Depletion of Bmp2, Bmp4, Bmp7 and Spemann organizer signals induces massive brain formation in *Xenopus* embryos

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Summary

To address the patterning function of the Bmp2, Bmp4 and Bmp7 growth factors, we designed antisense morpholino oligomers (MO) that block their activity in *Xenopus laevis*. Bmp4 knockdown was sufficient to rescue the ventralizing effects caused by loss of Chordin activity. Double Bmp4 and Bmp7 knockdown inhibited tail development. Triple Bmp2/Bmp4/Bmp7 depletion further compromised trunk development but did not eliminate dorsoventral patterning. Unexpectedly, we found that blocking Spemann organizer formation by UV treatment or β-Catenin depletion caused BMP inhibition to have much more potent effects, abolishing all ventral development and resulting in embryos having radial central nervous system (CNS) structures. Surprisingly, dorsal signaling molecules such as Chordin, Noggin, Xnr6 and Cerberus were not re-expressed in these embryos. We conclude that BMP inhibition is sufficient for neural induction in vivo, and that in the absence of ventral BMPs, Spemann organizer signals are not required for brain formation.

Key words: BMP, Chordin, Sizzled, Morpholino, Spemann organizer, Brain induction, Dorsoal-ventral patterning

Introduction

Bone Morphogenetic Proteins (BMPs) are growth factors identified by virtue of their ability to induce ectopic bone formation when introduced subcutaneously in mammals (Urist et al., 1979). BMPs are thought to be involved in multiple aspects of cell signaling and homeostasis, including germ cell formation, stem cell maintenance and cell differentiation (Hogan, 1996; Fujiwara et al., 2001; Ying et al., 2003). In particular, during early development BMPs are central players in establishing the dorsoventral (DV) pattern of the embryo. Many zygotic mutations affecting DV patterning have been isolated in *Drosophila* and zebrafish, the majority of which affect the BMP signaling pathway (Lall and Patel, 2001; Hammerschmidt and Mullins, 2002; De Robertis and Kuroda, 2004). Research in *Xenopus* has also provided important insights into the role of BMP signaling in embryonic patterning. This is illustrated by the discovery that Chordin and Noggin encode secreted BMP antagonists produced by the Spemann organizer (Piccolo et al., 1996; Zimmerman et al., 1996). The use of dominant-negative BMP receptors (dn-BMPR) or cleavage-mutant BMPs (cm-BMPs, precursor BMP proteins in which proteolytic processing sites are impaired) has provided valuable information on the requirements of BMP signaling for DV patterning (Hawley et al., 1995; Yamamoto et al., 2001; Munoz-Sanjuan and Brivanlou, 2002). However, these constructs do not allow the respective activities of individual BMP ligands to be distinguished, as they inhibit the activity of multiple BMPs. Therefore, gene-specific loss-of-function reagents are greatly needed in the field.

Three BMPs stand out as candidates for regulating DV patterning in *Xenopus*. These are Bmp2 and Bmp4, which can replace the function of the DPP morphogen in *Drosophila* (Padgett et al., 1993), and Bmp7, which when mutated in zebrafish results in dorsalized phenotypes (Hammerschmidt and Mullins, 2002). During gastrulation in *Xenopus*, *Bmp4* is expressed in a ventral domain diametrically opposed to the dorsal Spemann organizer. A number of secreted proteins are co-expressed with Bmp4 in this region, which has been designated the ventral gastrula center (De Robertis and Kuroda, 2004). Extracellular proteins expressed in the ventral center include Twisted Gastrulation (Tsg) (a co-factor of both Bmp4 and Chordin), the metalloproteinase Xolloid-related (which cleaves Chordin), the Chordin-related protein Crossveinless-2, the Bmp inhibitory pseudoreceptor Bambi, and the secreted Frizzled-related protein Sizzled (reviewed by De Robertis and Kuroda, 2004). Expression of ventral center genes coincides with high levels of BMP signaling, which can be monitored using phospho-specific antibodies directed against the carboxy-terminal region of the transcription factor Smad1, which is phosphorylated by BMP receptors (Faure et al., 2000; Kurata et al., 2001).

At the opposite pole of the gastrula lies the Spemann organizer, a source of secreted BMP antagonists and other factors that interact extracellularly with ventral center gene products to generate a BMP morphogenetic gradient (Harland and Gerhart, 1997; De Robertis and Kuroda, 2004). Traditionally, two main biological activities of the Spemann Organizer have been distinguished (Spemann, 1938; Niehrs, 2004). The head organizer located in the early blastopore lip induces head and trunk structures after transplantation, whereas the trunk-tail organizer located in the late dorsal lip

induces tail structures. Molecular investigations using Wnt pathway inhibitors or the multivalent inhibitor Cerberus have suggested that head formation requires the double inhibition of Wnt and BMP (Glinka et al., 1997), or the triple inhibition of Nodal-related, Wnt and BMP signaling (Piccolo et al., 1999; Niehrs, 2004). Inhibition of just the BMP pathway (by dn-BMPR, cm-BMPs or individual BMP antagonists) in wild-type embryos results in the development of partial secondary axes with trunk/tail structures that lack head and forebrain tissues.

Recent work has shown that at earlier stages, during blastula, two distinct dorsal centers are formed. On the dorsal animal and marginal zone, a blastula Chordin- and Noggin-expressing (BCNE) center is formed in cells that will later on give rise to the anterior CNS (Kuroda et al., 2004). In more vegetal dorsal cells, the Nieuwkoop center releases mesoderm-inducing *Xenopus* Nodal-related (*Xnrs*) growth factors and the antagonist Cerberus (Kuroda et al., 2004). Dorsal β-Catenin accumulation is triggered by sperm entry and can be blocked by irradiation with ultraviolet (UV) light during the first cycle (De Robertis et al., 2000; Weaver and Kimelman, 2004). Thus, the *Xenopus* DV pattern is currently thought to arise from a series of cell-cell interaction events involving four signaling centers, two at blastula and two at gastrula stage, that generate a gradient of BMP signaling (reviewed by De Robertis and Kuroda, 2004). Inhibition of BMP signaling is required for the initial neural induction in *Xenopus* (Harland, 2000; Stern, 2005). A second layer of regulation is provided by further inhibition of Smad1 activity by MAPK phosphorylation, which may mediate the neural-inducing effects of FGFs and IGFs (Pera et al., 2003; Sater et al., 2003; Kuroda et al., 2005).

In the present study, we developed BMP-specific antisense morpholino oligomers (MO) and used them to investigate the individual roles of Bmp2, Bmp4 and Bmp7 in early *Xenopus* development. In embryonic explants, inhibition of Bmp4 and Bmp7 signals caused neural differentiation in ectoderm, and differentiation of dorsal fates in mesoderm. Triple knockdown of Bmp2, Bmp4 and Bmp7 compromised trunk and tail development, giving rise to embryos with enlarged dorsal structures. However, these embryos retained a considerable degree of DV polarity. Unexpectedly, we found that knockdown of BMPs caused the formation of extensive head and brain structures in embryos in which dorsal development had been prevented by UV treatment. Similarly, striking radial brain structures were obtained in embryos ventralized by injection of β-Catenin MO. Formation of the Spemann organizer at gastrula stage, or of Nieuwkoop or BCNE centers at blastula stage, was not restored by BMP MOs in dorsalized embryos. Thus, in the absence of the dorsal organizing centers, inhibition of BMP signaling is sufficient to cause extensive head and CNS induction in embryos that would otherwise develop without any neural tissue at all.

Materials and methods

Morpholino oligomers and RNA injections

The 25-bp morpholino antisense oligomers for Bmp2, Bmp4 and Bmp7 were obtained from Gene Tools and consisted of the following sequences:

Bmp2 MO, 5′-GATCCCAGCGACCATTGTCAACCTG-3′;
Bmp4 MO, 5′-CAGCATTCGGTTACCAGGAATCATG-3′;
Bmp7 MO, 5′-TTACTGTCAAAGCATTCATTTTGTC-3′.

Morpholinos were resuspended in sterile water to a concentration of 1 mM, which was then further diluted to give a working solution of 0.25 mM. When co-injected with β-Catenin MO (Heasman et al., 2000) or Chordin MO [a mixture of two MOs targeting both pseudoalleles of Chordin (Oelgeschläger et al., 2003)], a mixture was prepared and embryos were injected four times radially at the two- to four-cell stage with 4 nl (3 ng MO/injection). For rescue experiments mouse *Bmp4* mRNA was microinjected at 25 pg in each blastomere at the four-cell stage.

Embryological methods

Microinjections and mRNA synthesis were performed as described (Kuroda et al., 2004). RT-PCR conditions and primers, as well as the protocol for whole-mount in situ hybridization, are described at http://www.hhmi.ucla.edu/derobertis/index.html, except for the modification that after hybridization all embryos (pigmented or albino) were bleached overnight (in 10% H_2O_2, 20% H_2O, 80% methanol) under intense neon light to enhance contrast. Digital photographs were taken with a Leica DC500 digital camera. For each embryo, at least three pictures were taken in successive focal planes and merged with Photoshop® CS.

Protein injections and western blots

Injections of recombinant mouse Fgf8b protein (R&D Systems) was carried out as described (Pera et al., 2003). Western blot analysis of endogenous levels of phospho-Smad1 in *Xenopus* whole embryos (Faure et al., 2000) used an anti-phospho-hSmad1 antibody at a 1 in 500 dilution (Persson et al., 1998).

Results

Generation of BMP-specific morpholino reagents

We generated MO reagents (Heasman et al., 2000) for Bmp2, Bmp4 and Bmp7. To overcome the pseudotetraploid nature of the *Xenopus laevis* genome (Oelgeschläger et al., 2003), a search of the EST database was performed and two pseudoalleles were identified for each of the three genes. Antisense MOs were designed to block translation of all BMP pseudoalleles by targeting 100% conserved 25-bp stretches, including the AUG initiation codon (Fig. 1A). In vitro, each MO specifically and efficiently blocked translation of its target mRNA (Fig. 1B). MOs were microinjected into the marginal region of each blastomere at the four-cell stage in order to test their effect on development (Fig. 1C).

Embryos depleted of Bmp4 exhibited the most severe phenotypes; at early tailbud stage, enlarged brain structures (marked by *Six3* in forebrain and eye, and *Krox20* in hindbrain) were observed (compare Fig. 1E and 1F). Bmp4 MO embryos appear larger and longer than controls because of an expanded archenteron cavity, presumably caused by increased convergence-extension (Myers et al., 2002). This dorsalized phenotype could be rescued by microinjection of mouse *Bmp4* mRNA (Fig. 1G), which has a different nucleotide sequence in the region targeted by *Xenopus* Bmp4 MO. At the swimming tadpole stage, a posteriorization of the proctodeum (anus) and a severe loss of ventral fin tissues could be observed (Fig. 1I,J). These tail phenotypes are consistent with a decrease of BMP signaling in *Xenopus* (see Fig. S1 in the supplementary material) and zebrafish (Wagner and Mullins, 2002). The onset of the Bmp4 depletion phenotype could be traced back to alterations in gene expression patterns at gastrula, the stage at which *Bmp4* expression is maximal in *Xenopus* (Dale et al., 1992). As shown in Fig. S2 (see supplementary material),

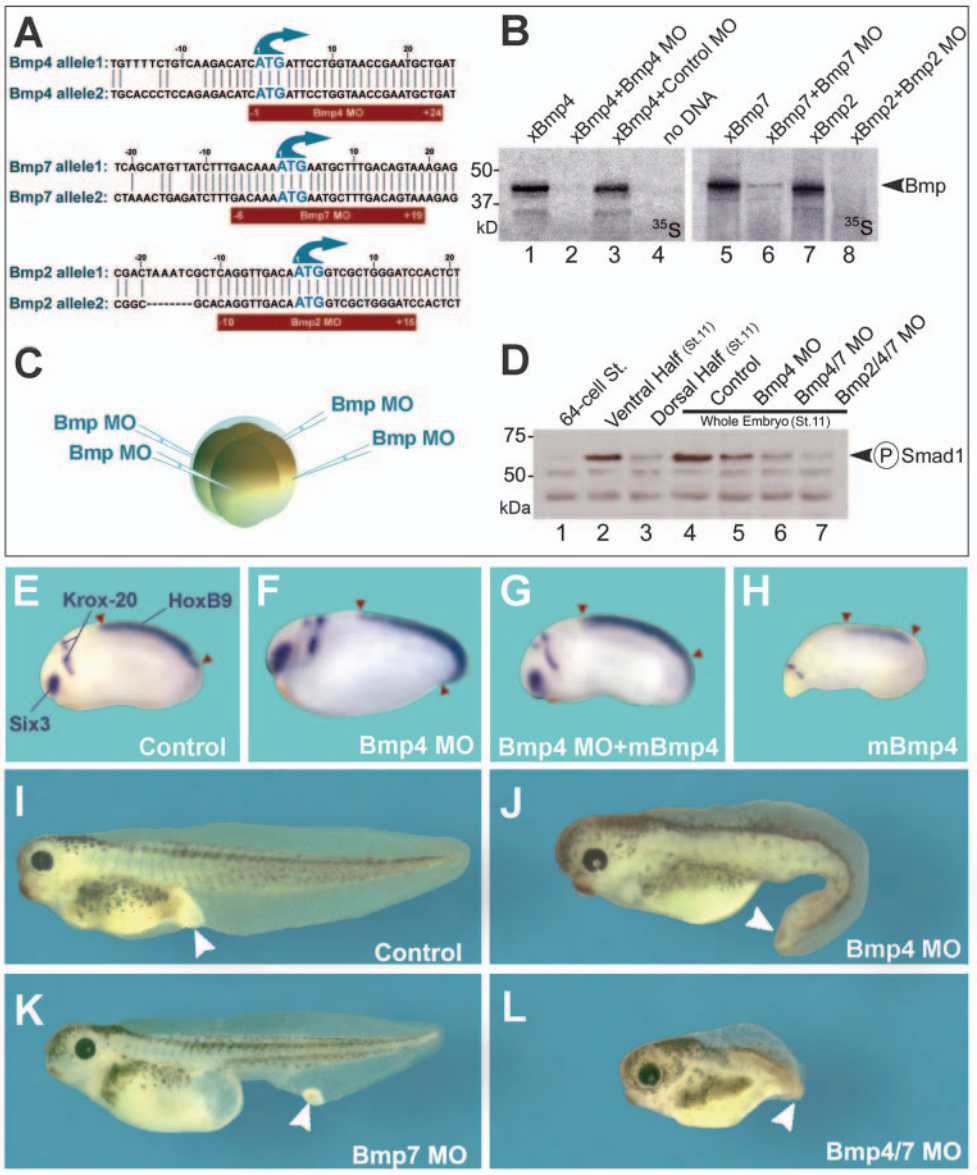

Fig. 1. Antisense MOs against Bmp2, Bmp4 and Bmp7 inhibit endogenous Smad1 phosphorylation and cause dorsalization and posterior truncations of *Xenopus* embryos. (A) Design of Bmp4, Bmp7 and Bmp2 antisense MOs that target both pseudoalleles expressed in the subtetraploid species *X. laevis*. (B) In vitro transcription/translation of *Bmp4*, *Bmp7* and *Bmp2* is specifically inhibited by the respective MOs. (C) MOs for Bmp2, Bmp4 and Bmp7 (at 12 ng each) were injected either alone or in combinations at the four-cell stage radially in each blastomere. (D) Endogenous carboxy-terminal Smad1 phosphorylation in stage 11 embryos is decreased by co-injection of multiple Bmp MOs. (E-H) Bmp4 MO-injected embryos (F) are dorsalized with enlarged heads (compare with control embryos, E). Red arrowheads delineate the spinal cord marker *Hoxb9* (>85%, *n*=60). (G) Bmp4 MO-dorsalized phenotype is rescued by microinjection of 100 pg of mouse *Bmp4* mRNA. (H) Microinjection of mouse *Bmp4* mRNA alone (100 pg) results in ventralized embryos with small heads, no eyes and reduced spinal cord structures (red arrowheads). (I-L) Bmp4-depleted embryos (J) develop into swimming tadpoles with no ventral fins and slightly larger heads than control embryos (I). Note the position of the anus, which is displaced (posteriorized) to the tip of the tail (white arrowhead; >72%, *n*=61). (K) Bmp7-depleted tadpoles develop with a partial loss of ventral fin and a posteriorized anus (white arrowhead; >79%, *n*=51). (L) Double knockdown of Bmp4 and Bmp7 results in tadpoles lacking tail structures (>90%, *n*=39).

knockdown of Bmp4 decreased expression of *Bmp4*, eliminated the ventral center marker *Sizzled*, and downregulated the BMP downstream target genes *Mix1* and *Vent1*. The neural plate, visualized through the pan-neural *Sox2* and the forebrain/midbrain *Otx2* markers, was expanded.

Embryos injected with the Bmp7 MO displayed similar but less severe phenotypes at the tadpole stage, with a partial loss of ventral fin tissue accompanied by posteriorization of the anus (Fig. 1K, arrowhead). At early tailbud, a dorsalized phenotype, less marked than that of Bmp4 MO, was observed (Fig. 2C,D). Bmp2 MO-injected embryos were also slightly dorsalized, but were less affected than Bmp7 MO-injected embryos (Fig. 2B). The expression domain of *Sizzled* in the ventral-most region at tailbud stage (Collavin and Kirschner, 2003) was found to be a particularly good reporter of BMP signaling. The anterior portion of this ventral domain was dependent on Bmp2 (Fig. 2A′,B′), whereas the posterior *Sizzled* expression domain required Bmp4 and Bmp7 signals (Fig. 2C′,D′). Taken together, these knockdown experiments are consistent with the notion that BMPs promote ventral development during *Xenopus* gastrulation.

Dorsalization in double and triple BMP knockdowns

To further characterize the requirements for Bmp2, Bmp4 and Bmp7, double and triple knockdowns were performed in all possible combinations and analyzed by in situ hybridization (Fig. 2). In addition, western blot analyses showed that phosphorylation of endogenous Smad1 at gastrula stage was increasingly inhibited by combined depletion of Bmp2, Bmp4 or Bmp7 activities (Fig. 1D). This suggested that BMP signaling in the *Xenopus* embryo results from the integration of multiple BMP signals that act in concert to promote ventral development. In agreement with this, more severe phenotypes were consistently observed in double or triple depletions than in single MO injections. For example, doubly depleted Bmp4/Bmp7 tadpoles were unable to develop tail structures (Fig. 1L).

Combinations of Bmp2, Bmp4 and Bmp7 MOs were

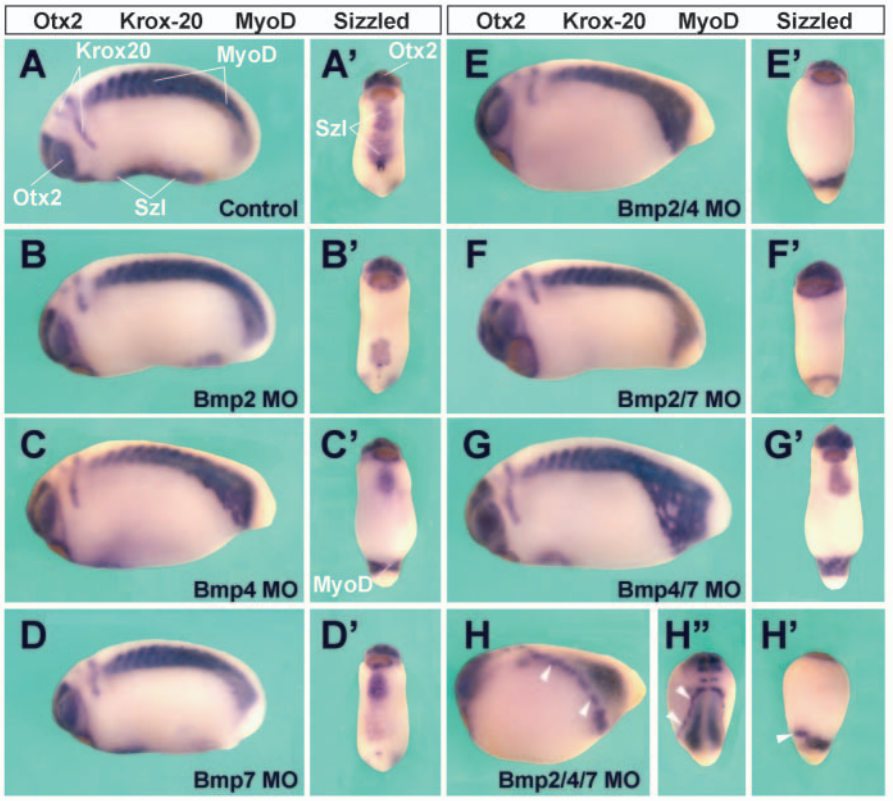

Fig. 2. Triple depletion of Bmp2, Bmp4 and Bmp7 results in greater dorsalization of the embryo than any single or double BMP knockdown. Embryos were fixed at tailbud stage and expression domains of four markers (*Otx2*, *Krox20*, *Myod* and *Sizzled*) were compared with control siblings (n=15 per experimental set). (A-H) Lateral view, anterior to the left. (A′-H′) Ventral view, anterior to the top. (H″) Dorsal view, anterior to the top. (A-D′) Uninjected controls and single Bmp2, Bmp4 and Bmp7 knockdowns. (E-G′) Double BMP knockdowns (2/4, 2/7 and 4/7). Note the dorsalized phenotype with increased *Otx2* expression, radial expression of *Myod* in the posterior, and ventral loss of *Sizzled*. (H-H″) Triple knockdown of Bmp2, Bmp4 and Bmp7 produces further dorsalization. Expression of *Krox20* in rhombomere 5 was seen over the entire circumference of the embryo down to the tip of the tail (white arrowheads).

injected and their effects on the expression domains of four markers genes, *Otx2*, *Krox20*, *MyoD* and *Sizzled*, analyzed at tailbud (Fig. 2). *Sizzled* expression in the entire ventral region was eliminated by Bmp2 MO in combination with either Bmp4 or Bmp7 MO (Fig. 2E′,F′). *MyoD*, a paraxial mesoderm marker, enveloped the tailbud ventrally, forming a ring in Bmp4 MO-injected embryos (Fig. 2C,C′) or any of the double BMP-depleted embryos (Fig. 2E-G′), indicating an expansion of paraxial tissue into ventroposterior mesoderm. *Krox20* was unaffected or modestly increased in any single or double depletion, but rhombomere 5 expression was radially expanded in triple Bmp2/Bmp4/Bmp7 MO-injected embryos (arrowheads in Fig. 2H,H″). Thus, triple Bmp2/Bmp4/Bmp7-depleted embryos were dorsoanteriorized and consisted mainly of tissues anterior to rhombomere 5. Surprisingly, the forebrain/midbrain marker *Otx2* was only moderately enhanced in Bmp-depleted embryos at tailbud stage, even in triple-knockdown embryos (Fig. 2H).

Differentiation of ectopic neurons marked by *N-Tubulin* was observed in the posterior ectoderm of Bmp4, Bmp2/Bmp4 and Bmp4/Bmp7 MO-injected embryos (Fig. 3). These neurons, which probably correspond to Rohon-Beard sensory neurons, followed the pattern of the underlying paraxial mesoderm (Fig. 2) (Mullins et al., 1996; Myers et al., 2002). Expansion of the Rohon-Beard neuron population has been reported in zebrafish mutant combinations that affect the BMP activity gradient in ectoderm (Barth et al., 1999; Nguyen et al., 2000). Fgf8 is known to decrease the activity of the BMP signal transducer Smad1 through an inhibitory phosphorylation of its linker region (Pera et al., 2003). Neuronal differentiation by lowering BMP levels strongly synergized with microinjection of the Fgf8 protein into the blastocoele cavity (Fig. 3E,F). This

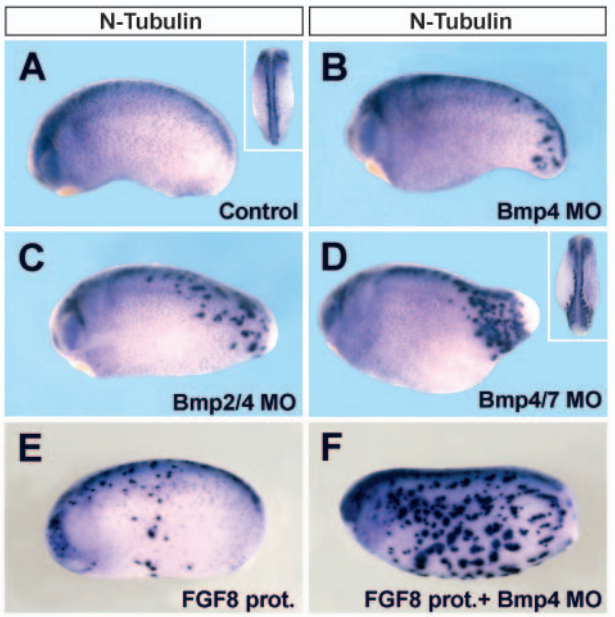

Fig. 3. BMP knockdown induces neuronal differentiation in posterior epidermis and cooperates with Fgf8 signaling. (A) The differentiated neuronal marker *N-Tubulin* is expressed in the CNS of uninjected control embryos at tailbud stage (inset: dorsal view, anterior to the top). (B-D) Inhibition of endogenous Bmp4, Bmp2/Bmp4 and Bmp4/Bmp7 activities results in ectopic neuronal differentiation in the posterior ectoderm (inset: dorsal view, anterior to the top). Comparable results were obtained with Bmp2/Bmp7 and triple Bmp2/Bmp4/Bmp7 MO injections (data not shown). (E,F) Control embryos or Bmp4 MO embryos were injected at the blastula stage with 1.5 ng of recombinant mouse Fgf8 protein into the blastocoele cavity and analyzed at tadpole stage for *N-Tubulin* expression. Fgf8 protein injection in wild-type embryos resulted in minor ectopic neural differentiation, which greatly synergized with Bmp4 depletion.

indicates that inhibition of BMP signaling can cooperate with FGF signals during neural induction.

We conclude from these results that the dorsalized phenotype observed in single Bmp2, Bmp4 or Bmp7 knockdowns is increased in double and triple MO injections. Although development of the tail is lost, even triple Bmp2/Bmp4/Bmp7 knockdowns retain a significant amount of DV and anteroposterior (AP) pattern.

BMPs are required for histotypic differentiation

We next analyzed cell differentiation in Bmp4/Bmp7-depleted ventral marginal zone (VMZ) and animal cap (AC) explants (Fig. 4A-H). RT-PCR analyses showed that VMZs depleted of Bmp4 or Bmp7 acquired dorsal fates, expressing muscle markers such as *Myf5* and neural markers such as *Ncam*, *N-Tubulin* and *Otx2* (Fig. 4A, lanes 2 to 4). In double Bmp4/Bmp7 knockdowns, midline mesodermal markers such as *Sonic Hedgehog* (*Shh*) and *Xnot* were induced, indicating that the VMZs differentiated into dorsal marginal zone (DMZ) tissues (Fig. 4A, lanes 5 and 6). Histological analyses revealed the formation of a large amount of neural and cement gland tissue accompanied by greatly increased cell numbers (Fig. 4, compare B with C). The ectodermal AC explant system provides the gold standard for neural induction studies in *Xenopus*. We found that although Bmp4 or Bmp7 MOs were sufficient to cause some neural induction alone, their combined knockdown was much more potent, activating the expression of neural markers such as *N-Tubulin* (Fig. 4D-G), *Ncam* and *Otx2* (Fig. 4H) in ectoderm.

We also found that, consistent with the involvement of BMPs in the specification of primordial germ cells (PGCs) in mice (Fujiwara et al., 2001), expression of *Xpat*, a marker for PGCs in *Xenopus* (Hudson and Woodland, 1998), was eliminated in embryos triply depleted of Bmp2, Bmp4 and Bmp7 (Fig. 4I-L). Knockdown of individual BMPs, and all double combinations, still retained at least some PGCs (Fig. 4J,K and data not shown). These loss-of-function experiments support the view that BMP signaling plays a key role in the differentiation of neural tissue, dorsal mesoderm and PGCs in *Xenopus*.

Bmp4 is epistatic to Chordin

In *Xenopus*, Chordin knockdown causes a ventralized phenotype that may result from excess BMP signaling (Oelgeschläger et al., 2003). To investigate this, we compared Bmp4 MO- or Chordin MO-injected embryos with double Bmp4/Chd MO-injected embryos (Fig. 5A-L). *Sizzled* expression, which provides an excellent readout for BMP signaling and ventral center formation, was greatly expanded in Chordin morphants at gastrula (Fig. 5G). Bmp4 MO- and double Bmp4/Chd MO-injected embryos were virtually devoid of *Sizzled* expression (Fig. 5D,J). At tadpole stages, a similar result was obtained: double Bmp4/Chd MO-injected embryos had the same dorsalized phenotype as Bmp4 MO alone (Fig.

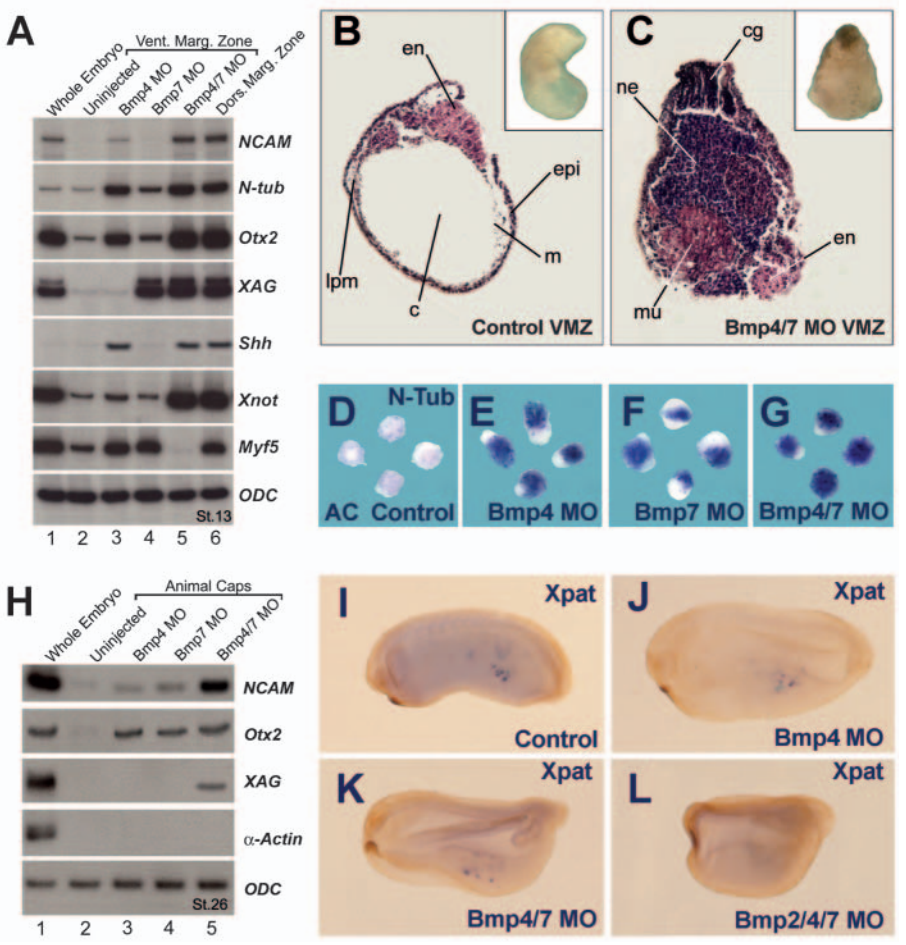

Fig. 4. BMP depletion causes ventral mesoderm to acquire dorsal fates, animal caps to become neural, and primordial germ cell losses. (A) RT-PCR analysis of Bmp4/Bmp7-depleted VMZ explants. Microinjections of Bmp4/7 MOs cause VMZ to adopt a DMZ fate with upregulation of neural markers, such as *Ncam*, *N-Tubulin*, *Otx2*, and dorsal midline markers, like *Shh* and *Xnot*. (B,C) Histological sections of control VMZ compared with Bmp4/Bmp7-depleted VMZ. Absence of Bmp4/Bmp7 signaling in VMZ induces massive cell proliferation accompanied by muscle differentiation (mu), and neural tissue (ne) and cement gland (cg) formation. Insets show external views of the explants. en, endoderm; epi, epidermis; m, mesothelium; c, coelomic cavity; lpm, lateral plate mesoderm. (D-G) Animal caps depleted of Bmp4, Bmp7, or both, contain neural tissue as shown by the expression of *N-Tubulin*, a marker of differentiated neurons (n=12 per experimental set). (H) RT-PCR analysis of animal cap (AC) explants. Inhibition of Bmp4/Bmp7 results in neural differentiation in animal caps as shown by the expression of *Ncam* and *Otx2* in the absence of mesodermal marker *α-Actin*. ODC provides a loading control and *Xag* is a cement gland marker. (I-L) Germ cells, marked by the probe *Xpat* (small dots in the endodermal region), fail to develop in embryos injected with Bmp2/Bmp4/Bmp7 MOs. Primordial germ cells still form, albeit in reduced numbers, in Bmp4 or Bmp4/Bmp7 MO-injected embryos (n=8 per experimental set).

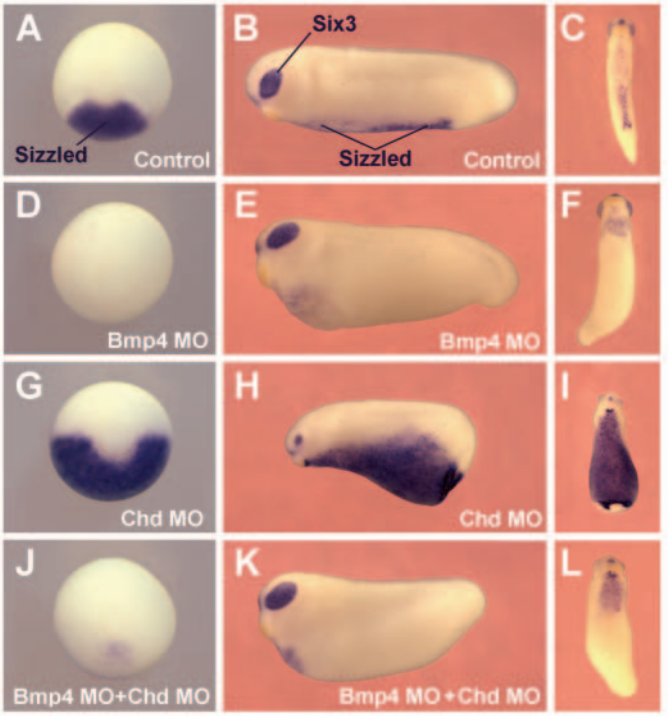

Fig. 5. Bmp4 antagonizes Chordin activity in the embryo. Four-cell stage embryos were injected, into each blastomere, with Bmp4 MOs (12 ng) or Chordin MOs (7 ng), or co-injected with a mixture of the two (n=15 per experimental set). (A-C) *Sizzled* is expressed in the ventral center at stage 12 (A) and on the ventral side (B,C) at tailbud stage. (D-F) Depletion of Bmp4 results in the lack of *Sizzled* expression at late gastrula stage (D) and in significant reductions at tadpole stage (E,F). Expression of *Six3*, an eye marker, is expanded. (G-I) Chordin depletion gives rise to a phenotype opposite to that seen in Bmp4 MO embryos, with increased *Sizzled* expression and reduced *Six3*. (J-L) Bmp4 is epistatic to Chordin in co-injection experiments.

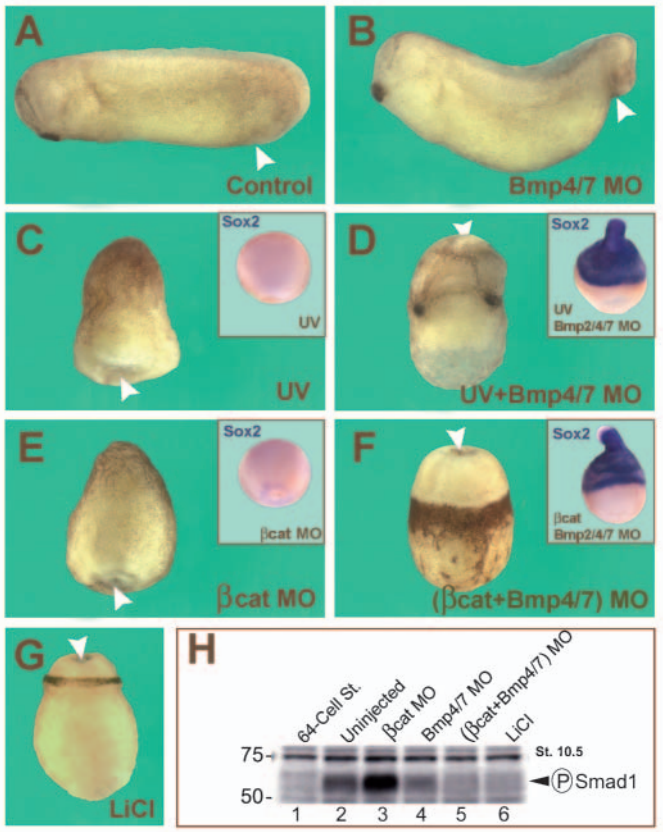

Fig. 6. Inhibition of Bmp2/Bmp4/Bmp7 signaling in UV-treated embryos or β-Catenin-depleted embryos results in radial brain formation. Bmp2/Bmp4/Bmp7 MOs were injected four times radially (12 ng each) into two-cell-stage UV-irradiated embryos, or were co-injected with β-Catenin MO (7 ng total) (n=45 or more per experimental set). White arrowheads point to the blastopore/anus. (A,B) Depletion of Bmp4/Bmp7 in wild-type embryos leads to tail defects, but leaves the head and trunk regions mostly unaffected [Dorso Anterior Index, DAI=6.6 (Kao and Elinson, 1988)]. (C) UV-treated embryos are radially ventralized and develop into ventral 'belly pieces' lacking all CNS and head structures (DAI=0.7). They are devoid of neural tissue, as shown by the lack of *Sox2* expression at stage 20 (inset). (D) UV-treated embryos injected with Bmp4/Bmp7 MOs are radially dorsalized, and lack trunk and tail structures (DAI=8.1). At stage 20, half of the ectoderm of UV-treated Bmp2/Bmp4/Bmp7 morphants expresses the pan-neural marker *Sox2* (inset). (E) β-Catenin MO-injected embryos, like UV embryos, develop into radially ventralized belly pieces (DAI=0.3). They are also devoid of neural tissue, as shown by the lack of *Sox2* expression at stage 20 (inset). (F) Depletion of Bmp4/Bmp7 activity in β-Catenin MO-injected embryos leads to dramatically hyperdorsalized embryos (DAI=9.5) with a radial cement gland (compare with B). At stage 20, half of the ectoderm of β-Catenin/Bmp2/Bmp4/Bmp7 morphants expresses the pan-neural marker *Sox2* (inset). (G) Embryos dorsalized by LiCl treatment (DAI=8.6). (H) Western blot analysis of endogenous phosphorylation of Smad1 at gastrula stage 11. Embryos ventralized by β-Catenin MOs have high levels of Smad1 phosphorylation, which require Bmp4/Bmp7 signals.

5, compare H with K). We conclude that *Xenopus* Bmp4 is epistatic to Chordin. *Xenopus* Chordin, as is the case in zebrafish (Hammerschmidt and Mullins, 2002), serves as a dedicated BMP antagonist.

Radial CNS in embryos lacking β-Catenin and BMP signals

While studying the effects of Bmp4/Bmp7 MOs in embryos ventralized with UV light, we made an unexpected discovery. UV treatment blocks the early nuclear β-Catenin signal, causing the development of ventralized embryos devoid of all axial and CNS structures (Fig. 6A,C) (reviewed by De Robertis et al., 2000). Inhibition of the early β-Catenin signal was accompanied by increased levels of endogenous Smad1 phosphorylation, indicating high BMP signaling levels (Fig. 6H, lane 3). Because UV-ventralized embryos overexpress *Bmp4* and its target gene *Sizzled* (see Fig. S3 in the supplementary material), we expected that the knockdown of Bmp4 and Bmp7 would be less efficient or, at most, equally efficient as in wild-type embryos (Fig. 6B). Instead, we found that in the UV background, Bmp4/Bmp7 or Bmp2/Bmp4/Bmp7 knockdown had a much greater effect, causing the formation of dorsalized embryos consisting almost exclusively of dorsoanterior head structures and deficient in ventroposterior tissues (compare Fig. 6B with 6D). Similarly, in embryos ventralized with β-Catenin MO (Heasman et al., 2000), Bmp4/Bmp7 MOs induced massive head-like structures

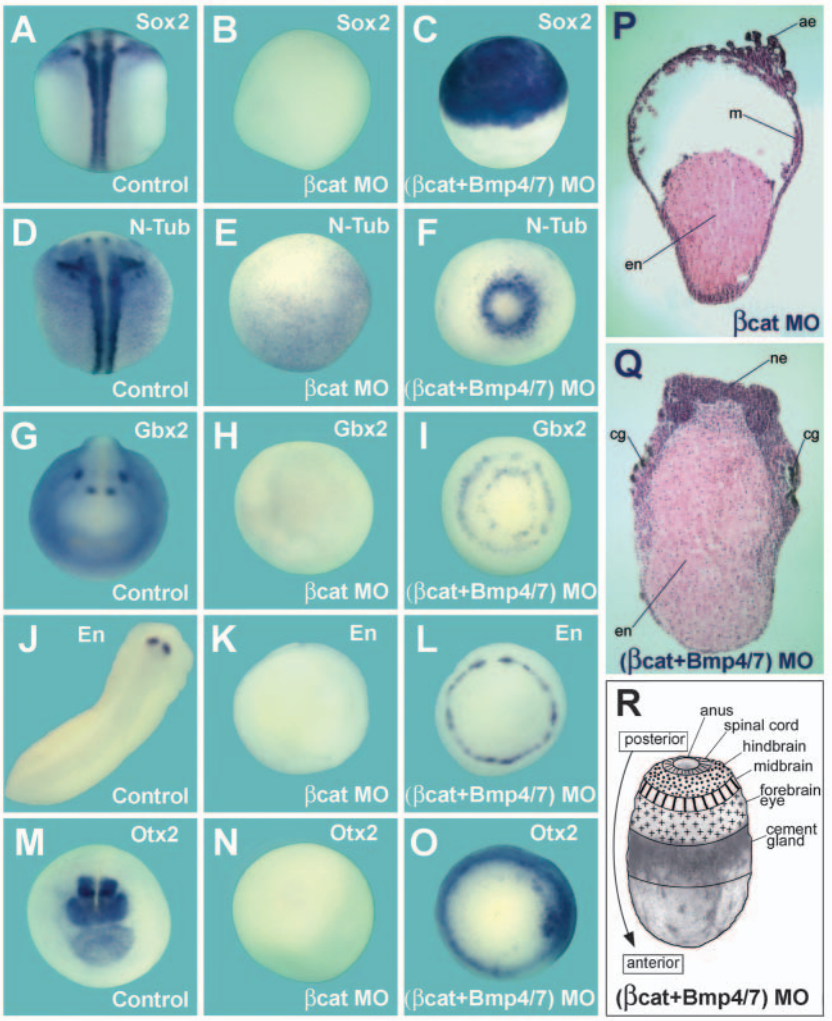

Fig. 7. Depletion of Bmp2/Bmp4/Bmp7 causes the formation of head-like structures with a radially patterned CNS in embryos ventralized by β-Catenin MOs. Wild-type, β-Catenin or triple β-Catenin/Bmp4/Bmp7 MO-injected embryos were tested for the expression of CNS markers (n=9 or more per experimental set). (A-C) Expression of *Sox2*, demarcating the CNS at stage 15 in a control embryo, is abrogated in β-Catenin MO-injected embryos, and greatly expanded in embryos depleted of β-Catenin and Bmp4/Bmp7. (D-F) Expression of *N-Tubulin* in differentiated neurons in the posterior CNS is abolished in β-Catenin MO-injected embryos, but can be seen radially around the blastopore in embryos lacking β-Catenin and Bmp4/Bmp7. (G-I) *Gbx2* expression, which marks rhombomere 1 and otic vesicles (Von Bubnoff et al., 1996), is undetectable in β-Catenin MO-injected embryos but can be visualized in two concentric rings in embryos depleted of β-Catenin and Bmp4/Bmp7. (J-L) *Engrailed-2* expression, which marks the midbrain-hindbrain boundary, is not present in β-Catenin MO embryos but is expressed as a ring in embryos depleted for β-Catenin and Bmp4/Bmp7. (M-O) Expression of the forebrain/midbrain marker *Otx2* is absent in embryos injected with β-Catenin MOs but is expressed circumferentially in embryos depleted of β-Catenin and Bmp4/Bmp7. (P) Histological section through a β-Catenin MO-ventralized embryo. ae, atypical ectoderm; en, endodermal tissue; m, mesothelium. (Q) Co-injection of β-Catenin and Bmp4/Bmp7 MOs revealed enhanced cell proliferation in ectoderm with neural tissue (ne) differentiation and radial cement gland formation (cg). (R) Schematic depiction of the anteroposterior polarity of the radial CNS formed in β-Catenin and Bmp4/Bmp7-deficient embryos deduced from in situ hybridization studies.

along the radius of the embryo, including the formation of a conspicuous radial cement gland (Fig. 6E,F). These head-like structures were much more extensive than those seen in embryos dorsalized by Lithium Chloride (LiCl) in a wild-type background (Fig. 6G). When Bmp2/Bmp4/Bmp7 and β-Catenin MOs were co-injected, extrusions containing the blastopore at their tip were also observed (see insets in Fig. 6D,F). The total amount of *Sox2*-positive neural tissue in β-Catenin/Bmp4/Bmp7- or β-Catenin/Bmp2/Bmp4/Bmp7-depleted embryos was comparable (Fig. 6D,F).

Using the pan-neural marker *Sox2*, massive neural inductions were observed in embryos co-injected with β-Catenin, Bmp4 and Bmp7 MOs, in striking contrast to the complete absence of the CNS in embryos in which only β-Catenin was inhibited (compare Fig. 7B with 7C). At least half of the ectoderm was transformed into histotypic neural tissue and cement gland (Fig. 7P,Q). Although this CNS was radially symmetric and lacked all DV pattern, it still expressed AP markers. Thus, BMP depletion has a potent neural-inducing effect in embryos ventralized by UV treatment or β-Catenin knockdown. The posterior CNS, marked by the neuronal marker *N-Tubulin* at this stage, was located next to the radial blastopore or future anus (Fig. 7D-F), whereas the forebrain/midbrain marker *Otx2* was found distal to it, adjacent to the radial cement gland (Fig. 7M-O). Hindbrain (*Gbx2*) and midbrain-hindbrain border (*En2*) markers were expressed at intermediate positions (Fig. 7G-L). These AP neural marker results are summarized in Fig. 7R. We conclude that BMP depletion has much greater effects on neural induction in ventralized embryos than in wild-type embryos. It appears that signals regulated by the early β-Catenin signal can partially compensate for the loss of Bmp4/Bmp7 in wild-type embryos.

Inhibition of Bmp4/Bmp7 does not induce expression of dorsal genes

Because ventralized embryos were so strongly affected by Bmp4/Bmp7 MOs, our expectation was that expression of dorsal genes, which is lost in β-Catenin MO and UV embryos, would be hyper-rescued. Surprisingly, we found that at the blastula stage neither Nieuwkoop center genes (such as *Xnr6*, Fig. 8A-D) nor BCNE center markers (such as *Pintallavis/Hnf3β* and *Chordin*, Fig. 8E-L) were induced in embryos depleted for β-Catenin and Bmp4/Bmp7. RT-PCR analysis confirmed that, as was the case for the single β-Catenin knockdown, all dorsal genes tested (*Chordin*, *Noggin*, *Xnr3*, *Siamois*, *Xtwn*, *Pintallavis/Hnf3β*, and *Cerberus*) were downregulated in β-Catenin/Bmp4/Bmp7 MO co-injections at the blastula stage (Fig. 8Q, lane 4). Even at gastrula, Spemann

organizer expression of *Chordin* (Fig. 8M-P) or *Goosecoid* (not shown) was not restored by Bmp4/Bmp7 MOs in embryos depleted of β-Catenin.

These findings show that the induction of radial CNS structures caused by depletion of Bmp2, Bmp4 and Bmp7 in ventralized embryos is independent of Nieuwkoop or BCNE center formation, and ultimately of Spemann organizer function. As the extent of neural induction caused by β-Catenin/Bmp2/Bmp4/Bmp7 MOs is much greater than in wild-type embryos, the results suggest that the dorsal organizing centers, in addition to providing dorsalizing growth factor antagonists, may be the source of additional ventralizing signals that are lacking in UV-treated or β-Catenin-depleted embryos, as explained below.

Discussion

In the present study, we have generated three new morpholinos that allow the requirements for Bmp2, Bmp4 and Bmp7 in *X. laevis* to be analysed. An important advantage of MO reagents

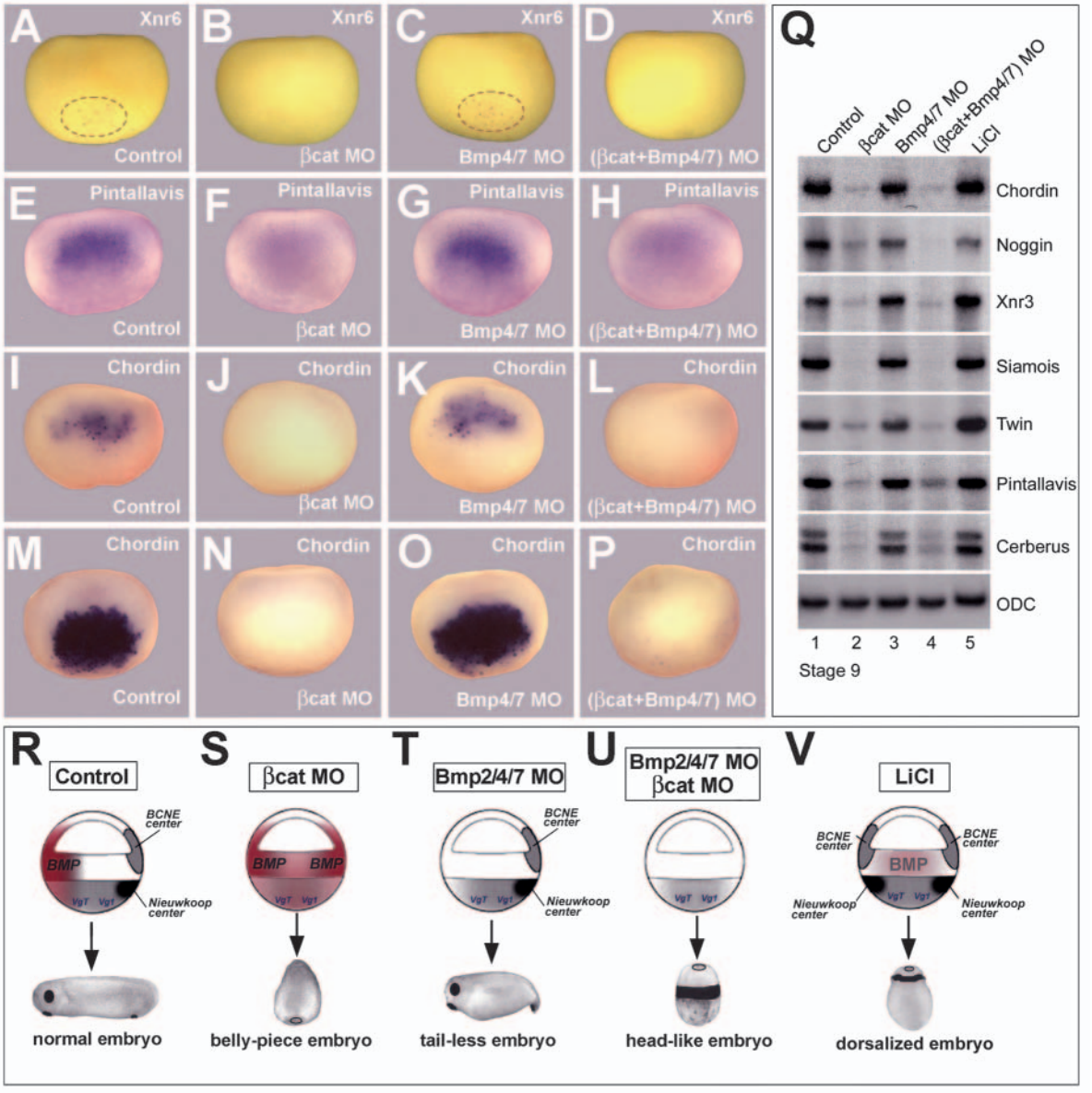

Fig. 8. Expression of Nieuwkoop, BCNE or Spemann organizer genes is not restored in β-Catenin/Bmp4/Bmp7-depleted embryos. (A-D) Expression of *Xnr6* in the vegetal dorsal side at blastula stage 9 in the Nieuwkoop center requires β-Catenin, is unaffected by knockdown of Bmp4/Bmp7, and is not re-expressed in β-Catenin/Bmp4/Bmp7 triple knockdowns. (E-L) Expression of the BCNE center genes *Pintallavis/Hnf3β* and *Chordin* at blastula stage 9 requires β-Catenin, is not affected by the depletion of Bmp4/Bmp7 and fails to be rescued in β-Catenin/Bmp4/Bmp7 triple knockdowns. (M-P) *Chordin* expression in the Spemann organizer at gastrula stage 10 is eliminated by β-Catenin depletion but is unchanged in Bmp4/Bmp7-depleted embryos. Note that embryos shown in panels D,H,L and P do not express any of the dorsal genes tested, yet still give rise to hyperdorsalized embryos with large head structures. (Q) RT-PCR analysis at stage 9. Dorsal markers are not expressed in embryos lacking β-Catenin or β-Catenin/Bmp4/Bmp7. (R-V) A simplified model highlighting the interactions taking place at blastula stage between BMP signals and the dorsal BCNE and Nieuwkoop centers.

is that simple co-injection experiments allow one to perform double or triple loss-of-function experiments that would be difficult to carry out by genetic means. These specific reagents are expected to be very useful in the analysis of development and were used here to demonstrate that BMPs play a crucial role in dorsoventral patterning and neural induction. Unexpectedly, we found that BMP knockdown had a much stronger effect in ventralized embryos devoid of Spemann organizer, in which it induced the formation of extensive head structures, including large amounts of brain tissue.

BMP loss-of-function in *Xenopus*

Individual knockdowns of Bmp2, Bmp4 and Bmp7 resulted in mild dorsalizations, characterized by an increase of dorsoanterior structures and a reduction of ventral and posterior markers. In particular, Bmp4 seems to play a crucial role in *Xenopus* development, as it does in mouse (Winnier et al., 1995; Hogan, 1996; Zhao, 2003), because its inhibition produced the strongest dorsalized phenotypes. All three BMPs had to be knocked down to obtain PGCs loss in *Xenopus*.

Ventral fin tissues were very sensitive to decreased BMP signaling levels. Both Bmp4 and Bmp7 MO-injected embryos had varying degrees of ventral fin truncations and a posteriorization of the anus (Fig. 1I-K), indicating that the posterior ventral-most region of the embryo requires maximal BMP signaling. This is in accordance with the co-expression of *Bmp4* and *Bmp7* on the ventral side of the closing blastopore (Hawley et al., 1995). In zebrafish, it is well established that the ventral fin requires high levels of BMP signaling (Mullins et al., 1996; Wagner and Mullins, 2002). Interestingly, Tucker and Slack (Tucker and Slack, 2004) have recently found that ventral fin cells derive from the ventral blastopore in *Xenopus*. In the mouse, defects in BMP signaling can cause siren (mermaid-like) phenotypes, in which ventral-posterior mesoderm development is impaired (Zakin et al., 2005).

Given that the transplantation of the dorsal organizer has potent effects but ventral grafts do not, ventral development has been viewed as subordinate to dorsal Spemann organizer signals. However, more recent studies indicate that diametrically opposite to the Spemann organizer, a ventral center, which expresses a number of secreted molecules, is formed at gastrula stage (De Robertis and Kuroda, 2004). The present loss-of-function study shows that Bmp4 is a key positive regulator of the expression of ventral center genes, such as *Sizzled* and *Bmp4* itself. Furthermore, epistatic studies showed that the ventralization caused by knockdown of Chordin can be compensated by loss of Bmp4 function (Fig. 5). In zebrafish, the strongest dorsalized phenotypes are caused by *Bmp2b* (*swirl*) and *Bmp7* (*snailhouse*) mutations (Hammerschmidt and Mullins, 2002). Double mutations in *bmp2b* and *bmp7* in zebrafish do not increase the phenotype (Schmid et al., 2000), whereas in *Xenopus* double and triple knockouts show increasing degrees of dorsalization (Fig. 2). This could be caused by a difference in transcriptional regulation, as zebrafish *bmp2b* or *bmp7* single mutants display greatly decreased *bmp2b*, *bmp4* and *bmp7* expression (Schmid et al., 2000). In *Xenopus*, however, Bmp2 MO does no affect *Bmp4/Bmp7* transcription (data not shown).

BMP inhibition and CNS formation

Does BMP inhibition play a role in neural induction in *Xenopus*? The early β-Catenin signal induces the BMP antagonists Chordin and Noggin in the BCNE center, and their transcripts are required for neural tissue formation in the absence of mesoderm (Kuroda et al., 2004). In addition, the early β-Catenin signal represses *Bmp4* transcription on the dorsal side of the embryo (Baker et al., 1999). We show that double Bmp4/Bmp7 depletion induced ectopic neurons in the posterior of the embryo, in the region in which paraxial mesoderm surrounds the tailbud. However, triple Bmp2, Bmp4 and Bmp7 knockdowns, although substantially dorsalized, did not have a significant increase in forebrain/midbrain neural tissues when compared with those of wild-type embryos at tailbud. By examining just these results, one would conclude that BMP levels are not involved in neural induction in *Xenopus*, as indeed has been proposed in other species (Stern, 2005). Even in *Xenopus*, the inhibitor Smad6 failed to induce neural tissue when injected into ventral ectoderm (Linker and Stern, 2004; Delaune et al., 2005). However, other experiments with an inducible construct of the inhibitor Smad7, indicate that ectopic neural tissue can be obtained by inhibiting BMP at pre-gastrula stages, but not at later stages of development (Wawersik et al., 2005). It has also been found that the use of multiple dominant-negative BMPRs greatly enhances secondary axis formation in the whole embryo (Yamamoto et al., 2001). Importantly, it has been shown that triple inhibition of Chordin, Noggin and Follistatin causes a catastrophic loss of dorsal structures, including the CNS (Khokha et al., 2005).

Neural induction can also be caused by Receptor Tyrosine Kinase (RTK) ligands, such as FGFs and IGFs, which can increase BMP signaling inhibition through inhibitory phosphorylation, via Ras/MAPK, of the linker region of Smad1 (Massague, 2003; Pera et al., 2003). This inhibitory phosphorylation is particularly importantly in dissociated animal caps, which undergo a sustained activation of endogenous Ras/MAPK signals (Kuroda et al., 2005). FGF signals may also induce neural tissue in Smad-independent ways (Delaune et al., 2005). Although inhibition of Bmp4/Bmp7 by itself does not induce much neural tissue in whole embryos, it greatly synergizes with Fgf8 protein microinjection, causing patches of neuronal differentiation throughout the ectoderm (Fig. 3). The loss-of-function results provided here for animal cap and VMZ explants confirm that decreasing endogenous BMP ligands promotes neural differentiation (Munoz-Sanjuan and Brivanlou, 2002).

Importantly, our results indicate that, in the intact embryo, regulatory mechanisms that limit the effects of depleting Bmp2/Bmp4/Bmp7 on neural differentiation must exist. When dorsal development is inhibited by ventralization by UV treatment or by β-Catenin MOs, a massive induction of brain structures is triggered by reduced BMP levels (Fig. 7B,C). In this experimental situation, the effects of BMP inhibition become obvious, so that an embryo that completely lacked a CNS would now develop radial neural structures that cover half the ectoderm. These results support a key role for BMP inhibition in CNS induction in the intact *Xenopus* embryo.

Brain induction in the absence of Spemann organizer

The most striking result presented here is that extensive CNS structures are generated by the knockdown of BMP signals in embryos ventralized by UV irradiation or β-Catenin depletion.

In amphibians, head and trunk induction are thought to be two separate processes, stemming from head and trunk-tail organizing centers at early and late gastrula, respectively (Spemann, 1938; Niehrs, 2004). It has been proposed that double inhibition of Wnt and BMP (Glinka et al., 1997), or triple inhibition of the Wnt, BMP and Nodal pathways (Piccolo et al., 1999), is required to form heads, whereas trunk formation only requires BMP antagonism. While these models are concerned with the role played by the organizer once it has formed, we now find that the Spemann organizer is dispensable for brain induction in embryos lacking both Spemann organizer and BMP signals (Figs 6, 7 and 8).

The diagrams shown in Fig. 8R-V attempt to explain these findings in terms of the cell-cell signaling thought to take place at blastula stage (before the actual formation of the Spemann organizer). We propose that, in *Xenopus*, brain formation results from the regulated antagonism between BMP signals and the two dorsal blastula signaling centers (the Nieuwkoop center and the BCNE center) that form under the influence of nuclear β-Catenin on the dorsal side (Fig. 8R) (De Robertis and Kuroda, 2004). Blocking early β-Catenin signals by UV irradiation or β-Catenin depletion (Fig. 8S) prevents the formation of all dorsal centers (the Nieuwkoop center, BCNE center and, ultimately, the Spemann organizer) and the expression of BMP antagonists. This leads to the unopposed activity of ventral BMPs, causing the development of ventralized embryos (also called belly-pieces) lacking all dorsal tissues, including CNS (Fig. 8S). Knockdown of BMP activity (Fig. 8T) in wild-type embryos by MOs principally affects development of the posterior ventral-most structures of the embryo, which arise from ventral mesoderm, giving rise to tail-less tadpoles.

Remarkably, simultaneous knockdown of both dorsal and ventral signals by co-injection of β-Catenin and Bmp4/Bmp7 or Bmp2/Bmp4/Bmp7 MOs results in radially symmetric embryos containing massive CNS structures (Fig. 8U). These hyperneuralized embryos retained AP pattern and in future it will be interesting to determine whether this is caused by posteriorizing inputs such as FGF, Wnt or retinoic acid. Radial head structures have also been reported in zebrafish triple mutants deficient for *bozozok*, *chordino* and *bmp2b* (Gonzalez et al., 2000). In *Xenopus*, radially dorsalized embryos can be obtained by LiCl treatment, but through a different molecular mechanism. LiCl-treated embryos have radial expression of BMP antagonists such as *Chordin* (Oelgeschläger et al., 2003), which inhibit ventralizing BMP signals and result in expanded dorsal structures (Fig. 8V). We note, however, that the radial CNS structures formed by LiCl treatment are much smaller than in those seen in β-Catenin/Bmp4/Bmp7-depleted embryos.

Lineage-tracing studies have shown that brain cell progenitors in *Xenopus* can be traced back to cells of the BCNE center (Kuroda et al., 2004). These cells transiently secrete Chordin and Noggin at blastula, forming an early domain devoid of BMP signaling, which, if cultured in isolation, can self-differentiate into anterior CNS tissue (Kuroda et al., 2004). In β-Catenin/Bmp2/Bmp4/Bmp7 morphants, lower BMP levels are attained throughout the embryo leading to hyperneuralization. Foley et al. (Foley et al., 2000) have proposed that in the chick, as gastrulation proceeds, an important function of the organizer, located in the anterior primitive streak in the chick embryo, would be to posteriorize forebrain progenitors located in the epiblast. These chick forebrain precursors (which are equivalent to the *Xenopus* BCNE cells) transiently express *Chordin* and at all stages migrate ahead of the organizer (Streit et al., 1998; Foley et al., 2000), and, consequently, are protected from its caudalizing signals. The posteriorizing signal produced by the primitive streak of the chick would correspond to the trunk/tail organizer in *Xenopus*. The results presented here are consistent with the proposal that neural induction precedes Spemann organizer formation. It appears that a principal role of the Spemann organizer of the gastrula is the maintenance and caudalization of neural tissue, rather than its initial induction. However, our results also differ from the current view of chick neural induction (Stern, 2004), as we find a crucial role for the inhibition of BMP activity in the initial induction of neural tissue in *Xenopus*.

Yet, one surprising finding is difficult to accommodate in this model: Bmp2/Bmp4/Bmp7-depleted embryos were dorsalized but still retained much of their DV polarity, whereas embryos without a Spemann organizer (β-Catenin depleted or UV-treated), which normally develop without a trace of a CNS, developed massive CNS structures in Bmp2/Bmp4/Bmp7 morphants. This indicates that the Spemann organizer is capable of partially compensating for the phenotypic effects associated with Bmp2/Bmp4/Bmp7 depletion, and that somehow it can mediate ventral development when Bmp2/Bmp4/Bmp7 signals are eliminated. If the organizer were responsible only for posteriorizing neural tissue, then its absence should mainly affect the AP pattern of the pre-existing CNS, but not trigger the massive formation of radial brain structures observed experimentally. We think the solution to this conundrum lies in the possibility that the Spemann organizer serves as a source of ventralizing signals, in addition to dorsalizing growth factor antagonists. Our ongoing research centers on the molecular identification of this ventralizing activity of the Spemann organizer.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/132/15/3381/DC1

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