Two lineage boundaries coordinate vertebrate apical ectodermal ridge formation

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Proximal–distal outgrowth of the vertebrate limb bud is regulated by the apical ectodermal ridge (AER), which forms at an invariant position along the dorsal–ventral (D/V) axis of the embryo. We have studied the genetic and cellular events that regulate AER formation in the mouse. In contrast to implications from previous studies in chick, we identified two distinct lineage boundaries in mouse ectoderm prior to limb bud outgrowth using a Cre/loxP-based fate-mapping approach and a novel retroviral cell-labeling technique. One border is transient and at the limit of expression of the ventral gene En1, which corresponds to the D/V midline of the AER, and the second border corresponds to the dorsal AER margin. Labeling of AER precursors using an inducible Cre showed that not all cells that initially express AER genes form the AER, indicating that signaling is required to maintain an AER phenotype. Misexpression of En1 at moderate levels specifically in the dorsal AER of transgenic mice was found to produce dorsally shifted AER fragments, whereas high levels of En1 abolished AER formation. In both cases, the dorsal gene Wnt7a was repressed in cells adjacent to the En1-expressing cells, demonstrating that signaling regulated by EN1 occurs across the D/V border. Finally, fate mapping of AER domains in these mutants showed that En1 plays a part in positioning and maintaining the two lineage borders.

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The coordinated activity of factors expressed in specific localized domains patterns the limbs along three asymmetric axes. A multilayered columnar epithelium, called the apical ectodermal ridge (AER), forms at what is thought to be the junction between dorsal and ventral ectoderm and produces growth factors that promote proximal–distal outgrowth. Signals from the presumptive limb mesenchyme are required to induce an AER, whereas signaling within the ectoderm apparently directs positioning and maturation of the AER [Irvine and Vogt 1997; Niswander 1997; Zeller and Duboule 1997]. Although mesodermal signaling molecules that can activate AER gene expression have been identified [Min et al. 1998; Sekine et al. 1999], the process of ridge assembly and localization is less well understood and only few molecules involved have been identified [Tickle and Altabet 1999].

During Drosophila appendage formation, interactions at boundaries between cells of differentially specified domains leads to the formation of specialized signaling centers that generate proximal–distal outgrowth. It has been hypothesized that a limb ectoderm dorsal–ventral (D/V) boundary exists that is analogous to the imaginal disc compartment border and is important for AER development [Irvine and Vogt 1997; Niswander 1997; Zeller and Duboule 1997; Loomis et al. 1998; Martin 1998; Tickle and Altabet 1999]. The relevance of such a boundary for AER formation was supported by the observation that ectopic limbs induced by FGF beads implanted into the interlimb flank region develop in precise alignment with the normal lungs [Cohn et al. 1995; Crossley et al. 1996; Vogel et al. 1996; Altabet et al. 1997]. In addition, transplant and dye labeling experiments in chick provided evidence for compartments within limb ectoderm, but the two experimental approaches identified different potential borders in the AER [Altabet et al. 1997; Michaud et al. 1997]. Chick–quail transplants showed a lineage restriction in the mid-
line of the AER when dorsal and ventral tissues were apposed, whereas dye labeling experiments showed dorsally and ventrally derived cells mixing within the AER, although cells never crossed into the opposing ectoderm domain [Altabef et al. 1997; Michaud et al. 1997]. The different results obtained in these two studies have not been reconciled and may be due to differences in the cell-labeling techniques and analysis with respect to region and time.

Unlike the direct induction of signaling centers along compartment boundaries in Drosophila, formation of the mature AER appears to involve the convergence of broadly distributed precursor cells towards the dorsal–ventral D/V boundary of the limb field [Altabef et al. 1997; Martin 1998]. Chick–quail transplantation studies have indicated that cells giving rise to the mature AER are initially located in a broad region of D/V ectoderm just prior to the formation of a thickened ridge [Michaud et al. 1997]. Consistent with this, in mouse, AER marker gene expression is initially observed in a broad domain of thickened ventral ectoderm (the pre-AER) and progressively becomes restricted to the triangular AER at the distal margin [Milaire 1974; Crossley and Martin 1995; Mahmood et al. 1995; Bell et al. 1998; Loomis et al. 1998]. Studies using diI labeling, however, showed mixing of AER precursors with cells destined to be non-AER ectoderm prior to limb outgrowth [Altabef et al. 1997]. Lineage analysis is necessary to establish whether there is a distinct population of committed AER precursors [Tickle and Altabef 1999].

Clues that distinctly specified dorsal and ventral limb domains play a role in AER formation have come from the finding that some molecules expressed in restricted domains along the D/V axis of the limb are required for proper AER morphology in addition to D/V patterning. Dorsal ectoderm expresses the secreted factor WNT7A, which promotes dorsal-type differentiation through induction of the transcription factor LMX1B in the underlying mesenchyme [Riddle et al. 1995; Vogel et al. 1995]. Loss of Wnt7a or Lmx1b function leads to double ventral identities within the developing AER [Tickle and Altabef 1999].

Results

En1 Expressing cells respect a transient lineage restriction in the AER

A recently developed Cre/LoxP system was used to examine whether En1-expressing cells are restricted from crossing the D/V boundary of the AER [Zinyk et al. 1998]. This approach involves expressing a site-specific recombinase transiently in specific cells, which induces a recombination event that leads to permanent expression of lacZ. The distribution of the marked cells and their progeny can then be detected at subsequent stages. Mice expressing Cre recombinase from an En1 knock-in allele [En1Cre] (Hanks et al. 1995) were bred with a universal lacZ reporter mouse strain [called R26R, Soriano 1999] and lacZ-expressing cells were examined at embryonic stages [Fig. 1A].

lacZ expression could be detected in En1Cre;R26R
was injected into the amniotic fluid of wild-type expressing retrovirus, pNK–lacZ (Gaiano et al. 1996), the ventral ectoderm and pre-AER. Prior to stage 4 (11.5 dpc) double transgenic limb buds as early as stage 1 (E9.5) in 1998; Gaiano et al. 1999). A replication-defective guide retroviral infection of limb ectoderm (Liu et al. 1998) was used to determine where the D/V border within the AER was located. Nevertheless, lacZ was expressed throughout the thickened remnant of the ridge (Fig. 1I). Examination of En1 expression between stage 2 (10.0 dpc) and stage 6 (12.5 dpc) showed that En1 RNA was detected in the ventral ectoderm and ventral AER prior to stage 4 [Fig. 1B,C] but was also present in the dorsal AER at later stages [Fig. 1D,E]. Thus, it is not certain whether dorsal AER cells initiate En1 expression after stage 4 or if dorsal AER cells are lost.

To provide further evidence for a lineage restriction within the AER that was independent of possible changes in gene expression, we performed a lineage analysis using ultrasound backscatter microscopy (UBM) to guide retroviral infection of limb ectoderm (Liu et al. 1998; Gaiano et al. 1999). A replication-defective lacZ-expressing retrovirus, pNK–lacZ (Gaiano et al. 1996), was injected into the amniotic fluid of wild-type embryos in utero at 8.5 dpc (prior to limb bud outgrowth), allowing surface ectoderm cells to be randomly infected and transmit lacZ expression to their descendants. Two or three days later, embryos were harvested and stained with X-gal. The level of infection varied greatly between embryos, with some being densely covered with blue cells and others having only a few scattered patches. The dispersal of the clustered cells on lightly infected embryos, with some being densely covered with blue cells and others having only a few scattered patches. The dispersal of the clustered cells on lightly infected embryos varied according to location within the limb bud. Patches of cells found proximally tended to be loosely associated and irregularly shaped [Fig. 2E, yellow oval]. By contrast, tightly clustered cells were found within the AER, appearing to align along the margin [Fig. 2A,E, red box].

For our analyses, we chose only embryos with few cell clusters in the limbs that included clusters aligned along the distal limb margin separated from other blue cells by ~10 cell diameters or more. Because lipophilic dye labeling experiments in chick have shown that extensive cellular rearrangements do not occur in the AER (Vargesson et al. 1997) and retroviral infection of mouse ectoderm at 9.0 dpc has shown that the progeny of infected cells remain in cohesive clusters (Sanes et al. 1986), the discrete cell clusters examined were assumed to be the progeny of a single or at most two infected parent cells. Limbs containing clusters of 6 cells or more [average of 8] were sectioned along the D/V axis of the limb bud to determine the distribution of cells with respect to the AER. lacZ-expressing AER cells in embryos dissected at 10.5 dpc (limb stages 2–3) were in all but one case (8/9 clus-
AER cells respect an additional dorsal boundary

A further unexpected finding was that in the retrovirally marked cell clusters examined, cells were generally found only within the AER and not also in the neighboring ectoderm. In a few exceptions, cells were seen in immediately adjacent ventral ectoderm (3/13) (Fig. 2H), and in one case in dorsal ectoderm (1/9). This suggested that additional borders might be present between the AER and the adjacent ectoderm domains. To investigate this possibility further, we used the Cre/LoxP system to label cells of the developing pre-AER or AER and follow their distribution with time. AER-specific gene expression was achieved using a fragment of the Msx2 promoter (Liu et al. 1994). Because this promoter is active over an extended time period of limb development, we used an inducible form of Cre (CreERT) (Feil et al. 1996; Brocard et al. 1997), which enabled us to modulate the time of initiation and the duration of Cre activity. Fusion proteins of a Cre recombinase and a tamoxifen-responsive estrogen receptor ligand-binding domain (ER7) can be activated in embryos by administering tamoxifen (tam) to the pregnant female following mating between a Cre transgenic and lacZ reporter mouse (Danielian et al. 1998). In control experiments, we found two injections of tam, at doses of up to 10.0 mg each within 12 hr of each other, caused no lethality. Furthermore, a dose of 5.0 mg generated robust reporter gene expression with Cre in an active state for a maximum of 24 hr.

Of nine transgenic founders produced with the Msx2–CreERT7 transgene (Fig. 3), eight transmitted the transgene, and four expressed CreERT in the AER, as determined by RNA in situ hybridization. The expression initiated at 9.5 dpc in the forelimbs and 10.0 dpc in the hindlimbs (stage 0.5/1) broadly in the ventral ectoderm and with development expression became restricted to the distal margin (Fig. 3A–C). The remaining studies were performed using one line (no. 27) that showed the strongest expression.

To examine the behavior of pre-AER cells with respect to the dorsal and ventral borders of the AER, 5.0 mg of tam was administered at 9.5 dpc, as expression of the transgene is initiating in the forelimbs and prior to initiation in the hindlimbs. The hindlimbs of embryos stained for lacZ ~30–36 hr following tam treatment (stage 2, 10.5 dpc) showed scattered blue staining in the ventral ectoderm and dense labeling in a broad band at the distal margin (Fig. 3D). In embryos treated with tam at 9.5 dpc and collected around 54–60 hr later (stage 4, 11.5 dpc), there was dense labeling of the narrow AER in hindlimbs, with additional scattered blue cells throughout the ventral ectoderm (Fig. 3E). The dorsal boundary of lacZ-expressing cells was clearly delineated at the dorsal edge of the AER at both stage 2 (10.5 dpc) and stage 4 (11.5 dpc) (Fig. 3D,E, arrow), whereas the ventral boundary was not, with blue cells extending proximally from the distal margin (Fig. 3D,E). Forelimbs showed a similar pattern of lacZ expression with the scattered ventral blue cells being located more distally. These results demonstrate that there is a restriction to cell movement at the dorsal limit of the AER from at least the beginning of formation of the pre-AER, whereas the ventral AER boundary is not determined at 9.5 dpc. The one case in the retroviral cell-marking studies in which a dorsal AER cluster extended into the dorsal ectoderm could reflect a polyclone or that the dorsal lineage restriction is not complete at 8.5 dpc. Interestingly, the presence of labeled pre-AER cells remaining in the ventral ectoderm following AER formation indicates that the initially specified population of AER precursors that express AER

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Figure 3. A border is present early at the dorsal AER margin [A–C] RNA in situ analysis of CreER expression in Mmx2–CreER transgenic embryos using a lacZ tag probe. Expression is seen in the ventral ectoderm at stage 1 [A], at the distal–ventral margin at stage 2 [B], and in the mature ridge at stage 4 [C]. Hindlimbs of embryos heterozygous for the Mmx2–CreER transgene and R26R, following injection with 5.0 mg of tam at 9.5 dpc and dissection after 30–36 hr [D] or 54–60 hr [E,G]. Labeled cells are seen in the ventral ectoderm and AER in stage 2 [D] and stage 4 [E] hindlimbs. [F] Cryosection across the D/V axis of a representative limb as in E, showing dense AER labeling in addition to blue cells in the adjacent ventral ectoderm. The dorsal AER margin is sharply delineated [D,F, red arrow]. Following injection with 5.0 mg of tam at 10.5 dpc, embryos collected after 30–36 hours showed intense β-gal activity in the AER, with a few positive cells in the ventral ectoderm at the extreme anterior and posterior margins of the handplate [G]. Sectioning across the D/V axis shows labeled cells restricted within the AER [H, arrow]. In all sections, distal is to the right and dorsal is up. [d] Dorsal, [v] ventral. [I] Diagrammatic representation of fate-mapping results. Injection at 9.5 dpc, when transgene expression is throughout pre-AER, results in blue cells scattered within the ventral ectoderm and densely in the AER at 11.5 dpc [top row]. When tam is given at 10.5 dpc, transgene expression is restricted to the AER, and labeled cells at 11.5 dpc are almost completely AER restricted. [J] Schematic diagram of the Mmx2–CreER transgenic construct. Cre-ER was inserted into a transgene construct containing the β-globin intron, LacZtag, and SV4opolyA, in which expression is driven by the Mmx2–AER promoter.

Misexpression of En1 throughout the AER inhibits limb formation

By analogy to patterning in Drosophila wing imaginal discs [Irvine and Vogt 1997], bisection of the vertebrate AER into dorsal and ventral domains by a lineage restriction provides a basis for signaling interactions to occur between these two domains that could be involved in AER morphogenesis. So far, vertebrate En1 is the only known gene to be expressed in only one of the AER D/V domains. Furthermore, the loss-of-function mutant studies in mice have shown En1 is required for proper AER formation. If ventral versus dorsal domains within the AER are required for its proper formation and En1 controls specification of the ventral domain, then misexpression of En1 in the dorsal AER should disrupt formation, or stability of, the D/V boundary and thereby should also disrupt AER formation, leading to limb abnormalities. To examine whether this is the case, En1 was misexpressed throughout the AER.

Of six Mmx2–En1 founder mice produced, one (no. 86) showed a dramatic limb phenotype characterized by severe truncation of both hindlimbs and deformed paws with absent digits and less severe syndactyly and oligodactyly in the forelimbs. Two additional founders (nos. 25 and 95) had normal-appearing limbs, but when bred to wild-type females gave pups with a phenotype similar to that seen in line no. 86. One founder transmitted the transgene but the litter had no phenotype and embryos did not show transgene expression. Two other founders gave normal litters and were not analyzed further. Analysis of several litters from founders nos. 25, 86, and 95 demonstrated that lines nos. 86 and 25 showed the phenotype in 100% of transgenic offspring, whereas only markers are not all committed to becoming part of the mature AER and do not maintain AER gene expression [Fig. 3I, top].

The autonomy of AER cells became apparent following administration of tam at later stages of limb development. Giving 5.0 mg of tam at 10.0 dpc resulted in blue cells in the ventral ectoderm and AER of stage 4 (11.5 dpc) hindlimb buds, as was described above for activation at 9.5 dpc [data not shown]. However, administration of tam at 10.5 dpc (hindlimb stage 2, forelimb stage 3), followed by lacZ staining 30–36 hr later (11.5 dpc, limb stage 4–5), resulted in blue labeling predominantly in the AER in both forelimbs and hindlimbs, with a well-demarcated ventral and dorsal boundary [Fig. 3G,H, arrow]. Only the most extreme anterior and posterior margins of the hand showed residual blue cells in the ventral ectoderm, with fewer such cells in forelimb as compared with hindlimb. This suggests that by 10.5 dpc, AER precursors have become committed to occupy the AER and no longer mix with ventral ectoderm, whereas pre-AER cells remaining in the ventral ectoderm do not maintain Mmx2 expression [Fig. 3I, bottom]. Furthermore, unlike the dorsal and D/V borders, the ventral boundary of the AER appears to be established after the AER becomes morphologically distinct.

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AER formation is perturbed when En1 is expressed throughout the AER

The severe phenotype of the Mxs2–En1 homozygous mutants suggested that misexpression of En1 had perturbed AER formation as it is reminiscent of the truncations produced in chick limbs following AER removal [Saunders 1948; Summerbell 1974]. Early limbs buds were therefore examined for expression of the AER marker gene Fgf8. In stage 1 transgenic Mxs2–En1 hemizygous limbs, Fgf8 was expressed normally in the ventral ectoderm of both forelimbs and hindlimbs [Fig. 5, cf. F and J to A]. By stage 2, the expression domain of Fgf8 in the forelimbs and hindlimbs of Mxs2–En1 hemizygous mutants showed irregular borders and an overall decrease in level as compared with wild types [Fig. 5, cf. G and K to B]. Fgf8 expression in stage 3 forelimbs became discontinuous, with the posterior portion being more affected [Fig. 5, cf. H, arrow, and C]. Hindlimb Fgf8 expression at stage 3 was more severely fragmented and showed clusters of expressing cells shifted dorsally or ventrally relative to the D/V margin [Fig. 5, cf. I, arrows, and C]. Forelimbs and hindlimbs became notched with further development, with Fgf8 expression present only along those portions of the distal handplate that had maintained proximal–distal outgrowth [Fig. 5, cf. I and M with D]. In addition, segments and patches of Fgf8 expression were seen anteriorly and at abnormal locations along the D/V axis in some hindlimbs [Fig. 5, cf. N with E]. The more marked disruption of Fgf8 expression seen in hindlimbs versus forelimbs correlates with the more severe hindlimb defects that are seen at birth. Sections of stage 2–3 forelimb buds across the D/V axis showed there was a range of AER phenotypes, from clearly visible but flattened ridges that contained patchy Fgf8 expression, to regions with no ridge or Fgf8 expression where limb outgrowth was reduced (not shown, see Fig. 6 for morphology). The large gaps in the handplate likely prefigure the missing bone elements seen in newborn paws, whereas small ectopic patches of ridge along the anterior margin could explain the occasional occurrence of ectopic digits.

In homozygotes, the hindlimb phenotype suggests early failure of AER formation. Although Fgf8 expression did initiate at stage 0.5/1 in such hindlimbs, by stage 2 there was virtually no Fgf8 expression. As expected, hindlimb bud outgrowth was severely compromised by 10.5 dpc [data not shown].

Expression of the transgene was examined using a

~60% of the transgenic offspring from line no. 95 displayed limb defects. All three lines were fully viable and fertile. Although our studies have concentrated on line no. 86, a similar range of defects was seen in affected transgenic animals from all three lines.

Homozygous transgenics had a more severe phenotype than hemizygotes, but in both genotypes the forelimbs were less severely affected than the hindlimbs [Fig. 4A]. Several litters of hemizygous and homozygous newborns [n = 25 animals] were examined in detail by bone and cartilage analysis. In the forelimbs of the homozygotes, the most common phenotype was loss of digits 3, 4 and/ or 5 [69%] [Fig. 4, cf. C with B]. Generation of only a single digit was seen in rare cases [3%]. The zeugopod was also affected with partial [8%] or complete loss of the ulna [8%]. In cases of partial ulna loss, the proximal portion is preserved, and the radius is bowed. The hindlimbs of hemizygotes frequently had a nearly complete absence of the autopod, with one malformed digit consisting of cartilaginous fragments distal to the zeugopod [67%] [Fig. 4, cf. F with E]. Occasionally, extra partial digits were seen in both forelimbs [10%] and hindlimbs [13%], which did not show any preferential anterior–posterior localization [Fig. 4H, arrowheads]. In addition, some hindpaws had ectopic digits protruding from the ventral handplate [Fig. 4J, arrow] or distal zeugopod [Fig. 4I, arrow] that contained poorly formed bony elements usually lost during processing [8% of limbs]. Homozygous transgenics were overall more severely affected, with forelimbs having only 1–2 digits [70%], or truncation below the humerus [10%, Fig. 4D]. 20% had more mildly affected forelimbs, with only digits 4 and 5 malformed. The majority of homozygotes lacked hindlimbs, with only small fragments of the femur present [80%, Fig. 4G]. Some homozygotes [20%] had a tibia and distal cartilaginous fragments, resembling the hemizygous phenotype.

Figure 4. Misexpression of En1 throughout the AER perturbs limb development. (A) Two-day-old mice hemizygous [center] or homozygous [right] for the Mxs2–En1 transgene have severe limb deformities, as compared to a wild-type [left]. Skeletal preparations of a wild type newborn mouse, showing the normal zeugopod, stylopod, and autopod [B,E]. Hemizygous transgenic showing loss of posterior digits 4–5 of the forelimb [C] and nearly complete absence of the autopod of the hindlimbs [F]. Severely affected homozygote with the forelimb truncated at the proximal stylopod [D] and truncation of hindlimbs at the proximal femur [G]. Ectopic outgrowths were seen infrequently and include extra digits within the plane of the handplate [H, arrowheads], protruding perpendicular to the ventral handplate [I, arrow], or from more proximal locations [J, arrow]. [h] Humerus; [r] radius; [u] ulna; [fe] femur; [t] tibia; [f] fibula.
Sections across the D/V axis of such limb buds showed genes was wavy instead of smooth (Fig. 6, cf. D and A). As Wnt7a function of the AER was reflected by decreased expression and distal limb notching, dysgenesis (data not shown). Consistent with loss of AER borders are disrupted in Msx2–En1 mutants

Both gene expression and fate mapping were used to examine changes in ectoderm specification and AER boundaries in hemizygous Msx2–En1 mutants. Forelimbs, in which AER formation is less severely perturbed, were examined in order to have some regions with morphological ridges to serve as a reference point. In forelimbs of wild-type 10.5 dpc embryos (stage 3 limbs), the dorsal boundary of the AER and Fgf8/Msx2 expression is coincident with the distal limit of Wnt7a expression, whereas the mid-AER border corresponds to the distal limit of En1 expression (Fig. 6A,B). The region lacking both En1 and Wnt7a expression is the dorsal AER (Fig. 6B). In Msx2–En1 transgenic 10.5 dpc forelimb buds labeled for En1 (endogenous and transgenic) as well as Wnt7a, the distal boundary of expression of both genes was wavy instead of smooth (Fig. 6C and A). Sections across the D/V axis of such limb buds showed En1 expression in the ventral ectoderm and throughout the flattened AER (described previously), but surprisingly, Wnt7a expression was not adjacent to this aberrant ridge (Fig. 6E). In regions along the anterior-posterior (A/P) axis where there was no thickened ectoderm visible, Wnt7a expression was also shifted proximally away from the En1 domain (Fig. 6F). Thus, the dorsal border of the AER, characterized by an abrupt morphological change from columnar AER cells to cuboidal dorsal epithelium that express Wnt7a, is perturbed in Msx2–En1 mutant limbs such that Wnt7a is not expressed adjacent to the ridges that form. In addition, the ridges have wavy, rather than straight, dorsal and ventral borders.

The position of the D/V border, as defined by endogenous En1 expression, was examined using lacZ expression from an En1 knock-in allele (En1 LacZ; Hanks et al. 1995; Matise and Joyner 1997). In wild-type 10.5-dpc forelimb buds, a rim of intense lacZ staining in the AER is readily apparent at the distal margin (Fig. 6C). In 10.5 dpc embryos heterozygous for En1 Lki and Msx2–En1, there did not appear to be significant lacZ staining at the distal margin and the distal border of expression was uneven (Fig. 6, cf. G with C). Sections across the D/V axis revealed that in some regions with flattened ridges, lacZ-expressing cells were not present in the ridge but were located more ventrally (Fig. 6H), whereas in regions without an AER, lacZ expression extended to the distal tip (Fig. 6I).

One possible reason for the apparent shift in En1 expression relative to the position of the AER is that endogenous En1 expression is initiated normally at early pre-AER stages but then is lost distally in mutants. To address this and to determine the initial position of the D/V border at 9.5 dpc, a similar analysis was performed in embryos heterozygous for En1 Cki, Msx2–En1, and the R26R reporter allele, thus marking cells that had expressed endogenous En1 before the transgenic misexpression of En1 perturbed limb development. In such

**Figure 5.** Fgf8 expression is abnormal in Msx2–En1 mutants. Whole-mount in situ hybridization with an Fgf8 probe in wild type (A–E) and, Msx2–En1 hemizygous forelimbs (F–I) and hindlimbs (J–N). Fgf8 expression initiates normally in Msx2–En1 mutant forelimbs and hindlimbs (F,I), compared with wild type (A). By stage 2, expression is patchy with irregular borders in both forelimbs (G) and hindlimbs (K) of Msx2–En1 transgenics. Fgf8 expression becomes discontinuous in stage 3–4 forelimbs (H, arrow, l), as compared with the smooth contour of Fgf8 along the distal margin of wild-type limb buds (C–D). Transgenic hindlimbs at stage 3 and later have fragmented AERs with portions that were not aligned along the D/V margin (L, arrows), and show dramatic decrease in the A/P extent of the handplate (M) compared with wild-type [D]. [N] Transgenic hindlimb bud at stage 4 with ridge fragments displaced ventrally [arrow] relative to the D/V margin as compared with Fgf8 staining in a wild-type limb bud (E). [d] Dorsal, [v] ventral, [A] anterior, [P] posterior.

AER borders are disrupted in Msx2–En1 mutants

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adjacent to the ectopic trally up to the D/V midline of the AER (Fig. 6A). Wnt7a is expressed in the dorsal ectoderm up to the dorsal margin of the AER (red arrow). lacZ expression in En1Lki/Msx2–En1 transgenics (Fig. 6M). In wild-type embryos, Wnt7a and En1 are expressed throughout a flat-tened ridge (between red and blue arrowheads). In other regions, no ridge is visible (F). The distal limit of Wnt7a expression (F, red arrow) is not adjacent to the shifted En1 domain (E, red arrowhead). lacZ staining in both En1Lki/Msx2–En1 (G–I) and En1Lki/R26R/Msx2–En1 (J–L) embryos has an irregular distal limit (G, I) and lacks dense lacZ staining at the limb bud margin (red broken line). In sections, ridges (outlined by red broken line) are no longer centered on the distal limit of endogenous En1 expression (H, green arrow). The distal limit of lacZ-positive cells in En1Lki/R26R/Msx2–En1 embryos is proximal (K, green arrow) to a flattened ridge (outlined by red broken line), or reaches the distal margin (L, green arrow) in a region lacking an AER. (M) Schematic showing disrupted ectoderm boundaries in Msx2–En1 mutant embryos. In wild-type limb buds (top right), the AER is centered on the distal limit of En1-expressing cells. In Msx2–En1 mutant embryos (middle right), in regions where a ridge is present the distal limit of lacZ and Wnt7a are shifted proximally. In regions lacking a ridge (bottom right), transgenic En1 extends further distally than cells expressing endogenous En1, and Wnt7a is absent from cells adjacent to the ectopic En1.

Discussion

AER formation requires assembling widespread precursor cells into a compacted linear structure at the proper position along the D/V axis of the embryo and maintenance of growth factor expression precisely in the definitive AER cells. Based on our experiments, we propose that cells of the dorsal and ventral pre-AER domains are pulled towards the lineage boundary at the dorsal margin of the pre-AER domain, which also provides a stable linear reference point for the assembling ridge (Fig. 7A). In addition, bidirectional constrictive pulling toward the D/V border, which is regulated by En1, generates a dome-shaped AER with its definitive central peak (Fig. 7C). Consistent with this, the distinctive triangular morphology is not maintained and the AER flattens after the D/V border is lost or if En1 is misexpressed in the dorsal AER. The borders that are then present between the mature AER and the adjacent ectoderm domains likely are important for maintaining AER position at the distal margin after the middle border is lost, as well as for providing the mechanical framework to generate a paddle-shaped limb bud (Dahmann and Basler 1999, Hogan 1999). Finally, AER morphogenesis and gene expression are dependent on cell–cell interactions at the dorsal and D/V borders, which are regulated, at least in part, by Wnts and En1. Cells in the ventral pre-AER that do not receive sufficient signaling lose AER specification and growth factor expression and become incorporated into the ventral ectoderm.

Lineage relationships in limb ectoderm

It has been proposed that compartment boundaries stabilize the position of patterning centers during morphogenesis, allowing for precise patterning of growing structures (Dahmann and Basler 1999). The fate-mapping experiments described here using Cre-mediated somatic recombination and retroviral cell labeling show that a
Lineage boundaries coordinate AER formation

Two stable borders are required for AER formation. A schematic of AER assembly in wild-type (A,C) and Msx2–En1 mutant (B) embryos. In wild-type limb buds, AER precursors (solid blue and green circles) are initially distributed widely in ventral ectoderm (A, left). These pre-AER cells then assemble along the dorsal (D) AER border. A portion of these precursors are not incorporated into the AER and remain as ventral ectoderm (open blue circles) (A, middle). The compacted, linear AER is generated as the entire domain shifts towards the D border concurrent with compression of the cells, symmetrically directed towards the D/V border, through increases in cell height and density (A, right, C). The ventral AER margin limits cell mixing after the mature AER has formed. In Msx2–En1 mutant embryos (B), the D and D/V borders are not stable. Cells attempt to assemble and compact along these displaced borders, resulting in a discontinuous, malaligned ridge (B, right).

Cellular interactions involving En1 and Wnts are important for AER formation

To determine whether it is critical for En1 to be expressed only in the ventral AER, transgenic mice were generated that express En1 throughout the AER. In hemizygous transgenics with moderate levels of En1 in the dorsal AER, improperly aligned, fragmented AERs formed that likely reflect pre-AER cells trying to assemble along abnormal borders (Fig. 7B). Indeed, the D/V border marked by the distal limit of endogenous En1 was irregular at 10.5 dpc and the AER in some regions was no longer centered on the position of the initial D/V border, but was shifted dorsally. Wnt7a expression was also shifted, but proximally away from the dorsal border of the AER, showing that the dorsal AER boundary was perturbed and that signaling downstream of En1 can influence the fate of adjacent En1 negative dorsal cells. In homozygous Msx2–En1 transgenics, in which En1 is expressed at higher levels and possibly in a more homogeneous manner, AER gene expression is lost at the pre-AER stage, suggesting that signaling between distinct dorsal and ventral cells is required to maintain AER gene expression.

Formation of a ridge dorsal to the normal D/V border in Msx2–En1 hemizygous transgenics suggests that a dorsally shifted AER is being induced adjacent to the new En1 boundary. The repression of Wnt7a adjacent to En1 cells indicates an early response to ectopic En1 repression of Wnt7a, which is characteristic of the normal dorsal AER. Fgf8, and the transgene itself

D/V lineage boundary restricts cell movement within the AER during its formation and the period of its peak function. By transient activation of a tamoxifen-inducible Cre recombinase, we were able to mark cells of the pre-AER to demonstrate that a boundary exists between the pre-AER cells and the dorsal ectoderm, but not the ventral ectoderm and pre-AER, at the time of AER marker gene initiation. Induction of Cre when a fully differentiated AER was present showed that the AER becomes a self-contained cell population.

Our studies in mouse have shown that the ventral border is established after the AER begins to form and, importantly, that both the middle and dorsal borders form early but the middle border is transient. If the same is true in chick, then it is possible that the mixing seen within the ventral AER in the diI labeling studies (Altabef et al. 1997) was due to analyses done at slightly later stages as compared to the chick–quail transplantation studies (Michaud et al. 1997), in which there was a sharp D/V boundary within the AER. Furthermore, our use of an inducible Cre recombinase in mouse enabled us to vary the times at which we initiated cell marking, permitting characterization of the location and dynamics of the multiple borders influencing AER formation.

Fate-mapping studies in the chick have attempted to address the lineage relationship of ventral ectoderm cells to the mature AER but were unable to resolve issues of early cell commitment. Using temporally and spatially regulated somatic recombination to study this question in mouse, we have shown that the initial population of pre-AER cells consists of cells not yet committed to an AER fate. As the ridge assembles, cell–cell interactions likely stabilize AER differentiation through positive regulatory signals, though precursors that don’t receive sufficient signal may revert to ventral ectoderm (Fig. 7A). Our mouse fate mapping experiments using Msx2–CreER<sup>T</sup> indicate that the AER precursors are restricted to the ventral ectoderm at stage 1. In contrast, diI labeling experiments in chick showed that some AER cells are located dorsally at an earlier stage (Altabef et al. 1997). Other studies suggest that dramatic shifts of ectoderm occur prior to limb outgrowth that most likely result in the pre-AER cells being localized to the ventral ectoderm at the time of AER marker gene induction and thus Cre initiation, in our studies (Geduspan and Solursh 1992; Michaud et al. 1997).

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through the Msx2 promoter, are likely induced in the new dorsal AER. The dorsally shifted transgene-derived En1 would then propagate these interactions at variable rates in different areas along the A/P axis, causing unstable borders that result in malaligned and fragmented ridges (Fig. 7B). The nearly complete failure of AER formation in Msx2−/−En1 homozygous transgenics could be due to homogeneous high levels of En1 in the dorsal AER inhibiting signaling interactions at the D/V boundary, or because more complete and rapid repression of Wnt7a and other Wnt genes in dorsal ectoderm abolishes the movements required for ridge assembly.

Previous studies showed that retroviral misexpression of En1 randomly throughout the chick limb results in AER loss and ectopic AER formation (Laufer et al. 1997; Logan et al. 1997; Rodriguez-Esteban et al. 1997). It was proposed that the two phenotypes were due to uniform En1 expression across the D/V border or patchy expression in the dorsal ectoderm, respectively (Laufer et al. 1997; Logan et al. 1997; Rodriguez-Esteban et al. 1997). However, the replication-competent retrovirus used infects randomly and spreads with time, so the various phenotypes could not be conclusively correlated with a particular pattern of ectopic En1 expression. En1 was hypothesized to act through repression of R-fng although the two genes are coexpressed in the ventral AER (Laufer et al. 1997; Rodriguez-Esteban et al. 1997). Furthermore, R-fng expression can barely be detected in mouse limb buds and null mutants have normal limbs (Moran et al. 1999). The expression pattern of R-fng in chick argues against it being an important molecule in boundary determination and AER formation, because the distal limit is located at the ventral border of the AER (Laufer et al. 1997; Rodriguez-Esteban et al. 1997, Moran et al. 1999). The expression pattern of R-fng in chick argues against it being an important molecule in boundary determination and AER formation, because the distal limit is located at the ventral border of the AER (Laufer et al. 1997; Rodriguez-Esteban et al. 1997), which we have demonstrated is only present after AER formation. Our studies show that the phenotypes generated by random retroviral En1 misexpression can be produced simply by specific expression of En1 in the dorsal AER.

The ectopic AERs that form late in En1 mutants are located at the distal border of ectopic ventral Wnt7a expression and are absent from Wnt7a−/−En1−/− double mutants [Cygan et al. 1997; Loomis et al. 1998]. This suggests that during normal AER formation, Wnt molecules, including Wnt7a, likely play a role in forming a ridge of cells at the dorsal border. Wnt3a has been shown in chick to be capable of causing ectopic ridges when misexpressed [Kengaku et al. 1998], and factors that have been shown to act downstream of Wnt3a are critical for mouse AER development (Galiszewski et al. 1999). Although Wnt3a is not expressed in mouse limb ectoderm, other Wnts are (Parr et al. 1993), and these may have overlapping functions with Wnt7a or be regulated by Wnt7a.

Consistent with our misexpression studies demonstrating a role for En1 in generating a dome-shaped AER, En1 null limb buds have broad, flattened ridges. Furthermore, En1Cre-based fate mapping has revealed that the D/V border is not sharply defined in En1 mutants [unpubl.]. Although En1 is clearly involved in maintaining a sharp D/V border and in signaling across the D/V border, other genes must act with En1 to define the ventral AER, as Wnt7a−/−En1−/− double mutants have an AER. The relatively normal AER formed in Wnt7a−/− and Wnt7a−/−En1−/− double mutants could also be accounted for by biological redundancy at the level of borders, and one sharp border might be sufficient to form a relatively normal AER. Genes responsible for setting up the initial limb D/V axis remain to be identified and may be responsible for the defects seen in chick mutants such as limbless, in which defective specification of the D/V axis includes an absence of En1 expression, as well as of Fgf8 (Grieshammer et al. 1996; Ros et al. 1996).

AER formation requires stable boundaries

In Drosophila limb development, organizers promoting outgrowth are generated through cell–cell interactions at perpendicularly oriented D/V and A/P compartment borders within epithelial imaginal discs. We have shown that vertebrate AER formation requires two lineage borders oriented parallel to each other. The different arrangement of boundaries could reflect the unique requirements for generating and maintaining signaling centers within a planar epithelial sheet in fly versus in a budding structure with multiple tissue layers in vertebrates. As limb ectoderm and mesoderm undergo dramatic expansion during development, it is essential that these two tissues remain appropriately juxtaposed and the AER stays localized at the distal tip during limb outgrowth.

Based on our results, we propose a model for vertebrate AER development in which prior to limb outgrowth at 9.0 dpc, the ectoderm is divided into domains of lineage restriction that express selector-type genes that have yet to be identified. At 9.0 dpc, Fgf10 induces expression of AER genes such as Fgf8 and Msx2 [Michaud et al. 1997; Bell et al. 1998; Loomis et al. 1998, Min et al. 1998; Sekine et al. 1999] in a population of AER precursors in ectoderm ventral to the dorsal lineage restriction. At 9.5–10.0 dpc, signaling from the dorsal ectoderm to the pre-AER and interactions within the ridge, controlled in part by En1, act to maintain expression of AER genes and to promote formation of a ridge with its apex at the D/V border. After 11.5 dpc when the D/V border is lost, the AER flattens and eventually regresses. The involvement of two borders in AER formation likely reflects the complexity of assembling a signaling center with precise topographical requirements concurrent with tissue outgrowth and patterning.

Materials and methods

Construction of transgenic vectors

The vector for transgenic misexpression was generated using a modified pKS bluescript backbone [Stratagene], in which the SacI site in the polylinker had been changed to SalI [C. Logan and A. Joyner, unpubl.]. A cDNA encoding a Cre–ER protein, kindly provided by P. Chambon [Feil et al. 1996], was
excised from pCre-ER T [using EcoRI digestion and the remaining plasmid containing a β-globin intron and poly(A) sequence was recircularized to form pSG6. The β-globin intron and SV40poly[A] on an 830-bp ClaI-XbaI fragment from pSG6 was inserted into the ClaI and XbaI sites, respectively, of pKS [SalI–SalI] to create pKS-IVS-poly(A). A SacI–KpnI fragment containing a polylinker and 825-bp lacZtag, kindly provided by D. Epstein [Epstein et al. 1996], was blunt-end ligated into the EcoRI site of pKS-IVS-poly(A) to create PEV II.

A 520-bp Msx2 AER-specific promoter on an EcoRI fragment, kindly provided by R. Maxson [Liu et al. 1994], was blunt-end ligated into the ClaI site of PEVII, creating Msx2–EV. The EcoRI fragment containing CreER T was then subcloned into the SnuBI site of Msx2–EV to create the final construct Msx2–CreER T. Similarly, the Apal fragment of an En1 C DNA [Joyner et al. 1985] was blunt-end ligated into the SnuBI site to generate Msx2–En1. For both Msx2–CreER T and Msx2–En1, the transgene construct was purified from the vector sequences by cleaving the plasmid with SalI, and transgenic animals were generated by zygote injection [Hogan et al. 1994] in Swiss Webster mice.

Transgenic Msx2–CreER T and Msx2–En1 mice and embryos were identified by phenotype and/or PCR on proteinase K-digested yolk sacs or tails. PCR was performed using upstream primers for either CreER T [5'-CATCTTGTACCTCCTGATGG-3'] or En1 [5'-GGATGTCGACGCAAAACG-3'] and a downstream primer against the lacZ tag [5'-TACCAACGGGTAG-3'], which amplified DNA fragments of 420 bp, and 350 bp, respectively.

**UBM injection of retrovirus**

Virus preparation and animal surgery were performed as described previously [Olsson et al. 1997; Liu et al. 1998; Gaiano et al. 1999; Turnbull 1999]. Viral stocks of pNK-lacZ [Gaiano et al. 1996] at 5 × 10⁷ cfu/ml were diluted 1:20 in L15 media [Cellgro] and supplemented with polybrene at a final concentration of 80 µg/ml. Virus solution (~0.5–1.0 µl) was injected into the amniotic cavity of 8.5-dpc embryos. lacZ-labeled cell clusters selected for sectioning at both 10.5 dpc and 11.5 dpc contained at least six and, on average, eight cells.

**Fate mapping using Cre-expressing mice**

The En1–Cre knock-in (En1Cki) construct was generated by inserting the Cre-recombinase gene cassette [Sauer and Henderson 1990] into a previously described En1 knock-in vector [Hanks et al. 1995, 1998; Broccoli et al. 1999] and electroporated into R1 ES cells. After germ-line transmission the neomycin cassette was removed by crossing to a transgenic mouse line expressing Cre-recombinase constitutively [Schwenk et al. 1995].

Proteinase K-digested DNA from yolk sacs or tails was genotyped by PCR using the upstream primer [5'-TTAACAGATCCT-CAGTCTGAGCCGTG-3'] and the downstream primer [5'-TCTCTGAGGTCATCTCTTACG-3'], which amplified a band of 300 bp. Rosa26Reporter (R26R) mice were kindly provided by P. Soriano and genotyped as described [Soriano 1999].

Tamoxifen (Sigma T-5648) was dissolved in corn oil [Sigma C-8267] at 37°C for several hr with periodic vortexing to a final concentration of 20 mg/ml. The tam solution was stored protected from light at 4°C. Intraperitoneal injections of 5.0–10.0 mg were administered to the pregnant female mice at 8.5–10.5 dpc. For the timing of tam injections, the morning on which the vaginal plug was detected was designated as 0.5 dpc. Injections done in the evening were considered to have advanced by 0.5 d.

Embryonic limb buds were staged according to the morphological criteria first described by Waneck et al. [1989], and modified by Bell et al. [1998] and Loomis et al. [1998].

**Whole-mount β-galactosidase histochemistry and RNA in situ analysis**

Embryos for in situ analysis were dissected in PBS and fixed overnight in 4% paraformaldehyde at 4°C. The probes used were to En1 [Wurst et al. 1994], Wnt7a [Parr and McMahon 1995], and Fgf8 [Crossley and Martin 1995]. RNA in situ analysis was performed essentially as described [Matise and Joyner 1997], except NBT (4.5 µl/ml)/BCIP (3.5 µl/ml) in NTMT was used as the AP substrate. For ectodermal analysis, Proteinase K treatment consisted of 0.2 µg/ml for 5 min at 37°C. Embryos for β-galactosidase analysis were fixed in 4% paraformaldehyde/PBS for 30 min–1 hr, depending on age, and processed as described [Liu et al. 1998].

**Histology**

Limbs from UBM-guided injection of retrovirus to be cryosectioned were incubated overnight at 4°C in 15% sucrose/PBS. Samples were then embedded in a solution of 7.5% gelatin/15% sucrose in a 0.12 M phosphate buffer [pH 7.4] and frozen in isopentane cooled to -55°C with dry ice. Samples were sectioned in the D/V plane at 8 µm. Embryos from whole-mount RNA in situ or β-gal analysis were immersed overnight in 15% sucrose followed by 30% sucrose for several hr. The embryos were then embedded in OCT and cut at 6–8 µm.

For skeletal analysis, newborn pups were incubated overnight in 1 M NaCl at 37°C to loosen skin, rinsed for 24 hr with PBS prior to removal of skin, and then processed as described [Luftin et al. 1992].

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**References**


