RESEARCH COMMUNICATION

Genetic interaction between Wnt/β-catenin and BMP receptor signaling during formation of the AER and the dorsal–ventral axis in the limb

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By conditional gene ablation in mice, we found that β-catenin, an essential downstream effector of canonical Wnt signaling, is a key regulator of formation of the apical ectodermal ridge (AER) and of the dorsal–ventral axis of the limbs. By generation of compound mutants, we also show that β-catenin acts downstream of the BMP receptor IA in AER induction, but upstream or parallel in dorsal–ventral patterning. Thus, AER formation and dorsal–ventral patterning of limbs are tightly controlled by an intricate interplay between Wnt/β-catenin and BMP receptor signaling.

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Significant progress has been achieved in the understanding of the molecular networks that control limb development (for review, see Capdevila and Izpisua Belmonte 2001; Niswander 2003). Three cardinal axes are established in the developing limb by coordinated interactions between distinct signaling centers; that is, the apical ectodermal ridge (AER) that controls proximal–distal elongation, the zone of polarizing activity (ZPA) that is responsible for anterior–posterior patterning, and the apical ectoderm of the limb bud that directs dorsal–ventral axis formation.

Wnt molecules are important regulators in diverse developmental processes, and control cell proliferation, differentiation, and survival (Wodarz and Nusse 1998; Eastman and Grosschedl 1999; Huelsken and Birchmeier 2001). Wnt ligands signal through different receptors of the Frizzled and LRP families and result in the stabilization of β-catenin, a central and essential component of the canonical Wnt-signaling pathway. β-Catenin then interacts with LEF/TCF transcription factors to control the expression of specific target genes (Behrens et al. 1996; Eastman and Grosschedl 1999; van de Wetering et al. 2002). In the absence of Wnt signals, the N terminus of β-catenin is phosphorylated by CKI and GSK3β, and phosphorylated β-catenin is subsequently ubiquitinated and degraded (Schwarz-Romond et al. 2002, and references therein). Mutations in tumors that affect these N-terminal phosphorylation sites lead to a stabilization of β-catenin and enhanced signaling activity (Polakis 2000).

Wnt signals participate in the patterning of limb (Capdevila and Izpisua Belmonte 2001). In the chick embryo, limb formation is initiated by Wnt-2b and Wnt-8c, which are expressed in the lateral plate mesoderm at the forelimb and hindlimb level, respectively, and signal through β-catenin to restrict the expression of Fgf-10 to the prospective limb mesoderm (Kawakami et al. 2001). FGF-10 then induces the expression of Wnt-3a in the limb ectoderm that is required for Fgf8 expression in the presumptive AER (Griesshammer et al. 1996; Kengaku et al. 1998; Kawakami et al. 2001; McQueeney et al. 2002). It was shown recently that the closely related Wnt3 takes over this function in the mouse (Barrow et al. 2003). Independent confirmation for such a mechanism was provided by the analysis of Lef-1/Tcf-1 mutant mice that lack Fgf8 expression in the limb ectoderm and do not form an AER (Galceran et al. 1999). Wnt-7a, which signals via a β-catenin independent pathway, is crucial for the dorsal–ventral patterning (Parr and McMahon 1995). Wnt-7a expression is restricted to the dorsal ectoderm by En-1, which is essential for ventral cell fate specification (Loomis et al. 1996).

Not only Wnts, but also BMP signals are important in AER formation and in dorsal–ventral patterning. Conditional inactivation of the Bmp receptor IA in mice impairs both AER formation and dorsal–ventral patterning of limb, and affects the expression of Fgf8 and En-1 [Ahn et al. 2001]. In the chick, expression of a constitutively active BMP receptor causes ectopic expression of Fgf8 and En-1 [Pizette et al. 2001]. BMP signals regulate the development of many tissues and organs, and extensive interactions between BMP and Wnt-signaling pathways have been reported [Kratochwil et al. 1996; Theil et al. 2002]. However, the hierarchies of Wnt/β-catenin and BMP signaling pathways have not been established in the limb, and it is unclear whether Wnts and BMPs control consecutive or parallel events during the formation of dorsal–ventral and proximal–distal axes in the limb.

To elucidate the interaction between Wnt/β-catenin and BMP signaling during limb formation by genetic means, we have used the Cre-loxP technology to ablate β-catenin and to express stabilized β-catenin in the ectoderm of the hindlimb. Loss of β-catenin results in defects in both AER formation and dorsal–ventral patterning, which are similar to those observed after ablation of the Bmp receptor IA. Moreover, we establish the epistatic relation of Wnt/β-catenin and BMP receptor IA using double mutants, and thus uncover an intricate interaction of the two pathways.

[Keywords: Limb ventral ectoderm, proximal–distal axis, FGF, Engrailed-1, Frizzled]
Results and Discussion

β-Catenin is essential for limb development

We used two distinct mutant alleles to introduce loss-of-function and gain-of-function mutations into the mouse β-catenin gene. The first allele contains two loxP sites that flank exons 3–6 (β-cateninlox/lox), upon recombination, no β-catenin protein is produced, and the recombined allele thus corresponds to a null mutation (Huelsken et al. 2001). The second allele contains two loxP sites that flank exon 3 (ΔN-β-catenin), after Cre-induced deletion of exon 3, stabilized β-catenin protein is produced that cannot be phosphorylated (Harada et al. 1999). To introduce conditional mutations into the β-catenin locus, a transgene that expresses Cre under the control of the Brn4 neural tube enhancer/promoter was used (Ahn et al. 2001; Heydemann et al. 2001). Cre activity was assessed with the help of a lacZ indicator strain. Brn4Cre-mediated recombination of a lacZ reporter gene was observed in limb-bud ectoderm (Fig. 1A; Ahn et al. 2001) and in the central nervous system (Zechnier et al. 2003). Immunohistochemistry demonstrated that β-catenin was absent in the ventral ectoderm of the hindlimb of loss-of-function mutants as early as the 25-somite stage, that is, prior to the initial formation of the AER [Fig. 1, cf. arrows in C and control in B]. Loss of β-catenin in dorsal ectoderm occurred later than in ventral ectoderm, and was observed at the 40-somite stage [data not shown]. We also observed heterogeneity in the extent of recombination, that is, some β-catenin positive cells remained in the ventral ectoderm of certain embryos (see below).

Brn4Cre-induced loss-of-function mutation of β-catenin resulted in severe malformations of the hindlimbs (Fig. 1D–L). In strongly affected individuals, the hindlimbs were completely lacking [Fig. 1E,G]. Others displayed a truncation of tibia and fibula, and an absence of digits I-IV (Fig. 1E,H). In the least affected individuals, only digit II and/or III were missing [data not shown]. Moreover, in hindlimbs of mildly affected mutants the ventral dermal pads were absent, and circumferential nails were detected [Fig. 1I,J]. Histological examination showed that nail plates were present on both dorsal and ventral surfaces of mutant hindlimbs [Fig. 1K,L]. Thus, mutant mice displayed an absence of ventral and a duplication of dorsal structures. Forelimbs of mutants displayed rarely minor anatomical changes. This is consistent with low and late activity of Cre in the forelimb ectoderm of Brn4Cre embryos [data not shown; Ahn et al. 2001]. The complete absence or the severe malformations of the hindlimb of Brn4Cre-β-cateninlox/lox mutant mice indicate that β-catenin plays an essential role during early stages of limb development.

β-Catenin acts downstream of BMP receptor Iα during AER formation

The severe truncations of limb along the proximal–distal axis in β-catenin loss-of-function mutants suggest a deficit in AER formation. To address this, the expression of genes in the hindlimb AER were analyzed in β-catenin gain- and loss-of-function embryos. In the loss-of-function mutants, Fg8, Bmp2, and Bmp4 were completely absent in strongly affected embryos at the 38–42-somite stage [data not shown]. A few remaining Fg8 and Bmp4-positive spots of cells were located at the distal rim and also at ectopic ventral positions in mildly affected animals [Figs. 2A,B, 3A–D; data not shown]. Serial sections indicate that remaining Fg8- or Bmp4-positive cells were β-catenin positive [Fig. 2D,E; data not shown]. In gain-of-function mutants, Fg8, Bmp2, and Bmp4 expression domains in the hindlimb
also observed in embryos that carry only the enlarged in the compound mutants (Fig. 3I,J); this was Bmp4, and the overall size of the limb were strongly in the area of the AER, the expression domains of Bmp4 and Fgf8, and that it acts in a cell-autonomous manner. Remarkably, these compound mutants expressed Fgf8, which is essential for the expanse of the presentation domains of Wnt3, was not changed in the ectoderm of mutant limbs (Fig. 4A,B). Fzd6 and Fzd8 were also present in both dorsal and ventral limb ecto-

Figure 2. β-Catenin is essential for AER formation. (A,B) Reduction of Fgf8 expression in hindlimbs of mutants carrying loss-of-function mutation of β-catenin; in comparison with wild type, at the 38–42-somite stage (arrowheads). (C) Strong ventrally expanded expression domain of Fgf8 in mutants harboring the gain-of-function mutation of β-catenin. Note ectopic expression of Fgf8 in dorsal limb ectoderm (arrowheads). (D,E,F) Expression of β-catenin in spots, but not in the surrounding ectoderm of mildly affected mutants carrying loss-of-function mutation of β-catenin, as detected by immunofluorescence at the 38–42-somite stage (β-catenin is indicated in green, DAPI nuclei staining in blue; the surface of the ectoderm is marked by a broken line). Note that the exposition time was reduced in F. Bars: A–C, 150 µm; D–F, 15 µm.

bud were markedly expanded [Figs. 2C, 3E,F; data not shown], and strong expression of β-catenin was observed in the cytoplasm and the nucleus of AER cells [Fig. 2F, arrow shows nuclear staining]. Moreover, a clearly discernable AER was either absent or strongly reduced in size in loss-of-function mutants [Figs. 2E, 3C,D]. In contrast, a dramatically expanded AER was observed in gain-of-function mutants [Figs. 2, cf. C and A, 3, cf. E,F and A,B]. We conclude that β-catenin is essential for the expression of AER-specific genes and for AER formation, and that it acts in a cell-autonomous manner.

Bmpr4Cre-induced loss-of-function mutation of the Bmp receptor IA results in AER deficits that are very similar to those we observed in Bmpr4Cre; β-cateninlox/lox mutants [Ahn et al. 2001]. For instance, the expression of Fgf8 and Bmp4 was absent or severely reduced, and the formation of the AER was impaired in Bmpr4Cre loss-of-function mutants [Fig. 3G,H]. This raises the question of the AER-specific genes and for AER formation, and that it acts in a cell-autonomous manner.

Bmpr4Cre; ΔN-β-cateninBmpr4IAlox/lox. Remarkably, these compound mutants expressed Fgf8 and Bmp4 strongly in the ventral ectoderm prior to AER formation (at the 30-somite stage; data not shown). At subsequent stages (for instance at the 42-somite stage), the area of the AER, the expression domains of Fgf8 and Bmp4, and the overall size of the limb were strongly enlarged in the compound mutants [Fig. 3I,J]; this was also observed in embryos that carry only the β-catenin gain-of-function mutation [cf. Fig. 3E,F]. We conclude from these results that increased β-catenin signaling can rescue the deficits in AER formation caused by loss-of-function mutation of the Bmpr4Cre. We can thus position β-catenin-mediated signaling to act genetically downstream of Bmpr4Cre during formation of the AER (see scheme in Fig. 3, right). Moreover, expression of Bmp4, a ligand of the BMP receptor IA, is controlled by β-catenin signals, and is enhanced in compound and in β-catenin gain-of-function mutants. β-Catenin therefore also participates in a positive feedback loop that amplifies BMP signaling.

To address the question of how BMP receptor IA signaling may control Wnt/β-catenin signaling in AER formation, we examined in Bmpr4Cre loss-of-function mutants, the expression of genes of canonical Wnt signaling. Expression of Wnt3, which is essential for AER formation [Barrow et al. 2003], was not changed in the ectoderm of mutant limbs [Fig. 4A,B]. Fzd6 and Fzd8 were also present in both dorsal and ventral limb ecto-
Bmp receptor IA mutants (Fig. 4C,D; data not shown). In contrast, Fzd1 expression was detected in the ventral ectoderm of control embryos, but not of Bmp receptor IA loss-of-function mutants (Fig. 4E,F, at the 26–31-somite stage). However, Fzd1 was present in β-catenin loss-of-function mutants (data not shown), indicating that BMP receptor IA, but not β-catenin signaling, is required for expression of this gene. In Bmp receptor IA loss-of-function mutants, we also examined the expression of a direct target gene of Wnt/β-catenin signaling, conductin (Jho et al. 2002; Lustig et al. 2002), using mice that carry a lacZ gene in the conductin locus. Expression of conductin was absent in the limb ectoderm of the Bmp receptor IA mutants (Fig. 4G,H). These data confirm that Wnt/β-catenin signaling is located downstream of BMP receptor signaling during induction of the AER, and that Fzd1 might be a crucial component in this cross-talk.

β-Catenin acts upstream of, or in parallel with, the BMP receptor IA during specification of the dorsal–ventral axis

Brn4Cre-induced loss-of-function mutation of β-catenin resulted in the absence of ventral and in the duplication of dorsal structures, which indicates a defect in dorsal–ventral patterning (Fig. 1I–L). The expression of En-1 in the ventral limb ectoderm was absent (Fig. 5C), and the expression domain of Wnt-7a was expanded into the ventral ectoderm of β-catenin mutants (Fig. 5, cf. D and controls in A,B; arrows indicate the borders of expression). Lmx1b was expressed only in the dorsal half of the limb mesenchyme in control mice, and its expression expanded into the ventral domain of the mutant limb bud (data not shown; Riddle et al. 1995). The expression pattern of En-1 and Wnt-7a was not changed in the β-catenin gain-of-function mutants (Fig. 5E,F); note that Brn4Cre is only active in the ventral ectoderm. BMP receptor IA signaling was also required for correct dorsal–ventral patterning of the limb and for correct expression of Wnt-7a and En-1.

**Figure 4.** β-Catenin signaling depends on BMP receptor signaling during AER formation. (A–D) Expression of Wnt3 and Fzd6 are not changed in the limb ectoderm of Bmp receptor IA loss-of-function mutants at the 30-somite stage. (E–H) Fzd1 and conductin are not expressed in ventral ectoderm of Bmp receptor IA loss-of-function mutants at the 30-somite stage. Note that conductin expression was analyzed in compound mice expressing lacZ under the control of the conductin promoter. Dorsal–ventral is as indicated. Bar, 50 µm.

**Figure 5.** β-Catenin acts upstream of, or in parallel to, the BMP receptor IA during dorsal–ventral patterning of limbs (see scheme at right). (A,C) En-1 is not expressed in ventral limb ectoderm of mutants carrying loss-of-function mutation of β-catenin at the 30-somite stage. (B,D) Wnt-7a is expressed ectopically in ventral ectoderm of β-catenin loss-of-function mutant limbs at the 30-somite stage. (E,F) En-1 and Wnt-7a expression domains in β-catenin gain-of-function mutants resemble the wild type. (G) En-1 is not expressed in ventral limb ectoderm of mutants carrying the loss-of-function mutations of Bmp receptor IA. (H) Wnt-7a is not expressed in both dorsal and ventral ectoderm of mutants carrying loss-of-function mutation of Bmp receptor IA. (I) En-1 is not expressed in the ventral ectoderm of compound mutants at the 30-somite stage. (J) Wnt-7a is expressed in ventral limb ectoderm of compound mutants at the 30-somite stage (cf. H). Dorsal ventral is as indicated. Bar, 50 µm.
of En-1 and Wnt-7a [Fig. 5G,H; see also Ahn et al. 2001]. To establish the epistatic interaction between β-catenin and BMP receptor IA signaling in dorsal–ventral patterning, we examined compound mutant embryos that carried a gain-of-function mutation of β-catenin and a loss-of-function mutation of the Bmp receptor IA for the expression of En-1 and Wnt-7a. Remarkably, in the compound mutants, En-1 expression in the ventral ectoderm was absent (Fig. 5I), and Wnt-7a was expressed in both dorsal and ventral ectoderm [Fig. 5J, arrow]. Thus, both ectodermal β-catenin and BMP receptor IA are required for correct specification of the ventral character in limb ectoderm. However, enhanced β-catenin signaling does not rescue the deficits caused by Bmp receptor IA loss-of-function mutation, suggesting that genetically, β-catenin acts upstream or in parallel to the BMP receptor IA during dorsal–ventral patterning (see scheme in Fig. 5, right).

It is known that the Wnt/β-catenin and TGFβ/BMP signaling pathways coordinately govern many developmental processes. During limb development, Wnt and BMP signals control the formation of the AER and participate in the establishment of the dorsal–ventral axis in the limb [Ahn et al. 2001; Pizzete et al. 2001; Barrow et al. 2003; this study]. The interactions between the two signaling systems in the limb were, however, not understood, and the epistatic relationship between BMP and Wnt signals remained unclear. Here, we have analyzed the interactions between Wnt/β-catenin and BMP receptor signaling during limb development using conditional mutagenesis, which allowed us to introduce loss-of-function and gain-of-function mutations of β-catenin, the central and essential mediator of canonical Wnt signaling. In addition, we generated compound mutant mice that carry both a gain-of-function mutation in β-catenin and loss-of-function mutations in Bmp receptor IA. Our analysis of these compound BrtnCre/ΔN-β-catenin: BmpRIA−/−; BmpRIA+/−; BrtnCre/ΔN−β-catenin: BmpRIA−/−; BmpRIA−/− mutant mice clearly demonstrates that β-catenin acts downstream of the BMP receptor IA in AER induction. β-Catenin-mediated signals do, however, control Bmp4 expression in the ectoderm, and are thus responsible for the formation of a positive feedback loop. In contrast, our data suggest that β-catenin acts upstream or in parallel to the BMP receptor IA during dorsal–ventral patterning. These intricate interactions between the Wnt/β-catenin and BMP-signaling pathways provide the molecular basis that connects the development of proximal–distal and dorsal–ventral axes in the limb, and might thus ensure a tight spatial-temporal control of signaling responses.

After submission of this manuscript, a publication appeared that demonstrated that Wnt3β/β-catenin-transmitted signals in the ventral limb ectoderm are essential for AER induction and maintenance as well as for the dorsal–ventral polarity, and it was suggested that Wnt3β-catenin signaling acts upstream of BMP signaling in both AER formation and dorsal–ventral polarity [Barrow et al. 2003]. These data are in agreement with ours with respect to the role of β-catenin in AER formation and dorsal–ventral patterning. However, they disagree with ours on the epistatic relationship between Wnt/β-catenin and BMP signaling.

**Material and methods**

**Mouse strains and embryos**

β-Catenin−/− mice, β-cateninIACS homozygous mice, BmpRIA−/− mice, and Brtn4Cre transgenic mice (pedigree bcre-32) were described previously [Harada et al. 1999; Ahn et al. 2001; Huelsken et al. 2001; Mishina et al. 2002]. To obtain loss-of-function mutations of β-catenin in the limb ectoderm, homozygous mice carrying β-cateninIACS alleles were crossed with Brtn4Cre transgenic mice that were heterozygous for the β-catenin−/− allele. To obtain the gain-of-function mutation of β-catenin in the limb ectoderm, heterozygous mice carrying the β-cateninIACS/− allele were crossed with Brtn4Cre transgenic mice. To obtain loss-of-function mutations of BmpRIA, homozygous mice carrying BmpRIA−/− alleles were crossed with Brtn4Cre mice that were heterozygous for the BmpRIA−/− allele. To obtain compound mutant embryos, heterozygous mice carrying the β-cateninIACS/− gain-of-function allele and the BmpRIA−/− loss-of-function allele were crossed with Brtn4Cre transgenic mice that were heterozygous for the BmpRIA−/− allele. Heterozygous mice carrying the conductinIACS/− allele [Lustig et al. 2002] and the BmpRIA−/− loss-of-function allele were crossed with Brtn4Cre transgenic mice that were heterozygous for the BmpRIA−/− allele. Mutant embryos were identified by PCR [Harada et al. 1999; Ahn et al. 2001; Huelsken et al. 2001; Lustig et al. 2002]. Cre-inducible lacZ reporter mice were described previously [Thorey et al. 1998].

**Histological techniques and in situ hybridization**

Wild-type and mutant embryos [n ≥ 5] were age-matched according to their somite numbers, and fixed in 4% formaldehyde in PBS. Immunohistochemical analyses were performed on 5-µm paraffin sections as described previously [Huelsken et al. 1994]. Skeletons of newborn pups were prepared and stained with Alcian blue 8GX and Alizarin red S as described [Hogan et al. 1994].

In situ hybridization of whole-mount or paraffin sections was performed using digoxigenin-labeled [DIG] RNA probes (Roche, see Huelsken et al. 2000). The anti-sense transcripts of mouse cDNA were as follows: Bmp4 [nucleotides 290–1780, X56848], En-1 [nucleotides 1041–1741, NM010133], Fgfl (Crossley and Martin 1999), Fgf2 [IMAPgf952G2216], Fdof [IRAKp961C2218], Mxl1 [Hill et al. 1989], Wnt3 [nucleotides 300–1120, NM009521], and Wnt7a [ICRFp522L0361]. The DIG label was detected by an anti-DIG Fab (Roche) coupled to alkaline phosphatase, using NBT/BCIP (Sigma).

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