En1 and Wnt7a interact with Dkk1 during limb development in the mouse

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Abstract

Wnt signaling plays an essential role in induction and development of the limb. Missing digits are one consequence of the reduced Wnt signaling in Wnt7a null mice, while extra digits result from excess Wnt signaling in mice null for the Wnt antagonist Dkk1. The extra digits and expanded apical ectodermal ridge (AER) of Dkk1-deficient mice closely resemble En1 null mice. To evaluate the in vivo interaction between En1 and the canonical Wnt signaling pathway, we generated double and triple mutants combining the hypomorphic doubleridge allele of Dkk1 with null alleles of En1 and Wnt7a. Reducing Dkk1 expression in Dkk1d/+Wnt7a/C0/C0 double mutants prevented digit loss, indicating that Wnt7a acts through the canonical pathway during limb development. Reducing Dkk1 levels in Dkk1d/dEn1/C0/C0 double mutants resulted in severe phenotypes not seen in either single mutant, including fused bones in the autopod, extensive defects of the zeugopod, and loss of the ischial bone. The subsequent elimination of Wnt7a in Dkk1d/dEn1/C0/C0/Wnt7a/C0/C0 triple mutants resulted in correction of most, but not all, of these defects. The failure of Wnt7a inactivation to completely correct the limb defects of Dkk1d/dEn1/C0/C0/Wnt7a/C0/C0 triple mutants indicates that Wnt7a is not the only gene regulated by En1 during development of the mouse limb.

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Keywords: Limb development; Wnt signaling; AER; Dkk1; En1; Wnt7a

Introduction

Wnt signaling is one of the key pathways in vertebrate limb development (Church and Francis-West, 2002; Martin, 2001). Early in limb development, canonical Wnt signaling through beta-catenin governs formation of the apical ectodermal ridge (AER), the signaling center that regulates limb outgrowth. Absence of Wnt3 or beta-catenin in limb ectoderm results in defective establishment of the AER (Barrow et al., 2003; Soshnikova et al., 2003). In mice deficient for both Lef1 and Tcf1, transcription factors mediating canonical Wnt signaling, limb development is arrested at the early limb bud stage (Galceran et al., 1999).

Wnt7a is expressed in dorsal limb ectoderm and governs formation of dorsal limb structures. In Wnt7a null mice, footpads develop on the dorsal as well as ventral surface, forming ‘double-ventral’ paws. Posterior digits are often missing, indicating that Wnt7a is required for maintenance of posterior limb tissue (Parr and McMahon, 1995). The loss of posterior limb tissue is preceded by reduced expression of Shh in the zone of polarizing activity (ZPA), which directs anterior–posterior specification during limb development.

Overactivation of Wnt signaling by expression of a gain-of-function mutation of beta-catenin results in dramatic expansion of the AER (Soshnikova et al., 2003). A null allele of the Wnt antagonist Dkk1 also leads to expansion of the AER (Mukhopadhyay et al., 2001). doubleridge is a hypomorphic allele of Dkk1, designated Dkk1d, with expression levels reduced to 10% of normal in developing limbs (MacDonald et al., 2004). Dkk1d/d mice develop a broad AER and also display postaxial polysyndactyly in the forelimbs, as seen in the Dkk1 null mice (Adamska et al., 2003, Mukhopadhyay et al., 2001).

The En1 homeobox transcription factor is expressed in the ventral limb ectoderm, where it represses expres-
sion of Wnt7a. En1 null limbs display an expanded AER, polydactyly, and syndactyly (Loomis et al., 1996; Wurst et al., 1994). In En1−/−Wnt7a+/− double mutants, there is no expansion of the AER or polysyndactyly, demonstrating that the limb phenotype of En1 null mice is mediated by ectopic expression of Wnt7a in the ventral limb ectoderm (Cygan et al., 1997; Loomis et al., 1998).

The limb abnormalities of En1 and Dkk1 mutant mice are strikingly similar. The similarity could result from direct regulation of one gene by the other or from similar effects of both on a common downstream pathway. To examine the in vivo interaction of effects of both on a common downstream pathway, we generated triple mutant Dkk1d/dEn1 mice (Hanks et al., 1995) to determine whether elimination of Wnt7a expression would compensate for the synergistic effects in the Dkk1d/dEn1−/− double mutants.

Methods

Mice

The doubleridge mutant arose in this laboratory and was previously described (Adamska et al., 2003; MacDonald et al., 2004). En1 null mice (Hanks et al., 1995) were obtained from Dr. A. Joyner. The Wnt7aUM null allele arose at the University of Michigan on an ICR background. The triple mutant mice were generated by intercrossing Dkk1d/dEn1+/−Wnt7a+/− mice. All experiments were performed on a mixed genetic background. Animals of the same genotype displayed similar phenotypes independent of the specific cross in which they were generated. All experiments were carried out with the approval of the University of Michigan animal use committee and in accordance with NIH requirements.

Genotyping

The En1 null allele was detected by amplification of a 200-bp band with primers specific for the LacZ sequence: LacZF, GCG ATG TCG GTT TCC GCG AG, and LacZR, GTA CCA CAG CGG ATG GTT CGG (A. Joyner, personal communication). The wild-type En1 allele was detected by amplifying a 125-bp fragment with the primers En1F, AGC CGG AGC GTA AAA GTC AG, and En1R, CAC GCT GTC TCC ATC GCT. Dkk1 was genotyped as described by MacDonald et al. (2004). The Wnt7aUM null allele was genotyped in a three primer assay containing the forward primer Wnt7aF, GAG CAT CTG CCA TTA GCA AG, and the reverse primers Wnt7aR1, GCA CAG CCA TCT CAT TAG CT, and Wnt7aR2, TGT GCA CTC AAG GCT CTT GA. Primers Wnt7aF and Wnt7aR2 amplify a 323-bp fragment from the null allele; primers Wnt7aF and Wnt7aR1 amplify a 175-bp fragment from the wild-type allele. Both fragments are obtained from heterozygous DNA.

Other methods

Embryos were genotyped by PCR of genomic DNA from embryonic membranes. E0.5 was considered to be noon of the day when the vaginal plug was found. Whole mount in situ hybridization was carried out with a digoxigenin-labeled probe (Bober et al., 1994) using BM Purple (Roche) as the substrate for alkaline phosphatase. Antisense mRNA probes for Dkk1 (Glinka et al., 1998), En1 (Wurst et al., 1994), Fgf8 (Crossley and Martin, 1995), Wnt7a (Parr and McMahon, 1995), Shh (Echelard et al., 1993), and Wnt5a (Yamaguchi et al., 1999) were prepared as described. Cartilage and bone were stained with Alcian blue and Alizarin red (Kimmel and Trammell, 1981). Images of cleared skeletons and whole embryos were captured with a DEI 750 Optronics digital camera and processed using Adobe Photoshop.

Results

Expression of Dkk1 and En1 in wild-type and single mutant mice

Expression of Dkk1 and En1 in developing limb of wild-type mice has been previously described (Loomis et al., 1998; Monaghan et al., 1999; Mukhopadhyay et al., 2001). At E9.5, En1 and Dkk1 are coexpressed in the ventral ectoderm, and Dkk1 is also expressed in the ventral mesenchyme. At E10.5, En1 continues to be expressed in the ventral ectoderm and is also strongly expressed in the ventral portion of the AER. At E10.5, Dkk1 transcripts are present throughout the AER and in the posterior and anterior mesenchyme of the limb bud.

To determine whether the expression of En1 is regulated by Dkk1, we analyzed mice homozygous for the hypomorphic allele. The expression of En1 was not altered in Dkk1d/d limbs at E9.5 (Adamska et al., 2003) or at E10.5, when En1 transcripts are present in the AER and the ventral ectoderm (Fig. 1A). Thus, no effect of Dkk1 deficiency on En1 expression was detected.

To determine whether En1 influences expression of Dkk1, we examined En1 null embryos. Expression of Dkk1 did not differ between mutant and wild-type limbs at E9.5, when there is no morphological difference between wild-type and En1 null limbs (Fig. 1B). At E10.5, Dkk1 was expressed in the expanded AER of En1 null limbs at a level comparable to wild type (Fig. 1C). Dkk1 was also present at comparable levels in anterior and posterior mesenchyme of mutant and wild-type limb.
expression of Dkk1 and in 8/10 Dkk1d/dEn1 null mice (shown). Thus, there is no apparent effect of En1 deficiency on Dkk1 expression.

**Novel limb phenotype due to combined deficiency of Dkk1 and En1**

In Dkk1d/d and En1−/− single homozygotes, limb abnormalities are restricted to the autopod (Figs. 2A–C). Postaxial polysyndactyly is more severe in Dkk1d/d mice than in En1−/−. To investigate genetic interaction between these genes, we studied double mutant mice obtained from several crosses between Dkk1d/d and En1+/- mice. All classes of progeny were recovered in the expected Mendelian frequencies. The double heterozygotes did not exhibit any abnormalities.

Double homozygotes displayed extensive and severe abnormalities of forelimbs and hindlimbs (Figs. 2E and F). In most cases, digits were not externally visible. The autopod and zeugopod of Dkk1d/dEn1+/- limbs were severely malformed. The ulna, radius, fibula, and tibia were thickened and bent, and ossification centers were severely malformed. The ulna, radius, fibula, and tibia were thick and bent, and the location of the deltoid tuberosity was shifted (Fig. 2D). Except for occasional ventral digits, the hindlimbs were normal (not shown). Dkk1d/dEn1+/- mice were identical to Dkk1d/d, with postaxial polysyndactyly of forelimbs (Adamska et al., 2003, and Fig. 2B). Syndactyly was sometimes limited to soft tissue, but bone was affected in other individuals.

Early development of limb abnormalities in Dkk1d/dEn1−/- double mutants

To study the molecular basis for the limb defects in double mutant mice, we examined the expression of markers for the three limb signaling centers: Fgf8 (AER), Shh (ZPA), and Wnt7a (dorsal limb ectoderm). In Dkk1d/d and En1−/− single mutants, delayed compaction of the AER is evidenced by expansion of Fgf8 expression in the ventral limb ectoderm. In double mutant limbs, the two Shh domains are separated by the expanded AER; while in En1 null limbs, the single Shh domain extends below the AER. Wnt7a is restricted to dorsal limb ectoderm in wild-type and Dkk1d/d limbs, but in En1−/- mice it is ectopically expressed in the ventral limb ectoderm below the AER (Adamska et al., 2003; Cygan et al., 1997; Loomis et al., 1998).

In En1−/- and Dkk1d/d single mutant embryos at E10.5, the Fgf8-positive AER encompasses half of the ventral forelimb ectoderm (Fig. 3A). In Dkk1d/dEn1−/- double homozygotes, Fgf8 expression extends through the entire ventral limb ectoderm from E10 through E11.5 (Figs. 3A and 6C). Shh expression in double mutant limbs is similar to that in Dkk1d/d at E10.5 (Fig. 3B), but at E11.5 Shh is limited to two weak expression domains in the posterior and ventral mesenchyme (Fig. 3C). Wnt7a is correctly excluded from the AER of double homozygotes but is abnormally expressed in the proximal region of the ventral limb bud (Fig. 3D).

The forelimbs of double homozygous embryos are visibly smaller than littermates at E11.5 (Fig. 3E). Apoptotic cells, detected by TUNEL staining, were limited to the AER, as in wild-type limbs. No increase in the intensity of TUNEL staining was observed, indicating that increased cell death is not responsible for the reduced size (Fig. 3F). The relative size of the mutant limb bud decreases as development progresses. By E12.5, there is a striking difference in shape and size of the limb in double homozygotes and their littermates (Fig. 3G).
Since Wnt5a null mice display shortening of limb bones similar to that in Dkk1^{dd}En1^{-/-} double homozygotes (Topol et al., 2003; Yamaguchi et al., 1999), we examined expression of Wnt5a at E11.5 (Fig. 3E). Although the shape of the limbs was altered in the double mutants, the level of Wnt5a expression appeared normal.

To determine whether the zeugopod malformations in neonatal limbs reflect earlier defects in condensation of the mesenchyme, E13.5 and E16.5 limbs were stained with Alcian blue and Alizarin red. At E13.5, the zeugopod elements were close to normal, while autopods already displayed severe deformations (Fig. 3H). At E16.5, dramatic deformations of the zeugopod were clearly visible (Fig. 3I). The relatively normal appearance of the initial mesenchyme condensation of the zeugopod in Dkk1^{dd}En1^{-/-} embryos at E13.5, compared with E16.5, indicates that Dkk1 and En1 are required for chondrogenesis during the intervening 3 days.

**Identification of a spontaneous null allele of Wnt7a**

Mice with phenotypes strikingly similar to Wnt7a null mice were identified in an ICR line maintained by brother–sister mating. Phenotypes included dorsal footpads, posterior digit loss, and infertility of both males and females. Amplification of genomic DNA from affected individuals, using primers flanking the four exons of Wnt7a, failed to yield a PCR product. These mice also displayed severe lethargy and died in utero before E15.5.
Wnt7a (Parr et al., 1998), demonstrated that exons 3 and 4 were deleted. A 54-kb deletion that includes exons 3 and 4 of Wnt7a, but does not disrupt adjacent genes, was detected by PCR analysis of 150 kb of genomic DNA from the region. The precise position of the deletion was confirmed by amplification of a 2-kb fragment from genomic DNA of homozygous affected mice using a forward primer terminating at 91,798,090 bp and a reverse primer terminating at 91,854,167 bp on mouse chromosome 6 (Ensembl v. 16.30.1 6 May 2003—Mouse Genome Assembly NCBI 30, http://www.ensembl.org/Mus_musculus). The new allele, designated Wnt7aUM, was used to investigate the genetic interaction of Dkk1 and Wnt7a.

Wnt7a and Dkk1 cooperate in establishing digit number

Dkk1<sup>dd</sup> mice were crossed to Wnt7a<sup>+/−</sup> mice and progeny were intercrossed or backcrossed to generate single and double mutants. Most of the Wnt7a<sup>−/−</sup> single mutant offspring, 30/38, lacked posterior digits in one or both forelimbs (Fig. 4B), consistent with previous reports (Parr and McMahon, 1995). In the Dkk1<sup>dd</sup> Wnt7a<sup>−/−</sup> offspring, the partial reduction of Dkk1 was sufficient to restore normal digit number in all of the animals obtained (37/37) (Fig. 4D).

In single mutant Wnt7a<sup>−/−</sup> limbs, decreased expression of Shh is evident at E10.5, and there is reduction of tissue in

Fig. 3. Development of limb defects in Dkk1<sup>dd</sup>En1<sup>−/−</sup> embryos. (A) No compaction of the AER in forelimb buds of double homozygous at E10.5. (B and C) Shh expression level is normal at E10.5 but dramatically reduced at E11.5. (D) Wnt7a expression is correctly excluded from the expanded AER of E11.5 hindlimb bud. (E) Normal expression of Wnt5a. (F) TUNEL staining reveals no increase in apoptosis at E10.5. (G) The autopod of Dkk1<sup>dd</sup>En1<sup>−/−</sup> limb is dramatically smaller than in the littermate controls at E12.5. The AER is marked by Fgf8 expression. (H) Alcian blue staining of cartilage in double mutants at E13.5 shows severe defects of mesenchyme condensation in the autopod and nearly normal zeugopod bones. (I) Alcian blue (cartilage) and Alizarin red (bone) staining of E16.5 forelimbs reveal dramatically misshapen zeugopod elements in the double mutant.
the posterior part of the limb bud (Fig. 4B). In Dkk1<sup>++</sup> Wnt7a<sup>−/−</sup> double mutants, the expression of Shh and the shape of the limb bud were corrected (Fig. 4D).

Single mutant Dkk1<sup>−/−</sup> mice have six digits in their forelimbs (Adamska et al., 2003). In Dkk1<sup>−/−</sup>Wnt7a<sup>−/−</sup> double homozygotes, digit number was restored to five, with an occasional small postaxial skeletal element (Figs. 4F and 5C, arrow).

Two phenotypes were not altered in double mutants. In Dkk1<sup>−/−</sup>Wnt7a<sup>−/−</sup> embryos, there was no correction of the ventrally expanded AER seen in Dkk1<sup>−/−</sup> mice (Figs. 4E and F). In addition, the ectopic dorsal footpads character-
istic of Wnt7a−/− mice were not affected by Dkk1 genotype (Fig. 4).

Partial correction of Dkk1, En1 phenotypes by removal of Wnt7a

The expanded AER, syndactyly, and polydactyly observed in En1 null limbs can be completely prevented by inactivation of Wnt7a (Cygan et al., 1997; Loomis et al., 1998). To determine whether inactivation of Wnt7a could correct the abnormalities of Dkk1ddEn1−/− limbs, we generated triple homozygous mice. All genotypes were recovered at the predicted frequencies (Table 1). The severe abnormalities were largely corrected in the triple mutants, including restored size of the limb, shape of the zeugopod elements, morphology of the digits, and presence of the ischial bone (Fig. 5B). Other abnormalities were not completely corrected (Fig. 5). Forelimbs of the triple mutants

Fig. 5. Loss of Wnt7a results in partial rescue of the Dkk1ddEn1−/− phenotype. (A) Dkk1ddEn1−/− mice display severe fusions of autopod elements, thickening and poor ossification of the ulna (u), radius (r), fibula (f), and tibia (t), proximal shifting of the deltoid tuberosity (dt), and loss of the ischial bone (i). (B) In triple Dkk1ddEn1−/−Wnt7a−/− mutants, zeugopod elements of the hindlimb are normal, the ischial bone is present, and fusions of autopod elements are less severe. The ulna and radius are partially corrected, forelimb digits are severely fused, and the first digit of the hindlimbs is missing. The olecranon process (o) is not fully attached to the ulna. (C) Dkk1ddWnt7a−/− limbs are characterized by a normal zeugopod and normal digit number in hindlimbs and forelimbs. A small postaxial element is sometimes present in the forelimb (arrow).
displayed severe bone and soft tissue fusions similar to these in Dkk1<sup>−/−</sup>En1<sup>−/−</sup> limbs, and nails were missing in some individuals. The bones of the zeugopod were often thick and misshapen like those of Dkk1<sup>−/+</sup>En1<sup>−/−</sup> forelimbs (Fig. 5B).

The olecranon process of the ulna was partly detached and sometimes free-floating (Fig. 5B). All of the triple homozygotes displayed loss or reduction of the most anterior digit of the hindlimb, a phenotype not observed in double mutants (Fig. 5B). In addition, the dorsal footpads characteristic of Wnt7a<sup>−/−</sup> mice were present in all four limbs of the triple mutants. Removal of Wnt7a thus compensated for some, but not all, of the abnormalities of Dkk1<sup>−/−</sup>En1<sup>−/−</sup> mice.

**Morphology of the AER in triple mutants**

To determine whether the AER is expanded in the triple mutants, we examined Fgf8 expression at E11.5 (Fig. 6). The AER of wild-type limbs is represented by a thin line of Fgf8 expression (Fig. 6A). In Dkk1<sup>−/−</sup> single mutants and Dkk1<sup>−/−</sup>Wnt7a<sup>−/−</sup> double mutants, the forelimb AER is broader than wild type, with strong Fgf8 expression in the well-defined dorsal and ventral borders (Figs. 6B and E). In Dkk1<sup>−/−</sup>En1<sup>−/−</sup> limbs, the greatly expanded AER occupies entire ventral portion of the limb and lacks a visible ventral border, with Fgf8 expression gradually decreasing in the proximal ectoderm (Fig. 6C). The AER abnormalities in the triple mutants are clearly less extensive than the Dkk1<sup>−/−</sup>En1<sup>−/−</sup> double mutant but are more severe than the Dkk1<sup>−/−</sup>Wnt7a<sup>−/−</sup> double mutant (Fig. 6D). The forelimb AER occupies approximately half of the ventral ectoderm, with two well-defined borders, while the expansion of the hindlimb AER is restricted to the most proximal limb region (Fig. 6D).

**Discussion**

Dkk1 and En1 act synergistically during mouse limb development

Mice deficient for Dkk1, an inhibitor of the canonical Wnt signaling pathway, develop a ventrally expanded AER, syndactyly, and polydactyly (Adamska et al., 2003; MacDonald et al., 2004; Mukhopadhyay et al., 2001). En1 null mice also display ventral expansion of the AER, syndactyly, and polydactyly, which are combined with dorsal–ventral patterning defects (Cygan et al., 1997; Loomis et al., 1998; Wurst et al., 1994). To investigate in vivo interactions between En1 and the canonical Wnt signaling pathway, we examined the phenotypes of double and triple mutant mice, as summarized in Table 2.

Combining the En1 null allele with reduced expression of Dkk1 in Dkk1<sup>−/−</sup>En1<sup>−/−</sup> mice increased the severity of autopod defects and produced novel abnormalities in other elements of the limb.

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**Table 1**

<table>
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<tr>
<th>Cross</th>
<th>Offspring Genotypes</th>
<th>En1&lt;sup&gt;+/−&lt;/sup&gt;</th>
<th>Wnt7a&lt;sup&gt;+/−&lt;/sup&gt;</th>
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<th>En1&lt;sup&gt;−/−&lt;/sup&gt;</th>
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<td>(n)</td>
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<td>10</td>
<td>6</td>
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<td>Dkk1&lt;sup&gt;−/−&lt;/sup&gt;En1&lt;sup&gt;−/−&lt;/sup&gt;-Wnt7a&lt;sup&gt;−/−&lt;/sup&gt; × Dkk1&lt;sup&gt;−/−&lt;/sup&gt;En1&lt;sup&gt;−/−&lt;/sup&gt;-Wnt7a&lt;sup&gt;−/−&lt;/sup&gt;</td>
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<td>7</td>
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<td>Dkk1&lt;sup&gt;−/−&lt;/sup&gt;En1&lt;sup&gt;−/−&lt;/sup&gt;-Wnt7a&lt;sup&gt;−/−&lt;/sup&gt; × En1&lt;sup&gt;−/−&lt;/sup&gt;-Wnt7a&lt;sup&gt;−/−&lt;/sup&gt;</td>
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<td>4</td>
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<td>16</td>
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</table>

Genotype frequencies did not differ from the Mendelian predictions indicated in the bottom row.
More than eight animals of each genotype were examined. The indicated phenotypes were present in Triple mutant d/d +/+ d/d Single mutants Dkk1 zeugopod bones. During this period, deficiency occurred later during the outgrowth of the appears that the effects of combined and poorly ossified. Since there was no delay in the initial of the zeugopod were dramatically misshapen, thickened, the limb. The skeletal elements of the autopod were expanded to include most of the ventral ectoderm of (Caricasole et al., 2003). abnormalities not present in the Dkk1d/+En1 Dkk1d/dEn1 mice have normal AER and develop normal limbs, but Dkk1d/+En1−/− double mutants exhibit zeugopod abnormalities not present in the En1−/− single mutant. Exacerbation of the En1−/− phenotype in this case is clearly not due to superimposition of two mutant phenotypes but reflects underlying interactions between En1 and the canonical Wnt signaling pathway.

**Table 2**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Dkk1</th>
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<th>Wnt7a</th>
<th>Ischial bone missing</th>
<th>Zeugopod abnormalities</th>
<th>Digit number</th>
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<td></td>
<td></td>
<td></td>
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<td>Hindlimb</td>
<td>Forelimb</td>
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<td>–</td>
<td>–</td>
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<tr>
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<td>+/+</td>
<td>–/+</td>
<td>–</td>
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<td></td>
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<td>+</td>
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<td>Triple mutant</td>
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<td>–/+</td>
<td>–/+</td>
<td>–</td>
<td>+</td>
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</table>

More than eight animals of each genotype were examined. The indicated phenotypes were present in ≥75% of individuals.

was expanded to include most of the ventral ectoderm of the limb. The skeletal elements of the autopod were severely fused, resulting in apparent digit loss. The bones of the zeugopod were dramatically misshapen, thickened, and poorly ossified. Since there was no delay in the initial mesenchymal condensation of zeugopod elements, it appears that the effects of combined En1 and Dkk1 deficiency occurred later during the outgrowth of the zeugopod bones. During this period, Dkk1 is known to be expressed in cells lining the perichordium and in cells involved in mineralization of bone (Monaghan et al., 1999). However, En1 transcripts have not been detected in developing bones (Davis and Joyner, 1988, and unpublished observations). The zeugopod phenotype may therefore reflect changes in gene expression early in development that only become evident later as the long bones grow.

Dkk1d/+ mice have normal AER and develop normal limbs, but Dkk1d/+En1−/− double mutants exhibit zeugopod abnormalities not present in the En1−/− single mutant. Exacerbation of the En1−/− phenotype in this case is clearly not due to superimposition of two mutant phenotypes but reflects underlying interactions between En1 and the canonical Wnt signaling pathway.

**Wnt7a maintains posterior limb tissue through the canonical Wnt pathway**

The phenotype of Dkk1d/dEn1−/− double homozygotes may reflect synergistic hyperactivation of Wnt signaling due to ectopic expression of Wnt7a combined with reduced expression of the antagonist Dkk1. It is not established whether Wnt7a acts through the canonical Wnt pathway during development of the limb. In the chicken limb, overexpression of Wnt7a did not affect formation of the AER, although treatment with Wnt3a did produce expansion of the AER (Kengaku et al., 1998). In the mouse, the loss of Lrp6 generates multiple defects that combine the effects of single mutations in Wnt1, Wnt3a, and Wnt7a (Pinson et al., 2000), suggesting that Wnt7a is acting through the canonical pathway. Direct biochemical interaction of Wnt7a with Lrp6 and Dkk1 was recently demonstrated in rat PC12 cells (Caricasole et al., 2003).

To evaluate genetically the interaction of Wnt7a with Dkk1 during limb development, we analyzed mice that were null for Wnt7a−/− and also had reduced expression of Dkk1. The limb abnormalities in Wnt7a null mice include loss of posterior tissue in the limb bud, missing posterior digits, and ventralization of the limbs with formation of footpads on dorsal and ventral surfaces (Chen and Johnson, 2002; Parr and McMahon, 1995; Parr et al., 1998). Although the expression domains of Wnt7a and Dkk1 do not overlap, their expression is closely juxtaposed in the AER. Digit loss was rescued by the modest reduction of Dkk1 in Dkk1d/+ Wnt7a−/− limbs. It has been proposed that Wnt7a maintains posterior limb tissue by up-regulation of Shh, which in turn promotes expression of Fgfs in the AER (Niswander et al., 1994; Parr and McMahon, 1995). Dkk1d/+ limbs do not display up-regulation of Shh expression, but the reduction of Shh expression seen in Wnt7a null limbs was prevented in Dkk1d/+ Wnt7a−/− mice. The effects of Dkk1 on Shh expression and digit loss in the Wnt7a double mutants provide evidence that derepression of the canonical Wnt pathway can compensate for Wnt7a deficiency during development of the mouse limb. This is consistent with the model that Wnt7a acts through the canonical pathway to regulate development of the posterior digits.

The defective dorsal or ventral polarity in Wnt7a null limbs was not corrected in the Dkk1/Wnt7a double mutant, consistent with the lack of Dkk1 expression in the dorsal limb. The present experiments thus do not address the mechanism of Wnt7a action on Lmx1b in the dorsal limb.

**Restriction of Wnt7a expression is not the only function of En1 during limb development**

Wnt7a is misexpressed in the ventral limb ectoderm in the absence of En1, leading to expansion of the AER. In En1−/− Wnt7a−/− mice, the lack of misexpressed Wnt7a prevents the expansion of the AER seen in En1 null mice (Cygan et al., 1997; Loomis et al., 1998). This observation has been interpreted as indicating that the sole function of En1 during embryonic limb development is repression of Wnt7a expression in the ventral limb ectoderm. If that were the case, then the limbs of triple mutant Dkk1d/d En1−/− Wnt7a−/− mice would be undistinguishable from
the Dkk1<sup>−/−</sup>Wnt7a<sup>−/−</sup> double mutants. In fact, removal of Wnt7a expression resulted in significant but not complete rescue of the complex Dkk1<sup>−/−</sup>En1<sup>−/−</sup> phenotype (Table 2). The defects of the hindlimb zeugopod and ischial bone were corrected, but the anterior digit was missing or severely reduced in the triple mutant. The forelimbs of the triple mutant were similar to Dkk1<sup>−/−</sup>En1<sup>−/−</sup> mice, with severe fusions of autopod elements, and thickened ulna and radius. Since some of the Dkk1<sup>−/−</sup>En1<sup>−/−</sup> defects were not mediated by Wnt7a, En1 must have additional target(s) in the limb, most likely within the canonical Wnt signaling pathway.

In summary, we used the hypomorphic double ridge allele of Dkk1 to investigate interactions of En1 and Wnt7a with the canonical Wnt signaling pathway during mouse limb development. Our data demonstrate that Dkk1 and En1 cooperate during several stages of limb development, and that their interaction is partially independent of Wnt7a expression.

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References


ing formation of the AER and the dorsal–ventral axis in the limb. 
Topol, L., Jiang, X., Choi, H., Garrett-Beal, L., Carolan, P.J., Yang, Y.,  
2003. Wnt-5a inhibits the canonical Wnt pathway by promoting GSK-  
defects in engrailed-1 mutant mice: an early mid-hindbrain deletion 
and patterning defects in forelimbs and sternum. Development 120,  
2065–2075. 
pathway underlies outgrowth of multiple structures in the vertebrate 