections were incubated with an anti-BrdU antibody (Sigma, St. Louis), and visualized with HRP-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, PA) and DAB substrate. Four separate

sections were analyzed from three different chimeric, and three different control, limb buds using the ImagePlus software (Media Cybernetics, MD). For TUNEL assay the apo TACS kit (R & D Systems, MN) was used.

### Whole-mount in situ hybridization

Whole-mount in situ hybridization was performed according to established methods (Conlon and Herrmann, 1992). The following probes were used: a 353 bp *Fgf8* probe (nucleotides 591 to 943, GenBank Accession Number D38752); a 580 bp *Bmp4* probe (nucleotides 431 to 1010, GenBank Accession Number X56848), and a 478 bp *Cd44* probe (nucleotides 1105 to 1582, GenBank Accession Number AJ251594). These probes were generated by PCR. The *Dlx2* probe was a gift from Dr J. Rubenstein (UCSF), the *Msx1* probe was a gift from Dr B. Hill (University of Edinburgh), *En1* was a gift of Dr K. Schughart (GSF-Forschungszentrum), and *Wnt7a* and *Shh* were donated by Dr A. McMahon (Harvard University). For histological analysis after whole-mount in situ hybridization or β-galactosidase staining the embryos were post-fixed in 2% glutaraldehyde, incubated in 15% sucrose overnight and then incubated in 30% sucrose for 4 hours. They were then embedded in 10% gelatin. Frozen gelatin blocks were sectioned into 25 μm sections. For double in situ hybridization and β-galactosidase staining, the embryos were fixed for 1 hour in ice-cold 4% paraformaldehyde and stained overnight at room temperature with 1 mg/ml Salmon-gal (6-Chloro-3-indolyl-β-D-galactopyranoside, Fluka Chemie AG), a pink chromophore. Then they were post fixed in 4% paraformaldehyde overnight and processed for in situ hybridization, omitting the methanol dehydration and peroxide bleaching steps. Proteinase K treatment was reduced to 5 μg/ml for 4 minutes at room temperature for both epithelial and mesenchymal mRNAs.

### Photography and image analysis

A Zeiss Axioplan or a Nikon DXMI200 microscope with a CCD camera was used.

## Results

### Gene targeting and preparation of chimeras

Initially two targeted *Fgfr2* mutations were assayed to produce homozygous mutant ES cells for chimera analysis. ES cells heterozygous for a point mutation of *Fgfr2b* (Eswarakumar et al., 2002), which in the homozygous mutant embryo exhibited abrogated limb development that was similar to the deletion reported by De Moerlooze et al. (De Moerlooze et al., 2000), were resistant to high doses of G418 (Mortensen et al., 1992). In the second mutation, *Fgfr2*<sup>Δ2</sup>, exon 9 was disrupted and exon 10 was deleted. Exon 9 encodes the IIIc domain of *Fgfr2*, but deletion of exon 10, which encodes the transmembrane domain, meant that no functional *Fgfr2b* or *Fgfr2c* protein could be made by homozygous mutant cells, which in this case were readily derived at high G418 concentrations. Homozygous ES cell clones carrying this mutation and a β-galactosidase reporter were used in this study. *Fgfr2*<sup>Δ2/Δ2</sup> homozygotes die around E10 and display a limb-less phenotype with placental defects (Arman et al., 1999). *Fgfr2*<sup>Δ2/Δ2</sup> ES cells with a PGK1-β-galactosidase reporter have been described previously in the context of a tetraploid fusion experiment, which separated limb ablation from the placental defects associated with loss of *Fgfr2* (Arman et al., 1999). Two *Fgfr2*<sup>Δ2/Δ2</sup> ES cell clones, derived from *Fgfr2*<sup>Δ2/+</sup> heterozygotes exhibited similar localization in multiple chimera experiments. As a wild-type control, we used R1 ES cells into which the same the β-

galactosidase reporter had been introduced (see Materials and methods). The limb defects caused by the mutant component of the chimera were likely to be caused by loss of the *Fgfr2b* variant, because loss of the *c* alternative causes defective bone growth and has no effect on limb morphogenesis (Eswarakumar et al., 2002).

### Defective limb morphogenesis in *Fgfr2 $\Delta 2/\Delta 2$* $\leftrightarrow$ *Fgfr2 $^{+/+}$* chimeras

First, to investigate the embryological effects of the mutation, we studied late gestation (E16 and E17.5) stage chimeras. Of 47 pups, 20 had obvious limb defects on at least one fore- or hindlimb, yielding 34 abnormal limbs. Pre-axial polydactyly was the most frequent defect (Fig. 1A-C), although some limbs exhibited fewer than the normal number of digits (Fig. 1D,F). Dorsally or ventrally displaced digits were the most common (Fig. 1A,C). In some limbs, the dorsoventral polarity of the displaced extra digits was apparently reversed (Fig. 1A,C,F). However, even in these cases, the general polarity of the chimeric autopod was normal and no dorsal footpads, or other signs of ventralization or dorsalization, were observed. The

chimeric autopod of most pups was fully developed and claws were present (Fig. 1A-C).

Most abnormal limbs were of full size, but in three pups small limb-like structures were present (Fig. 1D,E; arrows). One almost limbless chimera had only a single small limb rudiment, positioned in line with, but somewhat anterior to, the left forelimb field (Fig. 1E). These ‘mini-limbs’ had a three-part proximodistal structure similar to that of normal limbs, but they showed no skeletal tissue differentiation and their anteroposterior orientation was reversed.

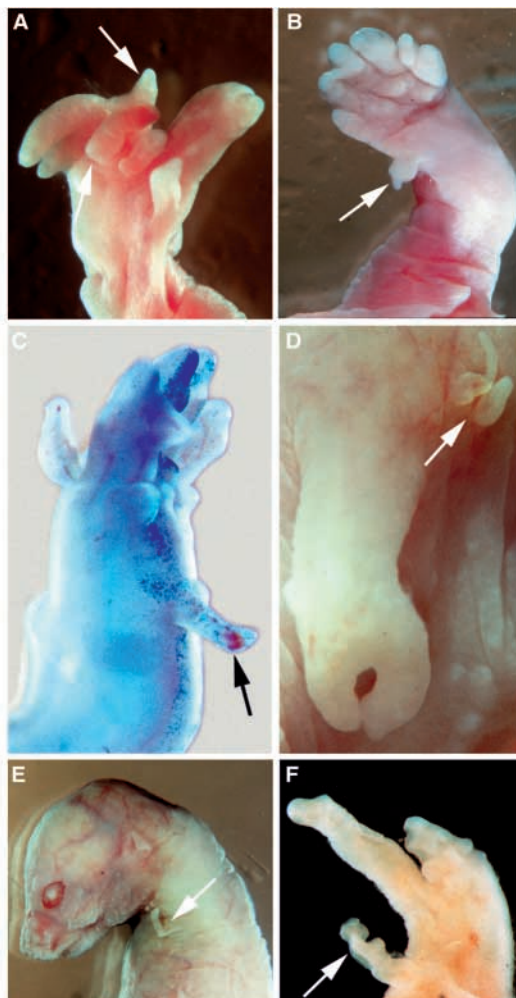
Skeletal preparations revealed defective morphogenesis in all three limb components, the stylopod, zeugopod and autopod. The humerus of two chimeras was partially duplicated, having a bony spike with a cartilage cap (Fig. 2C,D), whereas the zygopod of most mutant fore- and hindlimbs contained only one bone (Fig. 2B-D,F). A similar defect was observed in *Fgf4-Fgf8* double mutants (Sun et al., 2002). The autopod of most chimeric limbs was poorly mineralized (Fig. 2B-D,F), so the abnormalities shown in Fig. 1 do not show up well in the skeletal preparations. The three components of the limb develop consecutively; therefore, these phenotypes suggest that *Fgfr2* is active from early to late limb development.

### Mutant cells do not contribute to the AER

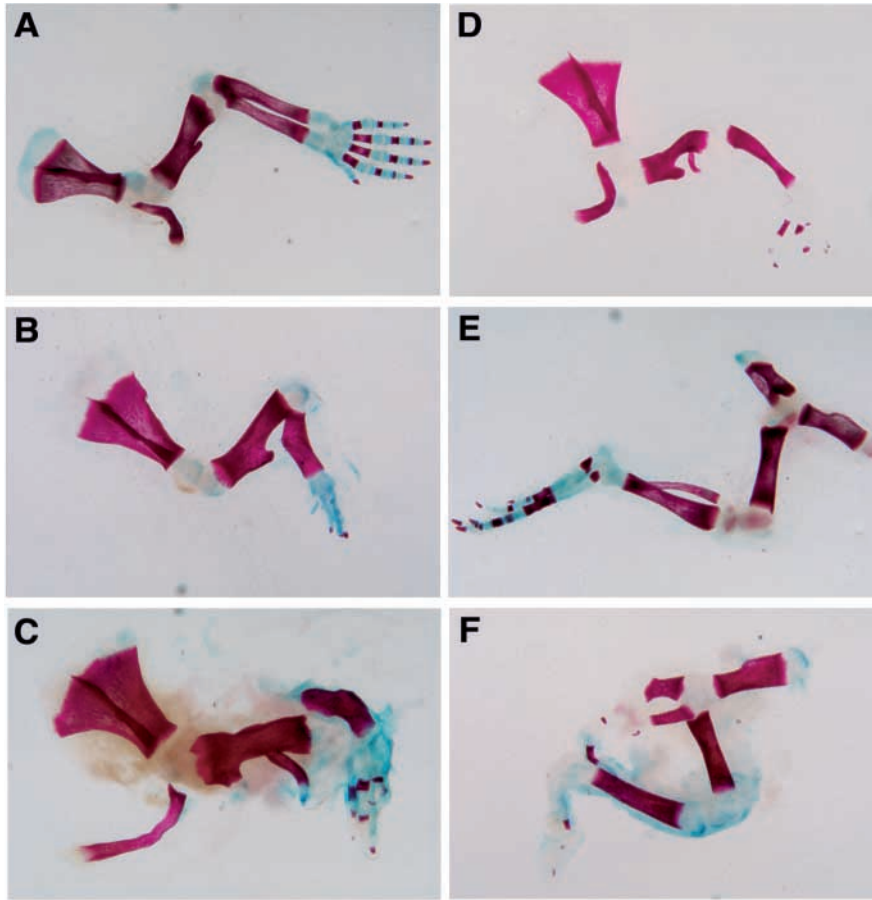
In order to determine the localization of *Fgfr2 $\Delta 2/\Delta 2$*  mutant cells at mid-gestation, we examined the distribution of  $\beta$ -galactosidase-positive cells in control *Fgfr2 $^{+/+}$*   $\leftrightarrow$  *Fgfr2 $^{+/+}$*  (Fig. 3A) and mutant *Fgfr2 $\Delta 2/\Delta 2$*   $\leftrightarrow$  *Fgfr2 $^{+/+}$*  chimeras (Fig. 3B,C). Three grades of chimerism were observed. Limb buds of low-grade chimeras ( $n=143$ ), which were predominantly  $\beta$ -galactosidase negative (not shown), and those of the control ( $\beta$ -galactosidase positive) chimeras (Fig. 3A), were phenotypically normal. Medium grade chimeras ( $n=82$ ) had abnormally shaped limb buds (Fig. 3B; compare Fig. 4A,B with 4C,D), whereas high-grade chimeras ( $n=91$ ), composed of a high proportion of mutant cells, had no limb buds (Fig. 3C) and resembled the loss-of-function mutations of *Fgfr2* or *Fgfr2b* (Arman et al., 1999; De Moerlooze et al., 2000). The abnormally shaped limb buds of medium grade chimeras (Fig. 3B), as well as the limb fields of high grade chimeras (Fig. 3C), contained significantly less  $\beta$ -galactosidase-positive mutant cells than other areas of the flank.

To assess the contribution of mutant cells to different tissues, limb buds of mutant and control chimeras were examined in histological sections. Medium-grade mutant limbs showed a lobular structure consisting of two or more parts (Fig. 4A-D; Fig. 5A,B). Control chimeras exhibited  $\beta$ -galactosidase-positive cells in the mesenchyme, in the surface ectoderm and in the AER (Fig. 4B). Limb buds of *Fgfr2 $\Delta 2/\Delta 2$*   $\leftrightarrow$  *Fgfr2 $^{+/+}$*  chimeras displayed numerous  $\beta$ -galactosidase-positive mutant cells in the mesenchyme, although, significantly, fewer mutant cells were detected in the surface ectoderm, and the AER, where present, was devoid of them (compare Fig. 4E and F).

To validate this observation,  $\beta$ -galactosidase-stained cells were counted in the trunk ectoderm, in the limb ectoderm and in the AER (Table 1). Significantly fewer mutant than wild-type cells colonized the limb ectoderm than the trunk ectoderm, nevertheless each sectioned limb bud contained them. In the AER of mutant chimeras, only one in ten sections contained a single *Fgfr2 $\Delta 2/\Delta 2$* -derived cell, whereas each



**Fig. 1.** Defective limb morphogenesis in perinatal chimeras. Fixed whole-mount preparations, with the exception of C, which is labeled for  $\beta$ -galactosidase. Arrows indicate digits or mini-limbs with reversed polarity. A-D are forelimbs, E and F are hindlimbs.

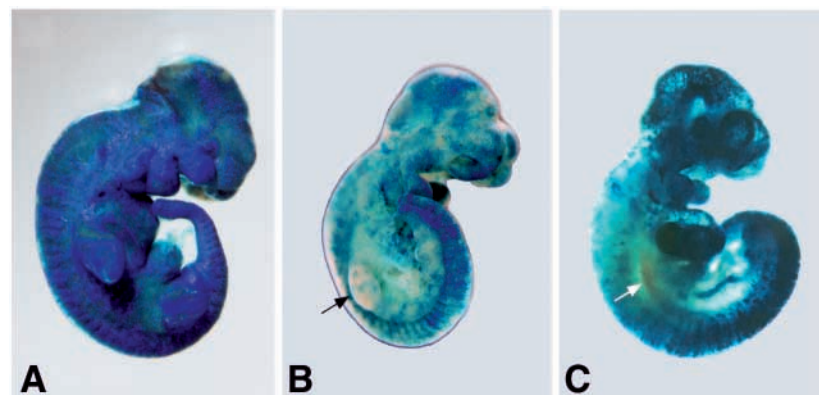


**Fig. 2.** Skeletal defects in perinatal chimeras. (A-C,E,F) Alcian Blue and Alizarin staining. (D) Alizarin staining only. A and E show control fore- and hindlimbs, respectively. B-D show chimeric forelimbs; F shows a chimeric hindlimb.

section of the control chimeras contained three to five  $\beta$ -galactosidase-positive cells in the AER.

### Interruption and displacement of the AER

Chimeric limb buds formed bi-lobed or multi-lobed structures (Fig. 5A,B). The lobes were mainly along the anteroposterior axis, but some limb buds had dorsally or ventrally placed lobes as well (Fig. 5B). To determine whether the AER-like structures visible at the distal tip of some lobes showed gene expression indicative of AER function, we carried out whole-



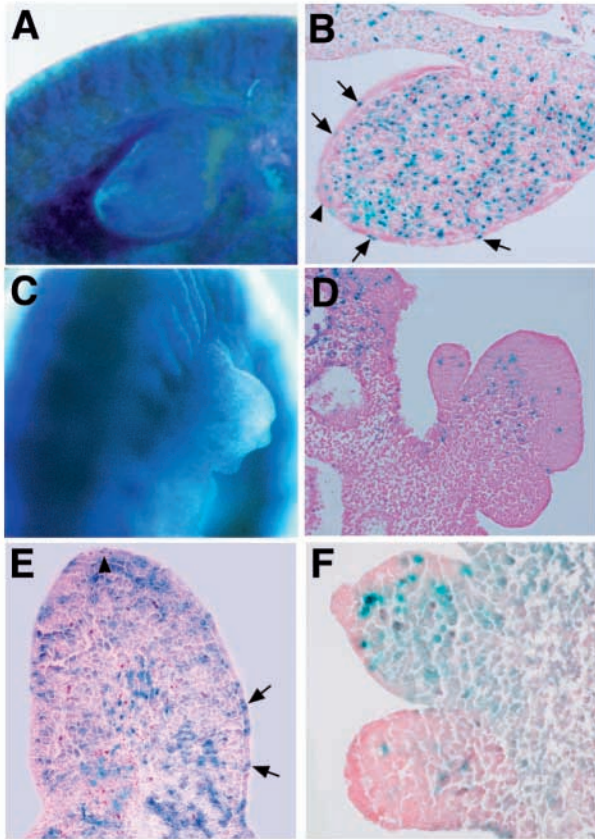
mount in situ hybridization with a number of AER markers, including *Fgf8* (Fig. 5C-F), *Bmp4* (Fig. 5G,H), *Dlx2* (Fig. 5I,J) and *Cd44* (Fig. 5K,L). The result revealed that gene expression characteristic of the AER was present, where a ridge-like projection could be seen. These lobes or projections in some limb buds were arranged along a line corresponding to the normal AER (Fig. 5D,H,L), whereas in others they were displaced from the apparent mid-line (Fig. 5E,F,L). In most of these areas, the hybridization signals were not significantly weaker than in the controls, despite fragmentation of the AER.

As AER signals induce proliferation of the limb mesenchyme, we reasoned that variation in the level of AER signaling might be responsible for the lobular structure of the chimeric limb. This could be explained either by local variation in the rate of cell proliferation, or by cell death. To distinguish between the two possibilities, cell proliferation and cell death was assessed by BrdU uptake and TUNEL assay, respectively, in sections cut in the anteroposterior plane (plane of the AER, if present; Fig. 5M,N). Immunohistochemical localization of BrdU revealed significantly fewer proliferating cells in the PZ under the trough areas than beneath the AER-containing peaks (Table 2). By contrast, there was no difference in the (low) levels of cell death exhibited by the limb buds of wild-type and chimeric mid-gestation embryos (Fig. 5M,N). We conclude that the lobular structure of the chimeric limb bud results from local reduction of mesenchymal proliferation under the AER-free areas, thereby diminishing proximodistal outgrowth.

### Gene expression in distal and posterior mesenchyme

It is known that AER-derived FGF signals regulate and maintain *Msx1* expression in the PZ (Sun et al., 2002), and *Shh* expression in the ZPA (Niswander et al., 1994). Consistent with these mechanisms, we found that in the limb buds of mid-gestation *Fgfr2 $\Delta$ 2/ $\Delta$ 2*  $\leftrightarrow$  *Fgfr2 $^{+/+}$*  chimeras, *Msx1* expression in the PZ mesenchyme was reduced where the AER was absent, i.e. in the notches between lobes (Fig. 6A,B), whereas *Shh* expression was reduced in the ZPA (Fig. 6C,D). We conclude that *Fgfr2*

**Fig. 3.** Mid-gestation chimeric embryos. (A) Control *Fgfr2 $^{+/+}$*   $\leftrightarrow$  *Fgfr2 $^{+/+}$*  chimera; (B) moderate and (C) high-level *Fgfr2 $\Delta$ 2/ $\Delta$ 2*  $\leftrightarrow$  *Fgfr2 $^{+/+}$*  aggregation chimeras. All were recovered at E10.5 and stained for  $\beta$ -galactosidase. Limb development is absent in the high-grade mutant chimera (C), and is impaired in the medium-grade mutant chimera (B; arrows).

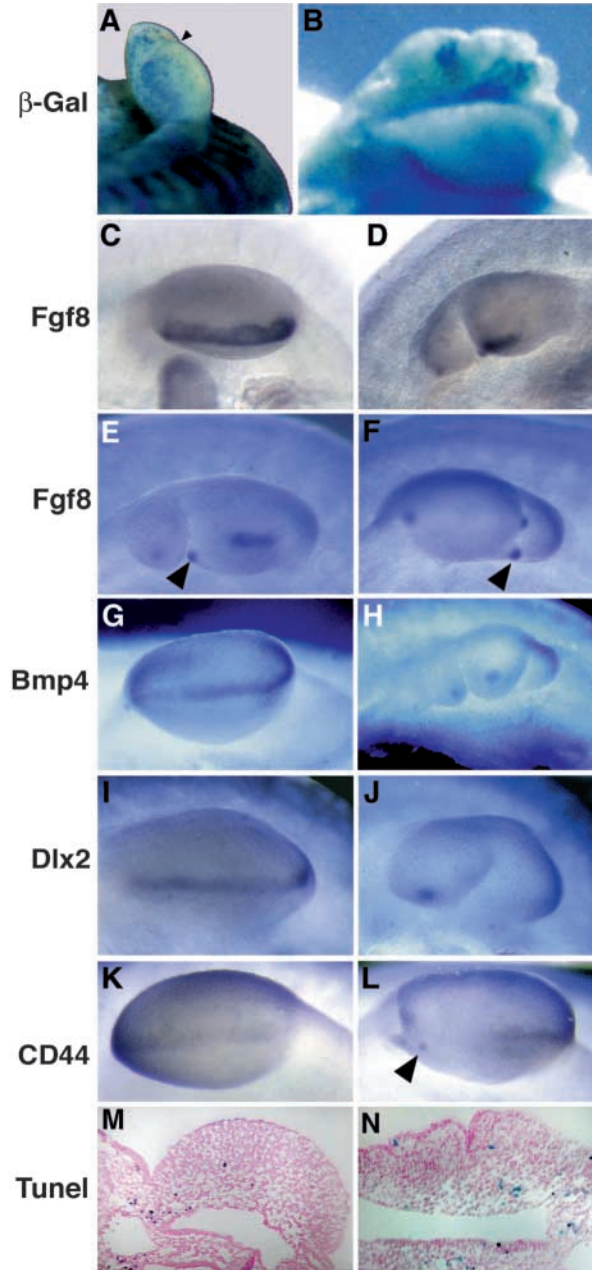


**Fig. 4.** Mutant cells do not colonize the AER. Localization of *Fgfr2*<sup>Δ2/Δ2</sup> mutant cells in chimeric limb buds, as shown by (A,C) whole-mount  $\beta$ -galactosidase staining and (B,D,E,F) histological sections of  $\beta$ -galactosidase-stained limb buds. (A,B,E) Control chimera; (C,D,F) mutant chimera. Arrowheads indicate the AER, arrows point to  $\beta$ -galactosidase-positive cells in the surface ectoderm. Note that in D and F there is little contribution of mutant cells to the surface ectoderm, and none to the AER.

plays a role in the FGF-mediated maintenance or induction of *Msx1* in the PZ, and of *Shh* in the ZPA.

#### Changes in the ventral and dorsal limb ectoderm

In the light of the observed gene expression patterns, which indicated local discontinuity of AER function, we wished to investigate whether cells of the dorsal and ventral limb ectoderm were also affected. We therefore investigated the localized expression of *En1* and *Wnt7a*, which are involved in specifying the distal ventral and dorsal limb patterns, respectively (Parr and McMahon, 1995; Loomis et al., 1996). Transcription of *En1*, a marker of the ventral limb ectoderm and AER (Fig. 7A), was significantly reduced in the mutant chimera (Fig. 7B). It was present around the distal edge of the chimeric limb bud, whereas in the more proximal ventral ectoderm, the signal presented only punctuate and striped hybridization signals (Fig. 7B). In three chimeric limb buds, small areas of ectopic *En1* expression were observed in the dorsal ectoderm (Fig. 7C), suggesting defects in recognition of the dorsoventral boundary. *Wnt7a* expression was much reduced; it was detected at low levels in the distal dorsal ectoderm, with some additional punctuate domains more



**Fig. 5.** Interruption and ectopic displacement of the AER, and low-level cell death in *Fgfr2*<sup>Δ2/Δ2</sup> ↔ *Fgfr2*<sup>+/+</sup> chimeras. (A,B)  $\beta$ -galactosidase staining of and (C-L) whole-mount in situ hybridization in control (C,G,I,K) and *Fgfr2*<sup>Δ2/Δ2</sup> ↔ *Fgfr2*<sup>+/+</sup> mutant chimeric (A,B,D,F,H,J,L) mice. (M,N) TUNEL assay for the detection of apoptotic cells in control (M) and *Fgfr2*<sup>Δ2/Δ2</sup> ↔ *Fgfr2*<sup>+/+</sup> mutant chimeric (N) mice. Staining and probes are shown on the left side of the images. Note the disruption and displacement of AER markers in mutant chimeras (arrowheads in E,F and L).

proximally (Fig. 7D,E). Ventral transposition of *Wnt7a* expression was, however, not observed in our material.

#### Exclusive expression of differentiation markers by wild-type cells

The observed effects on *En1* and *Wnt7a* gene expression domains correlate with morphological abnormalities of

**Table 1. Mutant cells do not colonize the AER of chimeric limb buds**

	Number of <i>lacZ</i> -positive cells/section (average)		
	Surface ectoderm	Limb ectoderm	AER
Wild-type	24	7	4
<i>Fgfr2</i> <sup>+/+</sup> ↔ <i>Fgfr2</i> <sup>+/+</sup> chimera	20	5	3
	37	8	4
	42 (30.8)	9 (7.25)	5 (4.0)
Mutant	12	3	0
<i>Fgfr2</i> <sup>Δ2/Δ2</sup> ↔ <i>Fgfr2</i> <sup>+/+</sup> chimera	10	3	0
	20	1	0
	18	3	0
	16	4	0
	24	1	0
	29	2	0
	28	2	0
	30	5	1
	16 (20.3)	4 (2.8)	0 (0.1)

Each line represents one section. In each section all β-galactosidase-positive cells were counted. The sections are consecutive 10 μm sections derived serial sectioning.

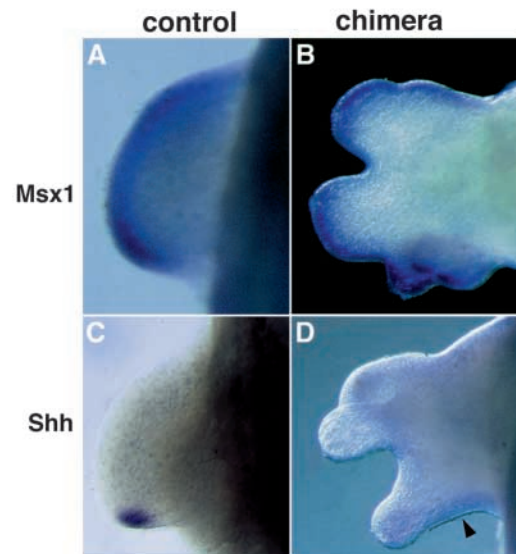
**Table 2. Proliferation defects in the progress zone of *Fgfr2*<sup>Δ2/Δ2</sup>↔*Fgfr2*<sup>+/+</sup> limb buds**

Relative number (N×10 <sup>3</sup> /μm <sup>2</sup> ) of BrdU-positive cells in the progress zone mesenchyme		
Wild-type limb bud	Trough areas of mutant chimeras	Non-affected areas of mutant chimeras
10.1	6.5	8.3
8.5	4.8	11.7
9.2	6.8	11.6
12.5	6.8	10.4
9.6	6.4	10.0
9.3	5.3	10.2
9.7 (9.8±1.3)*	6.1 (6.2±1.0)	9.7 (10.3±1.2)

\*Mean±s.d.

BrdU-positive cells were quantified in the distal limb bud mesenchyme. Cell number in areas where constrictions occur (notched areas) and in the non-affected areas was evaluated (see Materials and methods).

perinatal limbs, mid-gestation limb buds and their AER. It is therefore possible that *Fgfr2* is required for the normal function of these genes during establishment of the dorsoventral boundary. To test this hypothesis, we investigated the effect of lack of *Fgfr2* on *En1* and *Wnt7a* expression. In situ hybridization to detect *En1* and *Wnt7a* expression was performed at the inception of hind limb outgrowth (E9.5) in wild-type and homozygous non-chimeric *Fgfr2*<sup>Δ2/Δ2</sup> mutant embryos. Both markers were detectable in the wild type (Fig. 8A,C), but were absent from the mutant (Fig. 8B,D). This demonstrates that FGF signaling is upstream of *Wnt7a* and *En1* expression in the developing limb, and suggests that in our chimeras these genes were expressed exclusively by wild-type cells. The absence of mutant cells from the AER-like structures similarly suggests that the AER markers were also expressed by wild-type cells, and that the wild-type and mutant components were separately localized in the chimeric ectoderm.



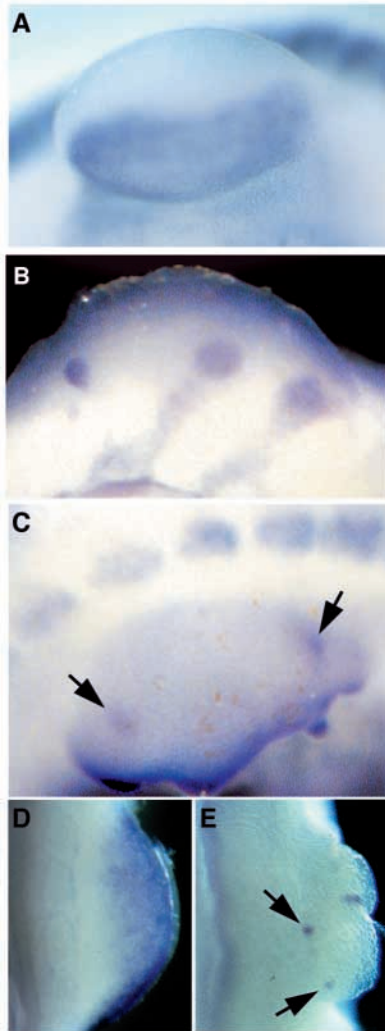
**Fig. 6.** Reduced expression of mesenchymal markers in the chimeric limb bud. Whole-mount in situ hybridization. (A,B) *Msx1* signals in the wild-type and chimeric progress zone mesenchyme. (C,D) *Shh* expression in the zone of polarizing activity (ZPA). (A,C) Wild type; (B,D) Chimera. Arrowhead indicates a faint *Shh* signal in the ZPA of the mutant chimeric forelimb bud.

To verify these interpretations we carried out a double-labeling experiment. Chimeric limb buds were stained with the pink Salmon-gal reagent to detect β-galactosidase-positive mutant cells. This was coupled with in situ hybridization, to identify *Fgf8* (Fig. 9A) or *En1* (Fig. 9B) expression. β-galactosidase-positive pink staining, representing the mutant, was observed in randomly placed patches on the surface of the chimeric limb bud. *Fgf8* hybridization could be seen in the fragmented and displaced AER, and did not overlap with the pink patches representing mutant ectoderm; Fig. 9A shows an example of a patch of pink (mutant) cells in a shallow trough adjacent to, but separate from, an *Fgf8*-positive fragment of AER. *En1*-positive patches and β-galactosidase-expressing mutant cells were also mutually exclusive in the ventral distal ectoderm, confirming that *En1* is expressed only in wild-type cells (Fig. 9B).

## Discussion

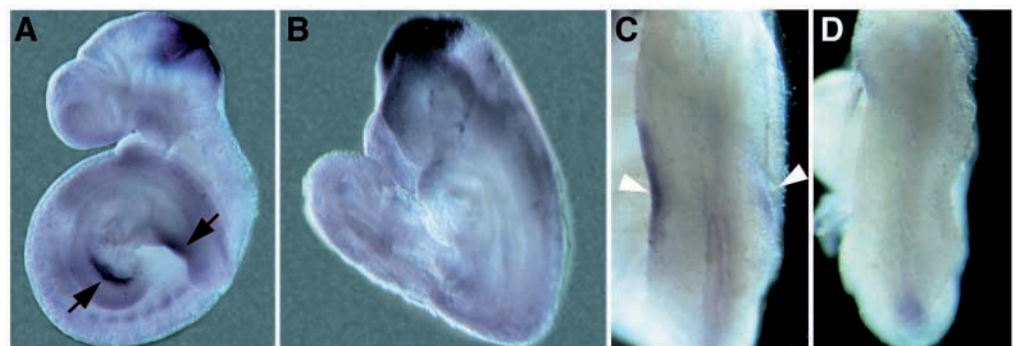
### The *Fgfr2*<sup>Δ2/Δ2</sup>↔*Fgfr2*<sup>+/+</sup> chimera reflects the activity of *Fgfr2b*

In this study, we have made chimeric embryos composed of a mixture of wild-type cells and cells homozygous for an *Fgfr2* mutation that abrogates synthesis of the functional receptor. *Fgfr2* has two transcriptional alternatives, *b* and *c*, that are created through alternative splicing of exon 8 or exon 9 (for a review, see Johnson and Williams, 1993). Both forms are expressed in the two-cell embryo, and at least one of the two is active in the trophectoderm (Haffner-Krausz et al., 1999). After implantation, from E8.5 onward, the *b* alternative is transcribed in the surface ectoderm throughout development, whereas the *c* form is present in various mesenchymal tissues (Orr-Urtreger et al., 1993). Although the *Fgfr2*<sup>Δ2</sup> mutation used

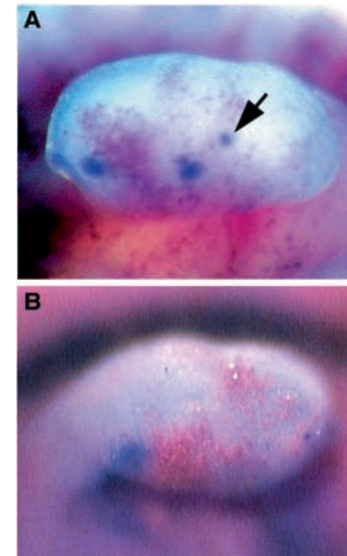


**Fig. 7.** Downregulation of *En1* and *Wnt7a* expression, and the ectopic localization of *En1*-positive cells in the dorsal ectoderm. (A-C) *En1* and (D,E) *Wnt7a* hybridization in wild type (A,D) and chimeras (B,C,E). B shows a ventral view, and C the dorsal view of *En1* hybridization in the chimeric limb bud. Arrows indicate patches formed by *En1* or *Wnt7a* in the dorsal ectoderm.

here affects both alternatives, the limb abnormalities observed are attributable to loss of *Fgfr2b*, which is specifically required for limb development and for multiple events in branching



**Fig. 8.** *En1* and *Wnt7a* expression in the limb fields of (non-chimeric) *Fgfr2*<sup>Δ2/Δ2</sup> E9.5 mutant embryos. A and B show *En1* (arrows), and C and D show *Wnt7a* (arrowheads) in situ hybridization. (A,C) Wild type. (B,D) Mutant embryos. Note that neither marker is expressed in the limb fields of the mutant.



**Fig. 9.** The mutually exclusive localization of (A) *Fgf8*- or (B) *En1*-positive wild-type cells (purple) and  $\beta$ -galactosidase-positive mutant cells (pink) is shown by double staining. Note, *Fgf8*- and *En1*-positive cells detected by in situ hybridization are observed in the AER;  $\beta$ -galactosidase staining with the pinkish Salmon-gal chromophore indicates mutant cells. Arrow indicates dorsally shifted ectopic AER fragment.

morphogenesis (De Moerloose et al., 2000), rather than to the loss of *Fgfr2c*, which affects skeletal growth but not patterning (Eswarakumar et al., 2002).

The limb abnormalities observed in our chimeras included patterning defects in all three proximodistal limb elements, i.e. in the stylopod, zeugopod and autopod, indicating that *Fgfr2b* is required throughout the entire period of limb outgrowth. Changes in the number and dorsoventral alignment of digits indicated effects on dorsoventral patterning, whereas the position of ectopic limb rudiments deviating from the site of normal limb outgrowth indicated defects in the anteroposterior axis. These observations on late gestation chimeras were consistent with the disrupted AER structure and with disruption of the known functions of genes whose expression patterns were altered at mid-gestation (Niswander, 2003). We therefore conclude that *Fgfr2b* is required for normal patterning during the development of all three limb axes. This is in accord with suggestions that the dorsoventral and proximodistal pre-pattern is established during early limb

development (Dudley et al., 2002; Sun et al., 2002). The displaced mini-limbs additionally indicate that the mutation affects the determination of limb position and polarity.

### ***Fgfr2* activity is required for AER differentiation**

Altabef et al. demonstrated that cells that form the AER originate from a population scattered within a broad area of early ectoderm (Altabef et al., 1997; Altabef et al., 2000). These converge on the limb bud as it grows out from the flank and form the AER at the dorsoventral compartment boundary of the trunk. It was therefore interesting and significant that our high-grade mid-gestation chimeras showed a lower level of mutant cells in the limb field than elsewhere in the trunk, even than where limbs failed to develop. Moreover in the abnormal limb buds of medium-grade chimeras, the proportion of mutant cells was lower in the limb than in the trunk ectoderm, and there were almost no mutant cells in the AER. This suggests that the movement of mutant ectodermal cells towards and into the incipient limb bud and AER is inefficient compared with the wild type in the absence of *Fgfr2*.

Although the surface ectoderm of the embryo transcribes *Fgfr2* before limb outgrowth (Orr-Urtreger et al., 1991), its loss in non-chimeric mutant embryos does not affect the basic structure of the embryonic integument (De Moerloose et al., 2000). Absence of mutant cells from the AER fragments is consistent with the interpretation that *Fgfr2b* is required for differentiation of the simple squamous surface epithelium into the pseudostratified structure of the AER. Double detection of mutant cells and AER-specific genes revealed that patches of *Fgf8* expression coincided with, and were restricted to, patches of morphologically distinguishable AER. We therefore suggest that the requirement for *Fgfr2* in AER differentiation is cell-autonomous. AER morphogenesis is correlated with the onset of AER-specific gene expression; Xu et al. (Xu et al., 1998) observed that the loss of *Fgfr2* results in failure of the mutant ectoderm to express *Fgf8* in response to *Fgf10* signals from the mesenchyme, and hence the failure of limb-bud formation.

### ***Fgfr2* affects multiple aspects of the limb pattern**

Many defects of our chimeras affected the dorsoventral limb pattern, which depends on the dorsoventral compartment boundary. This boundary is established in the prospective limb mesenchyme; it originates in the somatic or lateral mesoderm, from where it is transferred to the prospective limb ectoderm before limb outgrowth (Michaud et al., 1997; Altabef et al., 1997) and its timing is in good agreement with the timing of *Fgfr2b* expression (Orr-Urtreger et al., 1993). Thus, transcription of *Fgfr2b* in the surface ectoderm is in synchrony with the formation of the dorsoventral compartment boundary. Nevertheless, because the receptor is expressed in both the dorsal and ventral ectoderm, *Fgfr2* function may be necessary to enable, but not to specify, boundary formation. *Wnt7a* is an early effector of the distal dorsoventral limb pattern (Altabef and Tickle, 2002; Parr and McMahon, 1995). It induces *Lmx1b* in the dorsal limb mesenchyme, whereas *En1* in the ventral ectoderm restricts *Wnt7a* expression to the dorsal side (for a review, see Chen and Johnson, 1999). *Wnt7a* expression in *En1* mutants (Cygan et al., 1997), and forced *En1* expression in the entire limb bud (Kimmel et al., 2000), induced AER displacements similar to those described here.

Defective AER differentiation in our chimeras was correlated with reduced transcription of *Wnt7a* and *En1* in the dorsal and ventral ectoderm, respectively. Failure of expression of *Wnt7a* and *En1* in non-chimeric *Fgfr2* mutant embryos, at and before limb-bud outgrowth, suggests that FGFR2 signaling is upstream of the expression of these two genes. This result is consistent with a previous report on the role of FGF signaling in the control of *Wnt7a* expression (Altabef and Tickle, 2002). Ectopic expression of *En1* in the dorsal limb-bud ectoderm, and its restriction to the wild-type component of the chimera, indicates that *Fgfr2b* is required either to restrict *En1* gene expression to the ventral surface of the limb bud, or to prevent cells initiating on the ventral surface from migrating to the dorsal surface. We assume that the observed dorsal and palmar outgrowth of digits, and the reversal of dorsoventral polarity of some digits, were also due to these effects.

In spite of abnormal dorsoventral gene expression, the epidermis (hair, nails, foot pads) of late gestation limbs did not exhibit dorsoventral patterning defects. This indicates that partial loss of *Fgfr2* function did not affect later epidermal differentiation events. The abnormal position of the digits may have been due to the presence of mutant cells in the chimeric limb ectoderm. We have shown that mutant and wild-type cells form patches in the limb ectoderm. It stands to reason that patches of mutant ectoderm, which cannot form AER, when lodged at the ridge of the limb bud, hinder the accurate homing of wild-type cells to the dorsoventral boundary, and force them to differentiate at positions slightly dorsal or slightly ventral from it.

Defective *En1* and *Wnt7a* activation, and the hindrance of AER positioning by the mutant component of the chimera, may explain defects in the distal limb bud and in the autopod of later limbs. However, they do not explain the frequent loss of the radius, the bifurcation of the humerus and the de novo generation of ectopic mini-limbs. The first two may be consistent with abnormal mesenchymal proliferation and patterning due to reduced signaling from the limb ectoderm and AER. The mechanism of generation of ectopic limb rudiments is more difficult to understand, but presumably involves displacement of normal AER-forming cells away from the normal limb-forming site.

The chimeric defects observed here affect a succession of epithelial-mesenchymal interactions, which take place during limb development, from its earliest phase until the formation of digits and phalanges. Their failure in *Fgfr2*<sup>Δ2/Δ2</sup> ↔ *Fgfr2*<sup>+/+</sup> chimeras is indicated by the gene expression abnormalities observed at mid-gestation, and emphasizes the role of *Fgfr2b* in a whole series of these interactions. The most significant contributions made by this study are the findings: (1) that *Fgfr2* is involved in ectodermal cell movement towards and into the limb bud; (2) that it is required for the formation and function of the AER; and (3) that *Fgfr2* is upstream of *En1* and *Wnt7a*, and is involved in ventral restriction of the *En1* domain.

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