The role of *Xenopus dickkopf1* in prechordal plate specification and neural patterning

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**SUMMARY**

Dickkopf1 (dkk1) encodes a secreted WNT inhibitor expressed in Spemann’s organizer, which has been implicated in head induction in *Xenopus*. Here we have analyzed the role of dkk1 in endomesoderm specification and neural patterning by gain- and loss-of-function approaches. We find that dkk1, unlike other WNT inhibitors, is able to induce functional prechordal plate, which explains its ability to induce secondary heads with bilateral eyes. This may be due to differential WNT inhibition since dkk1, unlike frzb, inhibits Wnt3a signalling. Injection of inhibitory antiDkk1 antibodies reveals that dkk1 is not only sufficient but also required for prechordal plate formation but not for notochord formation. In the neural plate dkk1 is required for anteroposterior and dorsoventral patterning between mes- and telencephalon, where dkk1 promotes anterior and ventral fates. Both the requirement of anterior explants for dkk1 function and their ability to respond to dkk1 terminate at late gastrula stage. *Xenopus* embryos posteriorized with bFGF, BMP4 and Smads are rescued by dkk1. dkk1 does not interfere with the ability of bFGF to induce its immediate early target gene Xbra, indicating that its effect is indirect. In contrast, there is cross-talk between BMP and WNT signalling, since induction of BMP target genes is sensitive to WNT inhibitors until the early gastrula stage. Embryos treated with retinoic acid (RA) are not rescued by dkk1 and RA affects the central nervous system (CNS) more posterior than dkk1, suggesting that WNTs and retinooids may act to pattern anterior and posterior CNS, respectively, during gastrulation.

Key words: Blimp1, Hex, Shh, Retinoic acid, Prechordal plate, dkk1, Organizer, WNT, BMP, FGF, frzb, *Xenopus*

**INTRODUCTION**

During early amphibian development neural induction and anteroposterior (a-p) as well as dorsoventral (d-v) patterning of the neuroectoderm are regulated by inducers released from Spemann’s organiser and its derivatives. Many of the molecules secreted by the *Xenopus* organiser act by inhibiting signalling molecules of the BMP, WNT and Nodal-related families (Harland and Gerhart, 1997; Moon et al., 1997; Hsu et al., 1998; Piccolo et al., 1999). BMP inhibitors, like Chordin (Piccolo et al., 1996), Noggin (Zimmerman et al., 1996) and Follistatin (Hemmatti-Briavanou et al., 1994; Iemura et al., 1998), can induce ectopic trunks containing neural tube. WNT inhibitors like Frzb (Leys et al., 1997; Wang et al., 1997a), Dkk1 (Glinka et al., 1998), and Wif1 (Hsieh et al., 1999) can cooperate with BMP inhibitors in inducing secondary heads containing forebrain and eyes. Inhibition of Nodal signalling, e.g. by Cerberus (Hsu et al., 1998; Piccolo et al., 1999) or Antivin (Thisue et al., 2000) is sufficient to induce forebrain and eyes in the absence of mesoderm. While these studies suggest an important role for various TGF-β and WNT inhibitors in neural induction and patterning, a full understanding of their physiological relevance requires loss-of-function studies.

Dickkopf1 (dkk1) encodes a secreted WNT inhibitor (Dkk1), which acts upstream of the WNT pathway component dishevelled but whose mechanism of action is unknown (Glinka et al., 1998). Like *Xenopus dkk1*, mouse (Glinka et al., 1998), human (Fedi et al., 1999; Krupnik et al., 1999) and zebrafish dkk1 (Hashimoto et al., 2000) inhibit WNT/β-catenin signalling. dkk1 is expressed in the leading edge of involuting endomesoderm as well as in the prechordal plate during *Xenopus* gastrulation. Together with BMP inhibitors it induces secondary heads. Overexpression of dkk1 in *Xenopus* (Glinka et al., 1998) and zebrafish (Hashimoto et al., 2000) anteriorizes embryos, leading to shortened trunk and enlarged heads. Inhibition of Dkk1 in *Xenopus* using inhibitory antibodies leads to microcephalic embryos, typically exhibiting cyclopia (Glinka et al., 1998). While these studies suggest an important role for dkk1 in specification of rostral structures they raised a number of questions.

**Why does dkk1 induce two eyes and other WNT inhibitors only one eye?**

Coinjection of dkk1 mRNA with BMP antagonists routinely induces complete heads containing two well-formed eyes. In contrast, both frzb and dominant negative Xwnt8 together with BMP inhibitors induce only one ectopic eye (Glinka et al., 1998).
Is dkk1 required for prechordal plate fate specification?

dkk1 overexpression anteriorizes embryos but it is unclear if this corresponds to an effect of dkk1 on prechordal plate, or neuroectoderm, or both. In Xenopus, dkk1 superinduces anterior neural markers together with BMP inhibitors in animal cap ectoderm (Glinka et al., 1998) and in zebrafish overexpression of dkk1 promotes anterior neuroectoderm in antivin mRNA-injected embryos, which are devoid of most mesoderm (Hashimoto et al., 2000), indicating a direct effect on neuroectoderm. On the other hand, Xenopus dkk1 superinduces prechordal plate markers together with BMP inhibitors in ventral mesoderm (Glinka et al., 1998) and overexpression of dkk1 rescues mesendoderm formation in zebrafish mutant for bozozok (boz), a homeobox gene implicated in a pre-MBT WNT signalling pathway required for axis formation (Hashimoto et al., 2000). These latter results indicate an effect of dkk1 on mesendoderm.

Does dkk1 affect AP or DV neural patterning or both?

AntiDkk1 antibodies induce cyclopic embryos, indicating a requirement for dkk1 in anterior neural patterning. However, cyclopia can be caused by defects in ventral midline signalling, i.e. DV patterning, or by defects in AP patterning, or both (Durston et al., 1989; Chiang et al., 1996; Schier et al., 1996; Sampath et al., 1998). Our previous experiments did not distinguish between these possibilities.

When is dkk1 required for head induction?

Neural induction in vertebrates starts at early stages of gastrulation (Gawantka et al., 1998; Grinblat et al., 1998) but patterning occurs still during neurulation (Sasai and De Robertis, 1997). dkk1 expression starts in the early gastrula organizer but is maintained until late neurula in prechordal plate. It would be important to narrow down the critical period at which dkk1 is required and to infer the stages when WNT signals relevant to dkk1 action are active.

Which pathways does dkk1 interact with?

Regarding upstream regulation, zebrafish dkk1 expression is reduced in boz, one eyed pinhead (oep) and squint (sqt) mutants, suggesting that dkk1 is a target of pre-MBT (midblastula transition) WNT signalling as well as Nodal signalling (Hashimoto et al., 2000). Regarding downstream components, dkk1 functions as an inhibitory component of the posteriorizing WNT pathway, active after MBT. However, there are other secreted factors that also posteriorize embryos, including BMPs, FGFs and retinoic acid, and it is unclear if their pathways interact with dkk1.

Here we have addressed these questions, taking advantage of the availability of specific inhibitory Dkk1 antibodies that allowed us to test embryos for their requirement of dkk1 during early axis formation.

MATERIALS AND METHODS

Embryo culture and dissections

In vitro fertilization, embryo culture and staging were carried out as described previously (Gawantka et al., 1995). Operations on embryos were performed using a microknife and fused glass capillary on plastic dishes coated with 1% agarose in 0.5x Barth solution (Peng, 1991). Explants were cultured in the presence of antibodies in 96-well plates pretreated with BSA. Brains of 4-day embryos were excised by forceps in 1x Barth solution.

Antibodies

Antibodies for blastocoe1 injections were purified as described previously (Glinka et al., 1998). Embryos from stages 8-11 were injected with 150 ng antibody 14 or 15 into the blastocoe1 and allowed to develop for 3 days for phenotype analysis or fixed at stages 13, 15 or 30 for in situ hybridization. For incubation of explants, antibodies were dialysed overnight against 0.5x Barth solution, and utilized at concentrations of 3.2 mg/ml.

RNA and DNA injections

Synthetic capped RNAs for microinjection were obtained by in vitro transcription using the Megascript kit (Ambion). DNA templates were linearised, transcribed and mRNAs injected per blastomere as follows: pCSFzrb1, Norl, SP6; 0.2 ng; pSP64tBR, EcoRI, SP6, 0.25 ng; pRNkk1 Notl, SP6, 0.025ng; pT7TS-Xbhh BamHI, T7, 0.5 ng; pBMP4, Xhol, T3, 0.13ng; pCSXSmad1, SfiI, SP6, 0.5 ng; bFGF (Promega) was injected as protein into the blastocoe1 (25 pg/embryo). pCSmWnt3a DNA (Roelink and Nusse, 1991) was injected animaly at the four-cell stage at 0.05 ng in all blastomeres.

Whole-mount in situ hybridization, histological sections and TUNEL staining

Whole-mount in situ hybridization was performed as described by Harland (1991) with modifications (Hollemann et al., 1998). Histological 50 μm sections of albumin-gelatine embedded embryos were cut using a vibratome. TUNEL staining was carried out as described (Hensey and Gautier, 1998).

Luciferase assays and RT-PCR

For luciferase assays the BMP-responsive luciferase reporter construct pVent2-Luc (BRE) (Candia et al., 1997) and assays were carried out as described (Glinka et al., 1996). Embryos were injected with 25 pg pVent2-Luc and the following mRNAs: preprolactin (0.5 ng, control), BMP4 (0.25 ng), dkk1 (0.075 ng), frzb (0.5ng) or noggin (0.05 ng). RT-PCR assays were carried out as described (Gawantka et al., 1995). Gene-specific primers were as follows: H4, Xvent1 (Gawantka et al., 1995), Xbra (Glinka et al., 1996), Xvent2 (Onichtchouk et al., 1996), xcl (Salic et al., 1997).

RESULTS

dkk1 specifies the prechordal plate and is required for bilateral eye formation

Ventral coinjection of dominant-negative Bmp-2/4 receptor (IβR) mRNA (Suzuki et al., 1994) with dkk1 mRNA induces heads containing two eyes, while coinjected frzb (Leysn et al., 1997; Wang et al., 1997a) and IβR mRNAs induce heads with only one eye (Fig. 1A-C). To further test if this reflects a non-equivalence between these two WNT inhibitors we asked if frzb could rescue the phenotype resulting from the loss of Dkk1 function, the microcephaly induced by injected antiDkk1 Ab. This polyclonal antibody (anti15 Ab) was raised against a peptide epitope which is not conserved in dkk2-4 and not even
Role of *dkk1* in *Xenopus* It recognizes the extracellular form of Dkk1 and its effects can be completely blocked by preincubation with specific epitope-peptide as well as by extra Dkk1, attesting to its specificity (Glinka et al., 1998). Microinjection of anti15 Ab induces predominantly cyclopic embryos (Fig. 1D) while control Ab injection has no effect (not shown). However, while *dkk1* rescues head formation as shown previously, *frzb* injection only leads to a slight expansion of the cyclopic eye and forebrain but does not rescue bilateral eyes in anti15 Ab treated embryos (Fig. 1D-F). We conclude that *frzb* cannot substitute for the loss of *dkk1*, despite their common function as WNT antagonists.

Hedgehog emanating from the prechordal plate is thought to be a ventral midline signal necessary for splitting the eye field (Roelink et al., 1994; Ekker et al., 1995a,b; Macdonald et al., 1995; Chiang et al., 1996; Li et al., 1997). We therefore analyzed whether forced Hedgehog expression could rescue bilateral eye formation in anti*Dkk1* Ab-injected embryos. We used *banded hedgehog* (*bhh*), which has similar activities to *Shh* but is more potent (Ekker et al., 1995a). However, injected *bhh* mRNA does not rescue bilateral eye formation, although it elicits a potent hedgehog overexpression phenotype, most notably ventral eye defects (Fig. 1H), and it expands ventral midline tissue (not shown). It cannot be ruled out that *bhh* fails to rescue cyclopia because in order to do so its expression, unlike that of *dkk1*, needs to be localized.

We next tested the effect of *dkk1* and *frzb* on ectopic *sonic hedgehog* (*Shh*) (Ekker et al., 1995a) expression in the prechordal plate. In situ hybridization shows that secondary heads induced by *dkk1* expressed *Shh* in the ventral forebrain (*n*=32; Fig. 2A), as well as in the axial mesoderm rostral to...
the notochord, the position normally occupied by prechordal plate (n=26; Fig. 2C). This is unlike the secondary axes induced by frzb (n=22), where Shh expression did not reach up to forebrain level and remained in the outer tissue layer (Fig. 2B,D). Coinjected lacZ marker shows that frzb/tBRA expressing cells are capable of reaching a position ahead of the Shh expression limit, though, suggesting that specification, and not migration, is compromised. We examined the expression of another prechordal plate marker, goosecoid (gsc) (Cho et al., 1991) in early neurulae (stage 13). In dkk1-injected embryos all of the examined secondary heads (n=57) showed a zone of prechordal plate expression underlining and extending anterior to the neuroectodermal gsc expression (Fig. 2E, arrowheads). In contrast, in frzb-injected embryos the mesodermal and neuroectodermal gsc expression domains were always in register (Fig. 2F). We conclude that in secondary heads induced by frzb, unlike dkk1, prechordal plate cells, i.e. those expressing gsc and Shh simultaneously, are reduced and do not reach their proper position. This difference in formation of a proper secondary prechordal plate may explain the ability of dkk1 to induce bilateral eyes. However, Hedgehog signalling alone is not sufficient to rescue cyclopia induced by loss of dkk1, suggesting that additional ventral midline signals are affected in these embryos.

The above results indicated that dkk1 is sufficient to promote prechordal plate fate in conjunction with BMP inhibitors. We next asked whether dkk1 is also necessary for specification of this tissue. We analyzed the prechordal plate markers XHex (Newman et al., 1997), XBlimp1 (de Souza et al., 1999) and gsc, as well as the notochord marker XNot2 (Gont et al., 1993), in control neurulae, neurulae injected with antiDkk1 Ab, or neurulae overexpressing dkk1. Fig. 3 shows that all prechordal plate markers were reduced in Ab-injected neurulae and expanded in dkk1 mRNA-injected embryos. The expression domain of Xhex, which extends anterior of the prechordal plate into endoderm, was more sensitive to the Ab and to dkk1 overexpression than the posterior (black arrow in Fig. 3B,C). XNot2 expression showed that notochord was shortened and broadened by dkk1 overexpression (Fig. 3L). While the notochord marker XNot2 expanded following dkk1 injection it was hardly affected in Ab-injected embryos, suggesting that dkk1 is not required for notochord formation. We conclude that dkk1 is both necessary and sufficient for promoting prechordal plate specification.

dkk1 is required for a-p and d-v patterning of anterior neuroectoderm

dkk1 and AP patterning

To analyze the role of dkk1 in neural patterning we compared the expression of neural markers in embryos injected with antiDkk1 Ab, or injected with dkk1 mRNA or treated with retinoic acid (RA). RA is a posteriorizing agent, which like antiDkk1 Ab injection, can induce cyclopia and microcephaly (e.g. Durston et al., 1989; Sive et al., 1990; Ruiz i Altaba and Jessell, 1991a,b; Sharpe, 1991; reviewed in Sasai and De Robertis, 1997), and it was of interest to know whether both treatments act in a similar fashion.

We first analyzed the effect of dkk1 on AP patterning (Fig. 4). dkk1 RNA or DNA injection induces an expanded head region, including larger eyes and telencephalon. Expression of the telencephalic marker BF1 (Bourguignon et al., 1998), the anterior neural plate marker Xanf1 (Zaraisky et al., 1992), as well as XOtx2, whose anterior and posterior expression domains mark the cement gland and midbrain, respectively (Blitz and Cho, 1995; Pannese et al., 1995), was greatly expanded posteriorly and laterally following dkk1 injection (Fig. 4B,F,J). This can also be seen in dissected brains from tadpole embryos (Fig. 4O,S). In contrast, mesencephalon as well as hindbrain and otic placodes were reduced, depending on the injected dose, as indicated by reduced or absent En2 (Fig. 4B) (Hemmati-Brivanlou et al., 1991) and Krox20 (Bradley et al., 1993) expression (marking rhombomeres 3 and 4).

Fig. 3. dkk1 is required for prechordal plate formation.

(A,D,G) Uninjected controls. (B,E,H) Embryos injected with anti15 Ab into the blastocoel at blastula stage and (C,F,I) embryos injected radially at the four-cell stage with dkk1 mRNA. In situ hybridization with probes to XHex1 (A-C), XBlimp1 (D-F) and XNot2 + gsc (G-I). (A-F) Embryos from early neurula stage (stage 13) were cut sagittally; dorsal side up, anterior to the right. White arrowheads indicate the anterior and posterior limits of expression. (G-I) embryos from stage 13 are shown from the dorsal side, anterior side up. (J-L) The prechordal plates of control (J), anti15 Ab-injected (K), or dkk1 mRNA-injected embryos (L) are shown from gastrocoel, anterior side is up. Red arrows indicate the lateral limit of Shh expression. PCP, prechordal plate; NOT, notochord.
5, not shown). Likewise, the posterior domain of $XOt\text{tx}_2$ expression, which marks future mesencephalon and diencephalon caudally to the zona limitans, was significantly reduced (Fig. 4J). We conclude that $d\text{kk}1$ is able to anteriorize the neural plate.

Consistent with these gain-of-function results, we found that loss of $d\text{kk}1$ posteriorizes the neural plate. Injection into the blastocoel of anti14 control antibody, which recognizes intracellular Dkk1, had no detectable phenotypic effect ($n=45$), whereas microinjection of anti15 Ab led reproducibly to microcephaly (100%) and cyclopia (74%) ($n=120$). 5-22% of the embryos showed complete deletion of eyes and forebrain. Such embryos were lacking expression of $BF1$ and had reduced $Xanf1$ expression (Fig. 4C,G). $XOt\text{tx}_2$ expression shifted to the very anterior part of the brain and its posterior domain was expanded (Fig. 4K). While $En2$ expression at the mid-hindbrain boundary was unaffected (data not shown), this phenotype differs significantly from that induced following RA treatment, where brain structures are reduced up to hindbrain, as is highlighted by reduction of all markers, including $En2$ expression (compare Fig. 4C,D, P, Q, T, U). The loss of anterior neural structures in antiDkk1 Ab-injected embryos is not due to increased cell death, as monitored by TUNEL staining of neurula embryos (data not shown), and hence is due to repatterning. We conclude that $d\text{kk}1$ is required for specification of neural structures anterior of the mid-hindbrain boundary.

$d\text{kk}1$ and DV patterning

The role of $d\text{kk}1$ in prechordal plate formation makes an effect on DV patterning of the anterior neural plate very likely, since it is known that genetic or mechanic ablation of the prechordal plate affects ventral forebrain structures (Adelmann, 1936a,b). Indeed, analysis of dissected brains indicated that $d\text{kk}1$ RNA injection leads to an expansion and antiDkk1 Ab injection to a massive loss of ventral tel- and diencephalon (Fig. 4S,T). To further study DV patterning of the anterior neural plate we analyzed $XNot2$, which is also a marker of dorsal neuroectoderm, expressed bilaterally in the dorsal diencephalon, as well as of $Shh$, a marker for the ventral midline. $d\text{kk}1$ RNA injection leads to expansion of $Shh$ and a reduction of $XNot2$ expression, and conversely antiDkk1 Ab injection leads to expansion of $XNot2$ and reduction of $Shh$ expression (Fig. S). Thus, $d\text{kk}1$ is necessary and sufficient for ventralization of anterior neural plate. Similar to antiDkk1 Ab treatment, RA reduces ventroanterior structures, leading to a collapse and fusion of
XNot2 expression on the midline and to a reduction of Shh expression (Fig. 5D,H).

**Time window of dkk1 action**

To understand when *dkk1* acts in the specification of anterior neural tissue, we performed two types of experiment. First, we determined up to which stage anti-Dkk1 Ab is able to affect head structures. We dissected the head anlage during mid-late gastrula and cultured it in isolation. Such explants contain all three germ layers (anterior neuroectoderm, anterior endomesoderm) and they will develop into well-patterned heads (Fig. 6A-E). We dissected such explants at various stages and cultured them in the presence of anti15 or control Ab. While explants treated with control Ab developed into normal patterned heads (85%, *n*=44; Fig. 6D,E), anti-Dkk1 treated explants were cyclopic when cultured from late gastrula onwards (stage 12.5) (83%, *n*=57; Fig. 6B). However, when added from early neurula (stage 13) onwards, the Ab no longer had an effect and explants formed again well-patterned heads (87%, *n*=56; Fig. 6C). Second, we determined the stage at which head anlagen lose competence to respond to *dkk1*. We grafted animal caps of albino
embryos injected with dkk1 RNA to head anlagen from pigmented embryos of three stages, late gastrula (stage 12.5), early (stage 13.5) and mid neurula (stage 14.5). Only stage 12.5 explants (76%; n=21) had enlarged forebrain and big eyes compared to the control (Fig. 6G); the later stages were normal (100%, n=17; Fig. 6H). Thus, following both overexpression as well as inhibition, we find that dkk1 acts until late gastrula stage.

**dkk1 interacts with BMP and FGF signalling pathways**

**dkk1** functions as a WNT inhibitor in a variety of assays, in line with the view that its role in head formation pertains to its antagonizing the posteriorizing action of WNTs after MBT (reviewed in Niehrs, 1999). However, FGF, BMP and RA have been implicated in neural posteriorization in addition to WNTs (reviewed in Slack, 1994; Harland and Gerhart, 1997; Sasai and De Robertis, 1997). These factors may either act as posteriorizing signals independent of WNTs or they may act by ultimately activating a posteriorizing WNT pathway. In the latter case, their effects should be rescued by **dkk1**.

To analyze these relationships, embryos were posteriorized by incubation with RA (Fig. 7A), blastomere injection of **BMP4** (Fig. 7D) (Dale et al., 1992; Jones et al., 1992), **Smad1** (Fig. 7G) (Meersman et al., 1997), **Smad5** (Table 1) (Suzuki et al., 1997) or mouse **Wnt3a** (Fig. 7M) (Roelink and Nusse, 1991) mRNAs or by injection of bFGF protein into the blastocoel (Fig. 7J). As summarized in Table 1, **dkk1** mRNA rescued the posteriorization by all of these reagents, with the exception of RA (Fig. 7B). The WNT inhibitor **frzb** also efficiently rescued **BMP4-**, **XSmad-1-** and bFGF-treated embryos but, unlike **dkk1**, it failed to revert posteriorisation induced by **Wnt3a** (Fig. 7I), previously shown to be unaffected by **frzb** (Wang et al., 1997b). These results indicate that the posteriorizing effects of forced BMP and FGF expression can be compensated by a concomitant downregulation of WNT signalling and that the RA pathway acts independently of posteriorizing WNTs.

The fact that **dkk1** and **frzb** rescue BMP- and FGF-treated embryos raised the question of whether signalling of these growth factors may directly require WNT signalling. To test this we carried out animal cap assays and studied the induction of FGF and BMP target genes between blastula and gastrula stages by RT-PCR. Induction of the FGF target gene **Xbra** is known to be a direct response, which is not blocked by cycloheximide (Smith et al., 1991). Neither **dkk1** nor **frzb** interfere with **Xbra** induction by bFGF (Fig. 8A). This indicates that direct FGF signalling is unaffected by these

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**Table 1. dkk1 is able to rescue BMP4-, Xsmads-, mWnt3A- and bFGF-, but not RA-induced, posteriorization**

<table>
<thead>
<tr>
<th>Injected reagent</th>
<th>Posteriorized embryos (%)</th>
<th>Normal or anteriorised embryos (%)</th>
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<tr>
<td>10^-6 M RA</td>
<td>100</td>
<td>0</td>
<td>36</td>
</tr>
<tr>
<td>10^-7 M RA</td>
<td>81</td>
<td>19</td>
<td>40</td>
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<tr>
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<td>0</td>
<td>29</td>
</tr>
<tr>
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<td>16</td>
<td>29</td>
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<tr>
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<td>29</td>
<td>49</td>
</tr>
<tr>
<td><strong>BMP4/dkk1</strong></td>
<td>0</td>
<td>100</td>
<td>51</td>
</tr>
<tr>
<td><strong>BMP4/frzb</strong></td>
<td>8</td>
<td>92</td>
<td>51</td>
</tr>
<tr>
<td><strong>XSmad1</strong></td>
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<td>10</td>
<td>31</td>
</tr>
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<td>35</td>
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<td>26</td>
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<tr>
<td><strong>mWnt3A/frzb</strong></td>
<td>95</td>
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n, number of embryos injected.

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**Fig. 7. dkk1 rescues embryos posteriorized by bFGF, BMP4, XSmad1 and Wnt3A, but not by RA.** Where indicated, embryos were injected radially at the four-cell stage with dkk1 or frzb mRNA. (A,B) Embryos treated from stages 8-13 with 10^-7 M retinoic acid (RA). (D-F) Embryos were injected radially at the four-cell stage with **BMP4** or **XSmad1** mRNA. (J-L) Embryos were injected radially at the four-cell stage with dkk1 or frzb mRNA and at the blastula stage with bFGF protein into the blastocoel. (M-O) Embryos were injected animaly at the four-cell stage with mWnt3A plasmid DNA. (C) Uninjected control.
Fig. 8. Signalling of BMP but not FGF is sensitive to WNT inhibitors. (A) Xbra induction by bFGF is insensitive to WNT inhibitors. RT-PCR analysis of Xbra expression in animal caps. Embryos were uninjectected (Co) or injected animally at the four-cell stage with dkk1 or frzb mRNA. Animal caps were cut at stage 8, cultivated with or without bFGF and analysed for induction of Xbra at stage 10.5. (B) Induction of BMP target genes shows a transient requirement for WNT signalling. RT-PCR analysis of Xvent2, slz and Xvent1 expression in animal caps. Embryos were injected animally at the four-cell stage with BMP4 mRNA or BMP4 plus dkk1 or frzb mRNAs as indicated. Animal caps were cut at stage 8 and analysed for induction of mesodermal markers at stages 9, 10, and 11. –RT, minus reverse transcription control samples; H4, histone H4 for normalisation. (C) Induction of the Xvent2 promoter by BMP4 requires WNT signalling. Embryos were coinjected animally at the four-cell stage with the BMP-responsive reporter plasmid pVen2-Luc and either preprolactin mRNA (control, first column), or BMP4, dkk1, frzb or noggin mRNAs as indicated. At stage 10, luciferase assays were carried out with extracts of whole embryos in triplicate samples.

WNT inhibitors and that WNT-dependent posteriorisation by FGF occurs indirectly, e.g. by secondary induction of WNTs.

Surprisingly, in the analogous experiment with BMP4-injected animal caps, induction of all tested target genes, Xvent2 (Nichtchouk et al., 1996), sizzled (Salic et al., 1997) and Xvent1 (Gawantka et al., 1995) is sensitive to dkk1 and frzb (Fig. 8B). Xvent2 is an immediate early response gene to BMP signalling (Ladher et al., 1996; Rastegar et al., 1999) and its promoter contains a well-characterized BMP response element (Hata et al., 2000; Henningfeld et al., 2000). BMP4-induced activation of a luciferase reporter construct containing this promoter fragment (Candia et al., 1997) is also sensitive to dkk1 and frzb in stage-10 embryos (Fig. 8C). However, while dkk1 and frzb inhibit induction of BMP targets at blastula and early gastrula stage, the requirement for WNT signalling fades by midgastrula (stage 11; Fig. 8B). The results indicate that BMP, unlike FGF signalling, requires endogenous WNT signalling between the blastula and early gastrula stages.

DISCUSSION

dkk1 is able to induce entire heads when coexpressed with BMP inhibitors and injection of inhibitory antibodies leads to microcephaly in Xenopus embryos. These results, together with the expression in the anterior endomesoderm, indicate that dkk1 plays an important role in head induction. However, these findings raise questions regarding the role of dkk1 in different germ layers, the timing of its action and the pathways with which it interacts. Here we present a detailed analysis of the requirement for dkk1 during axial patterning. Our study provides compelling evidence for a physiological role of WNT signalling in neural and mesendodermal regionalization and characterizes the epistatic interactions of WNT with respect to BMP and FGF signalling during this process.

dkk1 and anterior endomesoderm formation

Our results indicate that dkk1 is necessary and sufficient to promote formation of the anterior endomesoderm. Specifically, we observe that prechordal plate markers such as XBlimp1, gsc, and XHex are reduced following antiDkk1 Ab injection. In addition, the anterior expression of XHex, labeling the anterior endoderm, is also reduced. These genes are superinduced and expanded following dkk1 overexpression but they are not induced ectopically, e.g. in ventral mesoderm or posterior chordamesoderm. This indicates that other factors in addition to WNT inhibitors are required for prechordal plate formation, most likely BMP inhibitors, since coexpression of dkk1 with BMP inhibitors can induce prechordal plate markers ectopically in ventral mesoderm (Glinka et al., 1998). Notochord formation does not appear to require dkk1, even though dkk1 overexpression expands notochord territory (this study) and rescues notochord in boz mutants (Hashimoto et al., 2000). While previous studies suggested that WNTs are able to ventroposteriorize dorsal mesoderm (Christian and Moon, 1993; Hoppler et al., 1996; Fredieu et al., 1997) and that simultaneous BMP and WNT inhibition is sufficient to convert mesoderm into prechordal plate (Glinka et al., 1997, 1998; Piccolo et al., 1999), our data provide the first evidence that WNT inhibition (via dkk1) is indeed required for this process in vivo.

The induction of bilateral eyes by dkk1 but not by frzb can be explained by the ability of the former to promote proper prechordal plate formation. While frzb also induces prechordal plate markers such as gsc, this induced tissue never reaches an anteroventral position. We also note that frzb, unlike dkk1, is unable to induce ectopic XHex expression when coinjected with bBR (data not shown). This difference in prechordal plate specification suggests that endomesoderm is antagonized by
more than one WNT and that dkk1 and frzb do not inhibit the same set of WNTs. This is supported by the inability of frzb to rescue antiDkk1 Ab-injected embryos. Indeed, we show that Wnt3a, which is required for posterior development in mice (Takada et al., 1994), is inhibited by dkk1 but not by frzb (Wang et al., 1997b). Interestingly, bhh mRNA injection was also insufficient to rescue cyclopic embryos following antiDkk1 Ab injection. This indicates that there are other ventral midline signals in addition to Hedgehog acting downstream of dkk1 that are required for splitting of the eye field.

Xenopus dkk1 is expressed in the deep endomesoderm of the gastrula, including both the prospective prechordal plate and anterior endoderm, and becomes confined to the prechordal plate in neurulae. In zebrafish and mouse, dkk1 is initially expressed in extraembryonic endoderm, the yolk syncytial layer and the anterior visceral endoderm (AVE), respectively, and later becomes confined to the prechordal plate (Glinka et al., 1998; Pearce et al., 1999; Hashimoto et al., 2000). It will be interesting to dissect the respective roles of dkk1 in these two tissues.

dkk1 and anterior neural patterning

Our data indicate that dkk1 is required for patterning of the entire anterior neural plate since both a-p as well as d-v markers are affected in Ab-injected embryos. dkk1 acts by anteriorizing the neural plate from midbrain onwards and by ventralizing it. This is consistent with the observation that WNTs not only posteriorize but also dorsalize neuroectoderm, promoting neural crest formation (Saint-Jeannet et al., 1997; Chang and Hemmati-Brivanlou, 1998; Dorsky et al., 1998; LaBonne and Bronner-Fraser, 1998). Thus, WNTs and their antagonists such as Dkk1 integrate the patterning of both neural axes. An important question is whether dkk1 patterns neuroectoderm only indirectly via its promoting endomesoderm formation or whether neuroectodermal cells directly respond to dkk1. Hashimoto et al. (2000) carried out an elegant experiment where they prevented anterior endomesoderm formation by antivin mRNA injection and asked whether dkk1 could still anteriorize neural plate. Indeed, in these embryos dkk1 anteriorized the neuroectoderm but could not rescue endomesoderm, indicating that ectodermal cells can respond directly to dkk1. Further evidence for a direct action of dkk1 on neuroectoderm is the observation that Xenopus animal caps, which are of purely ectodermal origin, express anterior neural markers in response to dkk1 mRNA injection (Glinka et al., 1998). Thus, dkk1 directly affects both neuroectoderm and endomesoderm.

The requirement for dkk1 both in prechordal plate and AP neural patterning argues against the notion that the prechordal plate is only involved in DV and not AP neural patterning. This was inferred from studying oep mutant embryos, which are cyclopic but contain forebrain structures, and where a mature prechordal plate does not form (Schier et al., 1997). The suggestion that prechordal plate only promotes ventral midline signalling and not anteriorization also conflicts with the findings that extirpation of anterior endoderm only affects heart induction while extirpating prospective prechordal plate deletes head structures (Schneider and Mercela, 1999). Furthermore, the prechordal plate is a potent neural-anteriorizing tissue (Mangold, 1933; Bradley et al., 1996; Foley et al., 1997; Pera and Kessel, 1997; Zoltewicz and Gerhardt, 1997). Close inspection of oep mutant embryos reveals a reduction of forebrain markers (Grinblat et al., 1998) and furthermore, prechordal plate markers are still expressed in oep mutant embryos during early gastrulation (Strähle et al., 1997).

The requirement for dkk1 in neural patterning supports the two-inhibitor model, which proposes that head formation involves simultaneous inhibition of BMP and WNT signals (Glinka et al., 1997; Niehrs, 1999). A requirement for BMP inhibition in head formation has recently been demonstrated by the finding that noggin/chordin double-homozygous mutant mice show severe forebrain defects and cyclopia (Bachiller et al., 2000). This is consistent with the notion that a BMP-dependent gradient of positional information is present across the entire gastrula ectoderm (Knecht et al., 1995; Wilson et al., 1997; Barth et al., 1999).

WNTs as posteriorizing signals

Classical experiments by Nieuwkoop, Saxen and Toivonen predicted that posteriorizing signals act during gastrulation to repattern an initially anterior neural induced state (Gilbert and Saxen, 1993; Nieuwkoop, 1997). A number of candidates for such signals have since been proposed, including WNTs (reviewed in Niehrs, 1999), FGFs (reviewed in Slack, 1994) BMPs (Dale et al., 1992; Jones et al., 1992) and RA (e.g. Durston et al., 1989; Sive et al., 1990; Ruiz i Altava and Jessell, 1991a,b; Sharpe, 1991; reviewed in Sasai and De Robertis, 1997). The requirement for dkk1 in anterior neural patterning provides compelling evidence for a physiological role of WNTs in posteriorization. Our data indicate that WNTs that antagonize head formation and interact with dkk1 are active until late gastrula. That neural patterning may mostly occur prior to neurulation is consistent with previous studies showing patterned expression of neural markers during early gastrulation (Gawantka et al., 1998; Grinblat et al., 1998). Various WNTs are expressed in tissues relevant to neural patterning. Xwnt8 is expressed in the lateroventral mesoderm and is a prime candidate for inhibiting notochord and prechordal plate fates (Smith and Harland, 1991). Xwnt1, -3A (Wolda et al., 1993) and -7B (Chang and Hemmati-Brivanlou, 1998) are predominantly expressed in gastrula ectoderm and/or neurula posterior neuroectoderm, suggesting that they are responsible for posteriorizing neural fates. They may act in either vertical (Nieuwkoop, 1997; Poznanski and Keller, 1997; Chen et al., 2000) and/or planar type signalling (Ruiz i Altava, 1993, 1998) to inhibit rostral and induce more caudal gene expression.

The results show that the posteriorizing action of FGFs may be due to their acting through WNTs because they can be rescued by dkk1 and frzb. Similarly, the requirement for WNT signaling during neural crest induction has been shown to be direct, whereas FGF-mediated neural crest induction may be indirect and mediated by WNT signals (LaBonne and Bronner-Fraser, 1998). Furthermore, the posteriorizing action of FGF in Noggin-treated animal caps is dependent on endogenous WNT signalling (McGrew et al., 1997). Finally, experiments with a dominant negative FGF receptorI argued against a posteriorizing role for FGF in early neural patterning (Kroll and Amaya, 1996). Thus, the active principle behind FGF-mediated posteriorization appears to be induced WNT signalling.
That BMPs antagonize anterior neural cell fates and head formation has long been known (Dale et al., 1992; Jones et al., 1992) and BMP inhibitors are indeed required for head formation since Chordin/noggin double homozygous mutant mice lose forebrain structures and are cyclopic (Bachiller et al., 2000). What is the relationship between early BMP- and WNT signalling? We show that BMP4-posteriorized embryos are rescued by dkk1. Furthermore, our results reveal that there is cross-talk between BMP and WNT signalling pathways until the early gastrula stage since induction of the immediate BMP target gene Xvent2 is sensitive to WNT inhibitors. A similar cross-talk between WNT and TGF-β pathways during gastrula induction of Xwnt8 expression has been demonstrated to be due to direct binding of Smad4 to Lef1 (Nishita et al., 2000). However, the WNT/BMP cross-talk appears to be transient since at stage 10.5 (Marom et al., 1999) or 11 (this work), BMP-induced target gene induction becomes independent of WNT antagonists. Yet, the relationship between BMP and WNT signalling is further complicated by the positive regulation of Xwnt8 expression by BMP4 after MBT (Marom et al., 1999). Furthermore, before MBT the WNT/β-catelin signalling pathway of the Nieuwkoop center appears to repress BMP4 expression (Baker et al., 1999). Thus, at least three interactions between BMP and WNT signals in early Xenopus can be distinguished. First, WNT signalling represses BMP4 in the early organiser (Baker et al., 1999). Second, in regions where BMP4 signalling is activated, it depends on concomitant WNT signalling between blastula and early gastrula stages. There is evidence for unidentified ventralizing WNTs that may be involved in this process (Itoh and Sokol, 1999; Kühl et al., 2000). Third, by midgastrula direct BMP4 signalling becomes independent of WNT signalling and is required for Xwnt8 expression (Marom et al., 1999). With regard to the co-inhibition of head induction by WNTs and BMPs, we favor a model where these pathways synergize during the latter phase to antagonize head formation. This synergy may either occur at the level of common or distinct target genes specifying rostral fates.

Unlike bFGF and BMP4, RA induces embryonic microcephaly in a fashion which cannot be rescued by dkk1. This indicates an independent posteriorizing pathway from that of WNTs. This is also supported by the way that RA and antiDkk1 Ab influence neural patterning. Both affect AP (e.g. Durston et al., 1989; Sive et al., 1990; Ruiz i Altaba and Jessell, 1991a,b; Sharpe, 1991; reviewed in Sasai and De Robertis, 1997) and DV patterning (Franco et al., 1999; this work) and induce microcephaly, but in RA-treated embryos neural structures including the midbrain are reduced while in antiDkk1 Ab-injected embryos the midbrain is slightly increased. Furthermore, loss-of-function studies in Xenopus and mouse indicated that retinoid signalling is required for patterning neural tissue posterior of the hindbrain (Blumberg et al., 1997; Dupe et al., 1999; Niederreither et al., 1999; Sharpe and Goldstone, 2000). This raises the possibility that WNTs pattern the neural plate between forebrain and midbrain and RA between hindbrain and spinal cord.

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