RESEARCH COMMUNICATION

Dkk1 and *noggin* cooperate in mammalian head induction

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Growth factor antagonists play important roles in mediating the inductive effects of the Spemann organizer in amphibian embryos and its equivalents in other vertebrates. Dual inhibition of Wnt and BMP signals has been proposed to confer head organizer activity. We tested the requirement of this coinhibition in *Xenopus* and mice. In *Xenopus*, simultaneous reduction of the BMP antagonists *chordin* and *noggin*, and the Wnt antagonist *dickkopf1* (*dkk1*) leads to anterior truncations. In mice, compound mutants for *dkk1* and *noggin* display severe head defects, with deletion of all head structures anterior to the mid-hindbrain boundary. These defects arise as a result of a failure in anterior specification at the gastrula stage. The results provide genetic evidence for the dual inhibition model and indicate that *dkk1* and *noggin* functionally cooperate in the head organizer.

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The primary body axis of vertebrate embryos arises and is patterned anteroposteriorly during gastrulation. Anterior signaling centers that are required for induction of head structures have been identified in gastrulae of all vertebrates. In amphibians, this induction is mediated by Spemann's head organizer, a group of cells of anterior mesendodermal fate that is localized in the gastrula upper dorsal blastopore lip (Harland and Gerhart 1997; De Robertis et al. 2000). In mouse gastrulae, anterior signaling centers, as defined by heterotopic transplantation, are the anterior visceral endoderm (AVE; Thomas and Beddington 1996; Tam and Steiner 1999) and the anterior mesendoderm (AME), whose progenitors are the early- and midgastrula organizers (Camus et al. 2000; Kinder et al. 2001). Subsequently, during early neural patterning, the anterior neural ridge induces and promotes forebrain, and inducing signals include FGF8 and Tlc (Rubenstein et al. 1998; Houart et al. 2002; for reviews, see Beddington and Robertson 1998; Davidson and Tam 2000; Perea-Gomez et al. 2001).

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To account for the molecular nature of region-specific organizers, a two-inhibitor model has been proposed, the hallmark of which is differential inhibition of Wnt and BMP growth factor signals. Inhibition of BMP signaling leads to formation of the trunk organizer and simultaneous inhibition of BMP and Wnt signaling promotes the head organizer (Fig. 1A). This model is based on the findings that overexpression of BMP inhibitors alone leads to trunk duplications, whereas coexpression of Wnt and BMP inhibitors leads to ectopic head formation (Glinka et al. 1997). Furthermore, genes encoding secreted Wnt antagonists such as *cerberus*, *frzb*, and *dkk1* are predominantly expressed in anterior mesendoderm, whereas all organizer derivatives express at least one of the

Figure 1. Functional synergy of anti-Wnts and anti-BMPs in anterior development of *Xenopus* embryos. (A) Two-inhibitor model: BMP and Wnt signaling pathways negatively regulate the Spemann organizer. Formation of the trunk organizer requires inhibition of BMP signals, whereas for formation of the head organizer both Wnt and BMP signals need to be inhibited. Differential expression in mesendoderm and chordamesoderm of secreted anti-Wnts (*Dkk1*, *Frzb1*, *Crescent*/*Frzb2*, and *Cerberus*) as well as anti-BMPs (*Noggin*, *Chordin*, *Xnr3*, and *Follistatin*) mediates regional-specific induction. (B) Specificity of *noggin* and *chordin* antisense Morpholino oligonucleotides (Mo): Embryos were injected at the two-cell stage with mRNA encoding either *noggin*-AP (5 ng) or *chordin*-AP (2 ng) and again at the four- to eight-cell stage with either *noggin* or *chordin* Mo (25 ng). Protein lysates were prepared at stage 13 and heated at 65°C for 30 min to inactivate endogenous alkaline phosphatase. The remaining AP activity was measured. (C) *Xenopus* embryos were injected at the four-cell stage in two dorsal blastomeres with a total of 50 ng control Mo or 25 ng each of *noggin* and *chordin* Mo as indicated. Zero percent ($n = 36$) of control Mo and 10% ($n = 34$) of *nog/chd* Mo injected embryos were eyeless. Embryos injected into the blastocoel of early gastrulae with 0.1 μ g of anti-*Dkk1* antibody (anti-*Dkk1*) showed 0% ($n = 32$) eyeless embryos. Coinjection of *nog/chd* Mo together with anti-*Dkk1* antibody leads to enhanced head defects (75% eyeless embryos; $n = 36$).

BMP antagonists *chordin*, *noggin*, *folliculin*, *Xnr3*, or *cerberus* (for review, see Niehrs 1999; Yamaguchi 2001). Although Nodal antagonists can also promote head structures (Piccolo et al. 1999), this may be an indirect consequence of blocking the formation of trunk mesoderm and its associated caudalizing signals, such as Wnt8. Rather, *cerberus-like/lefty* double mutants reveal a requirement of anti-Nodals to restrict primitive streak formation to the posterior end of mouse embryos (Perea-Gomez et al. 2002).

Consistent with the two-inhibitor model, inhibition of Wnt signals is required for anterior specification. Zebrafish mutants for the negative intracellular regulators of Wnt signaling *tcf3/headless* and *axin/masterblind* display anterior defects (Heisenberg et al. 2001; Kim et al. 2001; van de Water et al. 2001). Interference with the Wnt antagonist *dkk1* in *Xenopus* and mouse leads to head truncations (Glinka et al. 1998; Kazanskaya et al. 2000; Mukhopadhyay et al. 2001). Furthermore, *Six3* mutant mice lack forebrain structures, and this appears to be due to derepression of Wnt1 (Lagutin et al. 2003). These negative regulators of the Wnt signaling pathway may help establish a gradient of Wnt activity patterning the antero-posterior axis (Kiecker and Niehrs 2001; Houart et al. 2002).

A requirement for BMP inhibition for normal anterior specification is supported by mice double mutant for the BMP antagonists *noggin* and *chordin*, which show a range of anterior defects (Bachiller et al. 2000; Anderson et al. 2002). Likewise, zebrafish with reduced BMP activity have expanded anterior neural fates (Nguyen et al. 1998; Barth et al. 1999; Fekany-Lee et al. 2000).

Although these data strongly support the role of BMP and Wnt antagonists in promoting rostral cell fates, an important prediction in the above model for head induction that remains untested is that coinhibition of BMP and Wnt signaling is required for anterior specification. To test if BMP and Wnt antagonists act synergistically, we have created compound mutant mice for the Wnt antagonist *dkk1* (Glinka et al. 1998; Mukhopadhyay et al. 2001) and the BMP antagonist *noggin* (Smith and Harland 1992; McMahon et al. 1998). Analysis of these mice reveals a synergistic effect of both types of antagonists in the specification of anterior cell fates.

Results and Discussion

Dkk1 and anti-BMPs synergize in *Xenopus*

We initially tested if anti-Wnts and anti-BMPs act synergistically for head formation in *Xenopus*. Injection of Morpholino-antisense oligonucleotides (Mo) acting specifically against *noggin* (nog) or *chordin* (chd; Fig. 1B) alone does not result in head defects (data not shown), possibly because of the presence of pseudoalleles. Mild microcephaly was, indeed, reported for em-

bryos injected with a combination of two different Mos against *chordin*, which inhibit both pseudoalleles (Oelgeschläger et al. 2003). However, embryos coinjected with both *noggin* and *chordin* Mos show shorter trunks and minor microcephaly (Fig. 1C), consistent with results in the mouse, where they have redundant functions (Bachiller et al. 2000; Anderson et al. 2002). If limiting doses of a specific inhibitory anti-Dkk1 antibody (Glinka et al. 1998; Kazanskaya et al. 2000) are injected in addition to *nog/chd* Mo, the anterior phenotype is enhanced, leading to headless and eyeless embryos (Fig. 1C, anti-Dkk1 + *nog/chd* Mo).

Dkk1 and *Noggin* synergize in mouse head induction

We next tested the requirement for dual BMP/Wnt inhibition genetically in mice. *Dkk1* starts to be expressed in the AVE at embryonic day 6 (E6; data not shown), and in the anterior mesendoderm (AME) at E7 (Glinka et al. 1998). Homozygous *dkk1* mutant animals display lack of head and brain structures anterior to the mid-hindbrain boundary, as well as limb defects (Mukhopadhyay et al. 2001). *Noggin* is expressed in the node and AME at E7.5 (McMahon et al. 1998; Bachiller et al. 2000), where its expression partially overlaps with that of *dkk1* (Fig. 4A,B, below). In mice lacking *noggin*, normal gastrulation and anterior central nervous system patterning take place, although at later stages abnormalities in posterior spinal cord, somites, and limbs are observed (McMahon et al. 1998). Importantly, *dkk1* and *noggin* heterozygous mice are phenotypically normal and viable, and hence intercrosses were set up between them.

Figure 2. *Dkk1*^{+/-} *Nog*^{+/-} mice have head defects. Frontal (A,B,C,D) and lateral (A',B',C',D') views of wild-type (A,A'), mild (B,B'), strong (C,C'), and severe (D,D') newborn mutant animals. Lateral view of skeletal preparations from wild-type (A''), mild (B''), and severe case (D'') newborn heads reveal gradual loss of maxillary (mx), mandibular (mn), and other bones anterior to the parietal bone (p). Sagittal sections of wild-type (E) and severe (F) newborns show no mouth (arrowhead) or nasal structures (arrow) in the mutant. Skeletal preparation of limbs from wild-type (G) and severe *Dkk1*^{+/-} *Nog*^{+/-} (H) animals do not show any difference.

Only 98 *Dkk1*^{+/-} *Nog*^{+/-} animals of the expected 142 ($n = 571$) survived to adulthood and were fertile. All other newborns showed head defects, which ranged from reduced maxillary structures in mild cases (Fig. 2B–B'), loss of eyes (Fig. 2C,C'), to loss of rostral head structures anterior to the parietal bone in severe cases (Fig. 2D–D',E,F). The variability of penetrance of the anterior defects may be due to genetic modifiers. Our analysis has focused on the severe-class animals. No defects caudal to the neck were observed including the developing limbs, which express *dkk1* and *noggin* (Fig. 2G,H; data not shown; Brunet et al. 1998; Grotewold et al. 1999; Monaghan et al. 1999; Mukhopadhyay et al. 2001).

To understand where and when these head defects arise, we analyzed E10.5 embryos. A range of reduced telencephalic vesicles correlates with the head defects in newborns (Fig. 3A–E). At E9.5, *Fgf8* expression, which marks the commissural plate of the forebrain and the mid-hindbrain boundary (MHB; Crossley and Martin 1995; Shimamura and Rubenstein 1997), is only present in the MHB in severe *Dkk1*^{+/-} *Nog*^{+/-} mice (Fig. 3F,F'). Likewise, the forebrain markers *Pax6* and *Six3* are not expressed in severe *Dkk1*^{+/-} *Nog*^{+/-} mice (Fig. 3G,G',H,H'; Walther and Gruss 1991; Oliver et al. 1995). *Nkx2.1*, which marks the floor of the diencephalon and telencephalon (Lazzaro et al. 1991; Shimamura et al. 1995), is absent in the telencephalon in severe *Dkk1*^{+/-} *Nog*^{+/-} mice (Fig. 3I,I').

Marker analysis reveals that these anterior defects are evident at gastrula stages. At E6.5, expression of the AVE markers *Hex*, *Cerberus-like*, and *Lim1* (Crompton et al. 1992; Ang et al. 1994; Shawlot and Behringer 1995; Belo et al. 1997; Biben et al. 1998; Shawlot et al. 1998; Beddington and Robertson 1999; Martinez-Barbera et al. 2000a) is unaffected (Fig. 4D,D',E,E',F,F'). Expression of *Dkk1* was significantly down-regulated or absent in severe *Dkk1*^{+/-} *Nog*^{+/-} embryos (Fig. 4G,G'). This down-regulation is not observed in *Dkk1*^{+/-} embryos (data not shown). At E7.5, expression of the notochord and node markers *Hnf3 β* and *Shh* (Echelard et al. 1993; Monaghan et al. 1993; Ruiz i Altaba 1993; Ang and Rossant 1994; Chiang et al. 1996) is normal in *Dkk1*^{+/-} *Nog*^{+/-} embryos (Fig. 4H,H',I,I'). However, expression of *Hesx1* in AME and anterior neuroectoderm, and *Six3*, which marks the future forebrain (Oliver et al. 1995; Hermesz et al. 1996; Thomas and Beddington 1996; Martinez-Barbera et al. 2000b), are absent in *Dkk1*^{+/-} *Nog*^{+/-} embryos (Fig. 4J,J',K,K'). Of note, a mild constriction is visible at the anterior embryonic/extraembryonic border of *Dkk1*^{+/-} *Nog*^{+/-} embryos, which is characteristic of several embryos with defects in anterior patterning (Ang and Rossant 1994; Shawlot and Behringer 1995; Rhinn et al. 1998; Bachiller et al. 2000).

In intercrosses between surviving *Dkk1*^{+/-} *Nog*^{+/-} mice, homozygous *Dkk1*^{-/-} *Nog*^{-/-} mutant animals were never obtained, even when embryos were sampled at E6.5 ($n = 0$ out of 122, with 7 expected). This is puzzling because *noggin* starts to be expressed only at E7.5 by whole-mount in situ hybridization (Fig. 4B; McMahon et al. 1998; Bachiller et al. 2000). However, by reverse transcriptase PCR (RT-PCR), *noggin* transcripts are detectable already at E6.5 (Fig. 4C), consistent with a requirement for both genes during early gastrulation. To explore other genotypes, surviving *Dkk1*^{+/-} *Nog*^{+/-} mice were bred with *Noggin*^{+/-} or *Dkk1*^{+/-}. Both *Dkk1*^{-/-} *Nog*^{+/-} and *Dkk1*^{+/-} *Nog*^{-/-} mice were obtained in the expected Mendelian ratio. The *Dkk1*^{-/-} *Nog*^{+/-} mice did not show any head defects that are not already seen in *Dkk1*^{-/-} embryos (Mukhopadhyay et al. 2001; data not shown). In contrast, all *Dkk1*^{+/-} *Nog*^{-/-} embryos show anterior defects that are not observed in *Noggin*^{-/-} mice, ranging from cyclopia to severe reduction of head structures (Fig. 5A,A',B,B',C,C',I,I'). Similar to severe *Dkk1*^{+/-} *Nog*^{+/-} mice, *Fgf8* expression remained only in the MHB (Fig. 5D,D'). *Pax6* and *Six3* expression was absent in forebrain (Fig. 5E,E',F,F',G,G'). Unlike in severe *Dkk1*^{+/-} *Nog*^{+/-} mice, *Nkx2.1* was completely abolished in *Dkk1*^{+/-} *Nog*^{-/-} mice. No *Hesx1* expression was detected in E8.5 *Dkk1*^{+/-} *Nog*^{-/-} embryos (Fig. 5H,H'), indicating an early defect in forebrain development.

We set out to test whether dual inhibition of BMP and Wnt signaling is required for head induction. Our data

Figure 3. Brain defects in *Dkk1*^{+/-} *Nog*^{+/-} embryos. (A) E10.5 wild-type embryo showing telencephalic vesicle (tv) and midbrain/hindbrain boundary (*). (B–D) Gradual loss of telencephalic vesicles in *Dkk1*^{+/-} *Nog*^{+/-} embryos. (E) Severe cases lack forebrain. Whole-mount in situ hybridization of E9–E9.5 wild-type (F,G,H,I) and severe *Dkk1*^{+/-} *Nog*^{+/-} (F',G',H',I') embryos. (F) *Fgf8* is expressed in the commissural plate of the forebrain (arrowhead) and isthmus (arrow). (F') *Dkk1*^{+/-} *Nog*^{+/-} embryo showing normal expression in the isthmus but lack of commissural plate expression. (G) *Pax6* expression is localized in dorsal forebrain and rostral midbrain with a sharp boundary in the zona limitans intrathalamica (ZLI; arrowhead). (G') In *Dkk1*^{+/-} *Nog*^{+/-} embryos, no expression was detected. (H) *Six3* expression in the prospective forebrain and in future eyes is absent in *Dkk1*^{+/-} *Nog*^{+/-} embryos (H'). (I) *Nkx2.1* is expressed in the ventral diencephalon (arrowhead) and the floor of the telencephalon (arrow). (I') In *Dkk1*^{+/-} *Nog*^{+/-} embryos, expression remains only in the ventral diencephalon (arrowhead).

Figure 4. Anterior patterning defects in *Dkk1*^{+/-} *Nog*^{+/-} gastrulae. Whole-mount in situ hybridization for *Dkk1* (A) and *Noggin* (B) in E7.5 wild-type embryos shows overlapping expression in the anterior mesendoderm. (C) RT-PCR analysis of *Noggin* expression in E6.5 and E7.5 wild-type embryos. Whole-mount in situ hybridization of wild-type (D,E,F,G,H,I,J,K) and *Dkk1*^{+/-} *Nog*^{+/-} (D',E',F',G',H',I',J',K') embryos at E6.5 (D-G') and E7.5 (H-K'). Representative cases of severe mutant class embryos are shown, which occur at 30% frequency. (D,E,F) *Hex*, *Cerb-1*, and *Lim1* expression in AVE (arrowhead) and *Lim1* primitive streak expression (arrow) are unaffected in *Dkk1*^{+/-} *Nog*^{+/-} embryos (D',E',F'; *Hex*, *n* = 20; *Cerb-1*, *n* = 20; *Lim1*, *n* = 30). (G) *Dkk1* expression is down-regulated in *Dkk1*^{+/-} *Nog*^{+/-} mutants (arrow in G'; *n* = 3). (H,I) At E7.5, *Hnf3β* and *Shh* marking the node and the axial mesendoderm were normal in *Dkk1*^{+/-} *Nog*^{+/-} mutants (H',I'; *Hnf3β*, *n* = 6; *Shh*, *n* = 8). (J,K) *Hesx1* expression marking both anterior neuroectoderm and AME and *Six3* expression in the future forebrain are absent in *Dkk1*^{+/-} *Nog*^{+/-} mutants (J',K'; *Hesx1*, *n* = 2; *Six3*, *n* = 2). A mild constriction is observed at the anterior embryonic–extraembryonic border of *Dkk1*^{+/-} *Nog*^{+/-} mutants (arrowhead in H',K').

demonstrate that the Wnt antagonist *Dkk1* and the BMP antagonist *Noggin* act synergistically during anterior signaling in the mouse gastrula. One target gene is *Dkk1* itself, whose expression is down-regulated in *Dkk1*^{+/-} *Nog*^{+/-} animals, consistent with the finding that *noggin*, together with *chordin*, is required for maintaining gene expression in the AVE (Bachiller et al. 2000). Although our study does not directly address which tissue requires *Dkk1* and *Noggin*, it likely involves the AME: (1) All AVE markers analyzed are expressed normally in severe

Dkk1^{+/-} *Nog*^{+/-} embryos, with the exception of *Dkk1* itself, which is subsequently expressed in the AME. Likewise, *Hesx1* expression in the AME is abolished in these embryos. (2) *Dkk1*^{+/-} embryos containing mutant AVE but chimeric AME are normal (Mukhopadhyay et al. 2001). (3) *Noggin* is not expressed in the AVE but overlaps with *Dkk1* in the AME (McMahon et al. 1998; Bachiller et al. 2000). (4) The AME is the only tissue of mouse gastrulae that harbors full head-inducing activity in transplantation experiments (Kinder et al. 2001).

Figure 5. Head defects in *Dkk1*^{+/-} *Nog*^{-/-} embryos. Frontal (A,B,C) and lateral (A',B',C') views of newborn *noggin* mutant (*Nog*^{-/-} in A,A') and *Dkk1*^{+/-} *Nog*^{-/-} mutants (B,B',C,C') showing head defects, ranging from cyclopia (arrowhead in B) to complete lack of most anterior structures (C,C'). Whole-mount in situ hybridization of E9–E9.5 wild-type (D,E,F,G) and *Dkk1*^{+/-} *Nog*^{-/-} mutants (D',E',F',G'). (D) Expression of *Fgf8*, in the commissural plate (arrowhead) and isthmus (arrow), is only present in the isthmus in *Dkk1*^{+/-} *Nog*^{-/-} embryos (D'). (E) *Pax6*, marking the dorsal forebrain and rostral midbrain, is reduced anteriorly in *Dkk1*^{+/-} *Nog*^{-/-} embryos (E'). (F) *Six3* expression in prospective forebrain and future eyes is absent in *Dkk1*^{+/-} *Nog*^{-/-} embryos (F'). (G) Ventral diencephalon and the floor of the telencephalon are marked by *Nkx2.1* expression; in *Dkk1*^{+/-} *Nog*^{-/-} embryos both domains of expression are missing (G'). (H) Expression of *Hesx1* in forebrain is absent in E8.5 *Dkk1*^{+/-} *Nog*^{-/-} embryos (H'). Sagittal sections of E9.5 wild-type (I) and *Dkk1*^{+/-} *Nog*^{-/-} embryos (I') show reduction of forebrain (f). (h) Hindbrain, (m) midbrain.

Materials and methods

Genotyping and whole-mount in situ hybridization

Dkk1 (Mukhopadhyay et al. 2001) and *Noggin* (McMahon et al. 1998) heterozygotes were interbred to generate double-heterozygotes (*Dkk1*^{+/-} *Nog*^{+/-}) mutants by timed matings (Hogan et al. 1994). In the same way *Dkk1*^{+/-} *Nog*^{-/-} mutant embryos were generated by mating surviving *Dkk1*^{+/-} *Nog*^{+/-} mice with *Nog*^{+/-} mice. Embryos were staged as described previously (Kaufman 1992; Downs and Davies 1993). Both lines were kept in a C57BL/6 genetic background.

Adults, newborns, and embryos were genotyped by Southern or gene-specific PCR, using DNA from tail or visceral yolk sacs (Hogan et al. 1994). In some experiments, E6.5 and E7.5 embryos were genotyped after in situ hybridization using the whole embryos (Martinez-Barbera et al. 2000a,b).

Embryos were isolated in ice-cold phosphate-buffered saline (PBS), fixed overnight in 4% paraformaldehyde, and processed for whole-mount in situ hybridization as described for E9–E9.5 (Koop et al. 1996) and E6.5–E7.5 embryos (Lowe and Kuehn 2000). Color development was carried out using 4 mM nitroblue tetrazolium (NBT) solution and 0.05 mM 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in NTMT (100 mM NaCl, 100 mM Tris-HCl at pH 9.5, 50 mM MgCl₂, 0.1% Tween-20) with 2 mM levamisole.

PCR primers, RT-PCR, and antisense Morpholino oligonucleotides

PCR genotyping primers for *Dkk1* wild type were as described (Mukhopadhyay et al. 2001), and *Dkk1* mutant specific primers were forward, 5'-GAGAGGGCACAGCGATTAGGT-3'; reverse, 5'-TACCGTGGATGTGGAATGTG-3'. The *Noggin* wild-type and mutant specific primers were as described (McMahon et al. 1998). *Noggin* expression was analyzed in E6.5 and E7.5 wild-type embryos. Total RNA (RNAeasy kit, QIAGEN) was used for in vitro cDNA synthesis (Superscript II kit, GIBCO). The primers were: β -actin forward, 5'-GTGGGCGCTCTAGGCACCA-3'; reverse, 5'-CTCTTTGATGTACGCACGATTTC-3'; *Noggin* as described (McMahon et al. 1998). The antisense Morpholino oligonucleotides were *Noggin*, 5'-TCACAAGGCACTGGGAATGATCAT-3'; *Chordin*, 5'-GGACACTGCATTTTGTGGTTCCAA-3'.

Histology and skeletal preparations

Hematoxylin/eosin staining was carried out in newborns and E9.5 embryos, which were fixed overnight in 4% paraformaldehyde, dehydrated, embedded in paraffin, and sectioned at 6 μ m (Hogan et al. 1994). Cartilage and bone were stained with alcian blue and alizarin red (Wallin et al. 1994).

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***Dkk1* and *noggin* cooperate in mammalian head induction**

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