Neural induction requires BMP inhibition only as a late step, and involves signals other than FGF and Wnt antagonists

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Summary

A dominant molecular explanation for neural induction is the 'default model', which proposes that the ectoderm is pre-programmed towards a neural fate, but is normally inhibited by endogenous BMPs. Although there is strong evidence favouring this in *Xenopus*, data from other organisms suggest more complexity, including an involvement of FGF and modulation of Wnt. However, it is generally believed that these additional signals also act by inhibiting BMPs. We have investigated whether BMP inhibition is necessary and/or sufficient for neural induction. In the chick, misexpression of BMP4 in the prospective neural plate inhibits the expression of definitive neural markers (*Sox2* and late *Sox3*), but does not affect the early expression of *Sox3*, suggesting that BMP inhibition is required only as a late step during neural induction. Inhibition of BMP signalling by the potent antagonist Smad6, either alone or together with a dominant-negative BMP receptor, Chordin and/or Noggin in competent epiblast is not sufficient to induce expression of *Sox2* directly, even in combination with FGF2, FGF3, FGF4 or FGF8 and/or antagonists of Wnt signalling. These results strongly suggest that BMP inhibition is not sufficient for neural induction in the chick embryo. To test this in *Xenopus*, *Smad6* mRNA was injected into the A4 blastomere (which reliably contributes to epidermis but not to neural plate or its border) at the 32-cell stage: expression of neural markers (*Sox3* and *NCAM*) is not induced. We propose that neural induction involves additional signalling events that remain to be identified.

Key words: *Xenopus*, Chick, Neural induction, FGF, BMP, Wnt, Smad6, Default model

Introduction

The pioneering experiments of Spemann and Mangold in 1924 demonstrated that the dorsal lip of the amphibian blastopore could induce a secondary axis in the ventral side of a host embryo. The secondary axis formed contained cells not only from the grafted blastopore lip, which mainly contributed to dorsal mesoderm structures, but more importantly it included cells from the host that had been induced to acquire a neural fate (neural induction). Because of this the dorsal blastopore lip was called the (Spemann) organizer, and analogous structures have been found in all amniotes (Waddington, 1933; Waddington, 1937; Waddington, 1940; Nakamura and Toivonen, 1978; Kintner and Dodd, 1991; Blum et al., 1992; Beddington, 1994; Shih and Fraser, 1995; Hatta and Takahashi, 1996; Shih and Fraser, 1996; Tam et al., 1997).

The search for neural inducing factors was unsuccessful for decades, and it is only recently that some have been found. To date, all identified molecules with this activity in *Xenopus* have in common that they inhibit BMP signalling. This led to the widely accepted 'default model', which proposes that ectodermal cells are fated to become neural 'by default' but are normally inhibited from a neural fate by BMPs expressed throughout the ectoderm, from which they must be released for neural induction to occur (Hemmati-Brivanlou and Melton, 1997; Weinstein and Hemmati-Brivanlou, 1999; Muñoz-Sanjuán and Brivanlou, 2002). Consistent with this, the organizer expresses a number of BMP antagonists, which include *Chordin* (Sasai et al., 1994); *Noggin* (Lamb et al., 1993); *Follistatin* (Hemmati-Brivanlou et al., 1994); *DAN*, *Gremlin* and *Drm* (Hsu et al., 1998); *Xnr3* (Hansen et al., 1997); *Cerberus* (Bouwmeester et al., 1996); and *Dickkopf* (Glinka et al., 1998). In *Xenopus*, misexpression of any of these antagonists leads to neural induction in animal caps, while overexpression of BMPs has the opposite effect.

However, studies in other species (especially chick and mouse) have questioned this simple interpretation (Streit and Stern, 1999c). In the chick, the expression patterns of BMPs and BMP antagonists do not fit this model so neatly, and misexpression of neither BMPs nor BMP antagonists produces the effects expected from the model (Streit et al., 1998). In the mouse, mutants lacking the BMP antagonists Cerberus, Dickkopf-1, Noggin and/or Chordin still develop a nervous system (McMahon et al., 1998; Bachiller et al., 2000; Belo et al., 2000; Mukhopadhyay et al., 2001). These results suggest that BMP inhibition may not be absolutely required for neural induction and that other molecules may be implicated in the process. Recently, it has been shown that FGF signalling is required as an early step for neural induction in the chick (Streit et al., 2000; Wilson et al., 2000), consistent with earlier findings in *Xenopus* that BMP antagonists cannot induce neural markers when FGF signalling is blocked (Launay et al., 1996; Sasai et al., 1996). However FGF is not a sufficient neural inducer (Streit et al., 2000; Wilson et al., 2000), and it has been

proposed that cooperation of FGF with inhibition of Wnt signalling can repress BMP transcription (Bainter et al., 2001; Wilson and Edlund, 2001; Wilson et al., 2001), and FGF and related factors have been shown to phosphorylate the linker region of the BMP effector Smad1 (Pera et al., 2003). With this interpretation, BMP inhibition remains as playing a central role in neural induction.

Here, we have re-evaluated the participation of BMP and BMP antagonism during neural induction. We chose to manipulate the BMP pathway intracellularly in a cell-autonomous way, taking advantage of Smad6, the inhibitory Smad (Imamura et al., 1997; Hata et al., 1998). BMP signalling starts with the binding of extracellular BMP dimer to BMP receptor type II (BMPRII), which is then able to recognize BMP receptor type I (BMPRI) forming a tertiary complex, where BMPRII activates BMPRI by phosphorylation. In turn, active BMPRI recruits Smad1/Smad5/Smad8 proteins to the membrane and activates them by phosphorylation, which allows them to bind to Smad4; the complex then translocates to the nucleus to regulate transcription (von Bubnoff and Cho, 2001). A more divergent group of Smad proteins has been described: the inhibitory Smads (Smad6/Smad7/Smad10). Although Smad7 inhibits both activin/nodal-related and BMP/TGFβ signalling, Smad6 is a potent and specific antagonist of the BMP pathway. It acts by actively associating with and blocking BMPRI, as well as by competing with Smad4 to bind phosphorylated Smad1/5/8. In addition, Smad6 can inhibit an alternative BMP intracellular signalling pathway involving TCF3/Lef1/β-catenin (von Bubnoff and Cho, 2001).

We show that although forced BMP expression in the neural plate inhibits the expression of the definitive neural marker *Sox2*, it does not affect expression of the earlier marker *Sox3*. Moreover, BMP inhibition is not sufficient for neural induction, either in competent chick epiblast or in the prospective ventral epidermis of *Xenopus*. These results suggest that BMP inhibition is a relatively late step in a molecular cascade leading to the acquisition of neural identity. We tested the proposal of Wilson and Edlund (Wilson and Edlund, 2001) for the chick embryo by investigating whether combinations of FGF, BMP inhibition and Wnt inhibition might suffice; we find that no combination of these can induce neural tissue in vivo.

Materials and methods

Chick experiments

Fertilized hens' eggs (Brown Bovan Gold; Henry Stewart and Company) were incubated at 38°C to the desired stages. Electroporations were performed as described (Sheng et al., 2003). The coding region of chicken *Smad6* (a kind gift from P Szendro and G Eichele) (Yamada et al., 1999), chick Chordin (Streit et al., 1998), *Xenopus* truncated BMP receptor (Suzuki et al., 1994), *Xenopus* BMP4 (a kind gift from K. Howarth and P. Sharpe) and chick Cerberus (Zhu et al., 1999) were cloned into *pCAβ-IRES-GFP*. All DNA solutions for electroporation were at 1 μg/μl except cSmad6, which was used at 2 μg/μl. FGFs were delivered bound to heparin beads (prepared as described) (Streit et al., 2000). For each FGF, a concentration that did not induce *brachyury* was determined. FGF2 (Invitrogen 13256-029) induces *brachyury* at 50 μg/ml (5/5) and 2.5 μg/ml (12/12), but not at 0.5 μg/ml (0/7), and this concentration was therefore used for further analysis. FGF3 (R&D Systems 1206-F3) was used at 50 μg/ml. FGF4 (R&D Systems 235-F4) induces *brachyury* at 2.5 μg/ml (9/9) and 0.5 μg/ml (9/9), but not at 0.05 μg/ml (0/13), and this concentration was used for experiments. Different batches of FGF8b (50 μg/ml) produced different results when used at the same concentration. In the present set of experiments, FGF8b obtained from Sigma (F6926) was considerably more potent in inducing *Sox3* (and also induced mesoderm at the area pellucida/area opaca border, as marked by *brachyury* expression) than that obtained from R&D Systems (423-F8), which never induced *brachyury*. The latter was therefore used in our experiments at this concentration. Other secreted proteins were administered via pellets of cells: Noggin-expressing rat B1 cells (kind gift of R. Harland), or COS cells transiently transfected with Dkk1 (kind gift of E. Laufer), Crescent (kind gift of M. Marvin and E. Laufer) or soluble NFz8 (Deardorff et al., 1998) as described (Streit et al., 1998; Skromne and Stern, 2001). For combinations of these factors, pellets were made from a mixture of cells (1000 cells for each factor).

Anti-phospho Smad1 antibody was a kind gift of P. ten Dijke (Chang et al., 2002). In situ hybridization and whole-mount immunocytochemistry was performed as described (Stern, 1993; Streit and Stern, 2001).

Xenopus experiments

Xenopus oocytes were fertilized in vitro and the embryos staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1967). The *cSmad6* (a kind gift from P. Szendro and G. Eichele) (Yamada et al., 1999) coding sequence in pCS2+, FGF8 pCS2+ (a kind gift from R. Mayor) (Christen and Slack, 1997) and eFGF pCS2+ (*Xenopus* FGF4, a kind gift from J Slack) (Lombardo and Slack, 1997) were used to produce mRNA. Capped mRNA was made with mMessage mMachine (Ambion). Microinjection was performed as described (Marchant et al., 1998). The capped mRNA was injected into the animal zone of two-cell stage embryos, into the ventral marginal zone of four-cell stage embryos or into blastomere A4 at the 32-cell stage together with 5-10 ng lysine-fixable fluorescein dextran (FDX, 40,000 M_r; Molecular Probes) as a lineage tracer in most experiments. Animal caps were dissected with eyebrow knives at stage 8-10 with the embryos in 0.75× NAM, and were allowed to grow until their siblings reached stage 21. The accuracy of injection into the A4 blastomere was assessed by the fate of its progeny. Embryos in which labelled cells were found in regions other than the ventral epidermis were discarded without further processing. The proportion of embryos discarded was almost identical in control (18/51=35% embryos with incorrect labelling) and *Smad6*-injected embryos (29/79=37%). In situ hybridization was carried out as described (Linker et al., 2000).

Results

Inhibition of BMP signalling by Smad6 is not sufficient for neural induction

Smad6 is a universal inhibitor of BMP signalling through Smad1/Smad5/Smad8, without also inhibiting activin/nodal-related signalling through Smad2/Smad3 (Imamura et al., 1997; Casellas and Brivanlou, 1998; Hata et al., 1998; Nakayama et al., 1998; Bai et al., 2000; Ishida et al., 2000). We took advantage of this property to block BMP signalling in the chick embryo. To confirm that chick Smad6 is active in blocking BMP signalling, we first electroporated an expression construct (*pCAβ-cSmad6-IRES-GFP*) into one side of a stage 3+ embryo and allowed it to grow until the neural tube had formed and closed (stage 9-10). The embryo was then stained with an antibody against phospho-Smad1 (Chang et al., 2002), which revealed that the electroporated side of the neural tube contained a significantly lower level of activated Smad1 (Fig. 1A-D). As an additional test, we injected chick *Smad6* mRNA (400 pg-1 ng) into the ventral marginal zone of four-cell stage *Xenopus* embryos and grew these to tadpole stages (Fig. 1E). Out of 207 injected embryos, 196 (95%) formed a secondary

axis when compared with 0/72 embryos injected with the same concentration of *GFP* mRNA (Fig. 1F,G). These results confirm that chick Smad6 is active as a BMP inhibitor in both *Xenopus* and chick embryos.

To test whether BMP inhibition by Smad6 is sufficient to cause competent epiblast cells to acquire expression of early (*Sox3*) or definitive neural markers (*Sox2*), we electroporated *pCAβ-cSmad6-IRES-GFP* in a discrete domain in the inner third of the area opaca of a stage 3+ chick embryo, at approximately the level of Hensen's node (Fig. 1H). After incubation for 16-22 hours, no expression of *Sox3* (0/7; Fig. 1I-K), *Sox2* (0/16; Fig. 1L-N) or the mesodermal marker *brachyury* (0/22; Fig. 1I,L) was detected in the electroporated region. As a positive control, we tested whether chick *Smad6* construct can indeed neuralize *Xenopus* animal caps, as previously reported (Hata et al., 1998; Nakayama et al., 1998). Injection of *cSmad6* mRNA into the animal pole of two-cell stage embryos, followed by culture of their isolated animal caps leads to strong expression of *Sox3* (*cSmad6*: 40/40; *GFP* control: 0/23; Fig. 1O-Q).

Despite published reports that Smad6 should inhibit all BMP signalling (reviewed by von Bubnoff and Cho, 2001), it is conceivable that some escapes inhibition in our experimental setup. To overcome this, we misexpressed a dominant-negative form of the BMP receptor (Suzuki et al., 1994), Chordin or Noggin, or a combination of all of the above, with Smad6. In all cases (dnBMPR 0/6; Noggin 0/6; Chordin 0/2; Smad6+dnBMPR+Noggin+Chordin 0/9), no expression of *Sox2* was seen in the electroporated region (Fig. 2).

Our results strongly suggest that BMP inhibition, even under conditions that are likely to abolish all BMP signalling, is not sufficient to induce expression of either early (*Sox3*) or definitive (*Sox2*) neural markers in competent epiblast cells.

BMP inhibition is required as a late event in formation of the neural plate

The above experiments suggest that BMP inhibition is not sufficient for neural induction – but is it necessary? To address this, we electroporated *Xenopus* BMP4 (*pCAβ-XBMP4-IRES-GFP*) into the prospective neural plate of stage 3+ embryos, and analysed the effects in time course. After 12 and 15 hours of incubation, the early marker *Sox3* is not affected (0/14 at 12 hours, 0/8 at 15 hours; Fig. 3A,B,G,H), while the later marker *Sox2* is strongly downregulated in the neural plate (9/9 at 12 hours, 10/10 at 15 hours; Fig. 3C,D,I,J). At 20 hours, both *Sox3* (10/10, Fig. 3M,N) and *Sox2* (14/14; Fig. 3O,P) are downregulated. At this time, histological analysis showed that neural plate morphology is lost in the electroporated region (not shown). By contrast, control embryos electroporated with *GFP* show no downregulation of either marker at any time point (0/11; Fig. 3E,F,K,L,Q,R). These results show that BMP inhibition is necessary for expression of definitive neural plate markers and for neural plate formation, but does not appear to affect the early steps of this process.

BMP inhibition is not sufficient for neural induction even in combination with FGFs and Wnt antagonists

Recent work has implicated FGF signalling as an early step in neural induction in the chick (Henrique et al., 1997; Alvarez et al., 1998; Storey et al., 1998; Streit et al., 2000; Wilson et al., 2000; Wilson et al., 2001), in combination with BMP inhibition. One group has proposed that FGF signalling can cooperate with Wnt antagonism to inhibit BMP activity and thus induce neural fates (Wilson and Edlund, 2001; Wilson et al., 2001). To test this hypothesis, we misexpressed *Smad6* with FGFs (FGF2, FGF3, FGF4 or FGF8), with a combination of

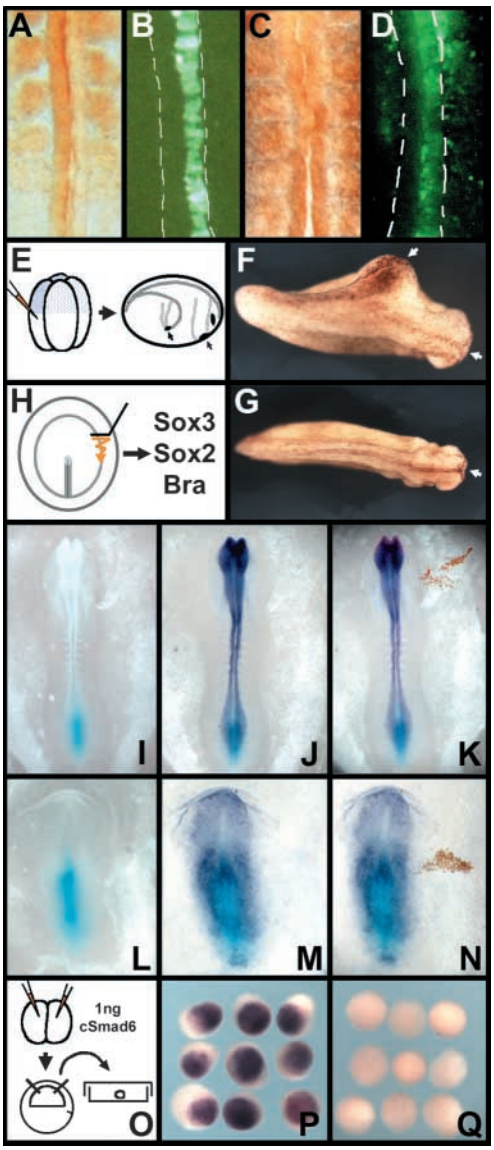

Fig. 1. *Smad6* does not induce neural markers in the chick. (A-G) Experiments to test the activity of Smad6. *Smad6-IRES-GFP* was electroporated into one half of the chick neural tube (A,B). Staining against phospho-Smad1 (A) reveals that activation of Smad1 has been inhibited in the electroporated cells (green in B), while in control GFP electroporated embryos (C,D) phospho-Smad1 is not altered (C). *cSmad6* injection into the marginal zone in *Xenopus* (E) induces a secondary axis (F), while GFP-injected controls appear normal (G). (H-N) *Smad6* does not induce neural markers. Electroporation of *Smad6* into competent area opaca epiblast at stage 3+ (H) does not induce *Brachyury* (I,L; light blue), *Sox2* (J) or *Sox3* (M) (purple). In this and subsequent figures, electroporated cells were visualized by staining with anti-GFP antibody (K, N; brown). (O-Q) Positive controls. *cSmad6* or GFP was injected at the two-cell stage and animal caps isolated at early gastrula (O). *Smad6* (P) can neuralize animal caps, while GFP cannot (Q), as assessed by *Sox3* expression.

Wnt antagonists (Dkk1, Crescent, NFz8 and Cerberus) or all of these together. As shown previously (Streit et al., 2000), FGF8 alone induces *Sox3* (5/6; 83%) but not *Sox2* (0/8; Fig. 4A) in the epiblast of the area opaca. FGF8 still fails to induce *Sox2* when misexpressed with *Smad6* (0/11; Fig. 4F,H,I), consistent with the finding that FGF8+Chordin fail to induce *Sox2* (Streit et al., 2000). Similar results are seen when FGF8 is misexpressed together with three Wnt antagonists (Dkk1, Crescent and NFz8) (0/7; Fig. 4C-E), or when any of the same Wnt antagonists is misexpressed individually (Dkk1, 0/9; Crescent, 0/5; NFz8, 0/7; Fig. 4B,D). More dramatically, even ectopic expression of FGF8+Smad6+all three Wnt antagonists is unable to induce *Sox2* in competent epiblast (0/10; not shown), and the same is seen when Cerberus is also included in the combination (FGF8+Smad6+Dkk1+Crescent+NFz8+Cerberus: 0/11; Fig. 4G,J-K). These experiments show that BMP inhibition is insufficient for neural induction, even in combination with FGF8 and/or Wnt antagonism.

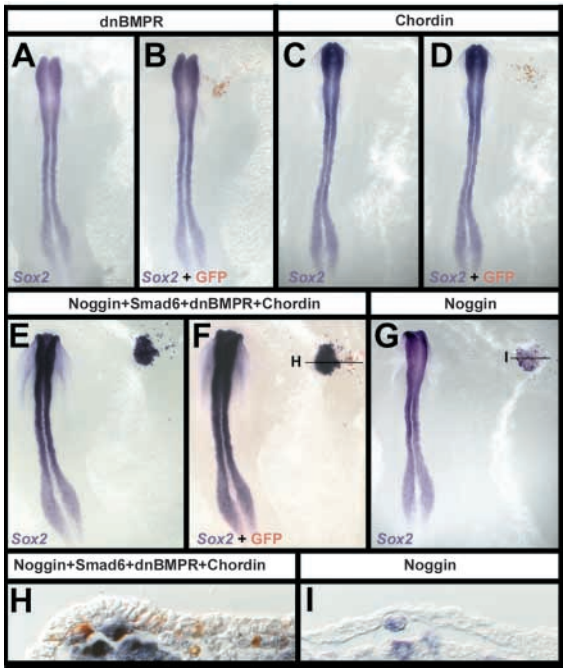

Fig. 2. BMP inhibition is not sufficient for neural induction. Inhibition of BMP by misexpression of *dnBMPR* (A,B), *Chordin* (C,D), Noggin (G) or *dnBMPR*+*Chordin*+Noggin+*Smad6* (E,F) is not sufficient to induce *Sox2*. (H,I) Sections through the embryos in F,G at the levels indicated. All cell pellets produce background staining after *Sox2* in situ hybridization (E-I); sections are therefore necessary to show absence of expression in the epiblast.

It has been suggested (Wilson et al., 2000) that FGF3, rather than FGF8, is the endogenous signal, and these authors' experiments were conducted with FGF2. We therefore tested these two FGFs as well as FGF4 in the same assays (Fig. 5). Neither FGF2 (0/7) nor FGF3 (0/7) alone, nor either factor in combination with *Smad6* (FGF2+Smad6: 0/5; FGF3+Smad6: 0/4) or *Smad6*+Wnt antagonists+Cerberus (FGF2: 0/6; FGF3: 0/5) induced *Sox2* (Fig. 5A-P) at concentrations of FGF that did not induce *brachyury* (see Materials and methods). A more complicated result was obtained with FGF4. At 0.05 μg/ml this factor alone does not induce *brachyury* or *Sox2* (0/13; Fig. 5Q,R). However, it induces both markers when co-expressed with *Smad6* (6/6 for *brachyury*, 5/6 for *Sox2*; Fig. 5S-U). When Wnt antagonists and Cerberus are misexpressed with FGF4 and Smad6, the induction of both markers is abolished (0/6; Fig. 5V-X).

Taken together, these results show that FGF2, FGF3, FGF4 or FGF8 are all unable to induce *Sox2* expression in the absence of mesoderm, even when any of them is misexpressed together with BMP inhibitors and Wnt inhibitors.

BMP inhibition by Smad6 is not sufficient for neural induction in *Xenopus*

The above results in the chick are in direct conflict with the dominant 'default model' (Hemmati-Brivanlou and Melton, 1997; Weinstein and Hemmati-Brivanlou, 1999; Muñoz-Sanjuán and Brivanlou, 2002) that was based on experiments in *Xenopus* embryos. In these experiments, mRNA encoding BMP antagonists is usually injected at the animal pole of early embryos (one- to four-cell stage),

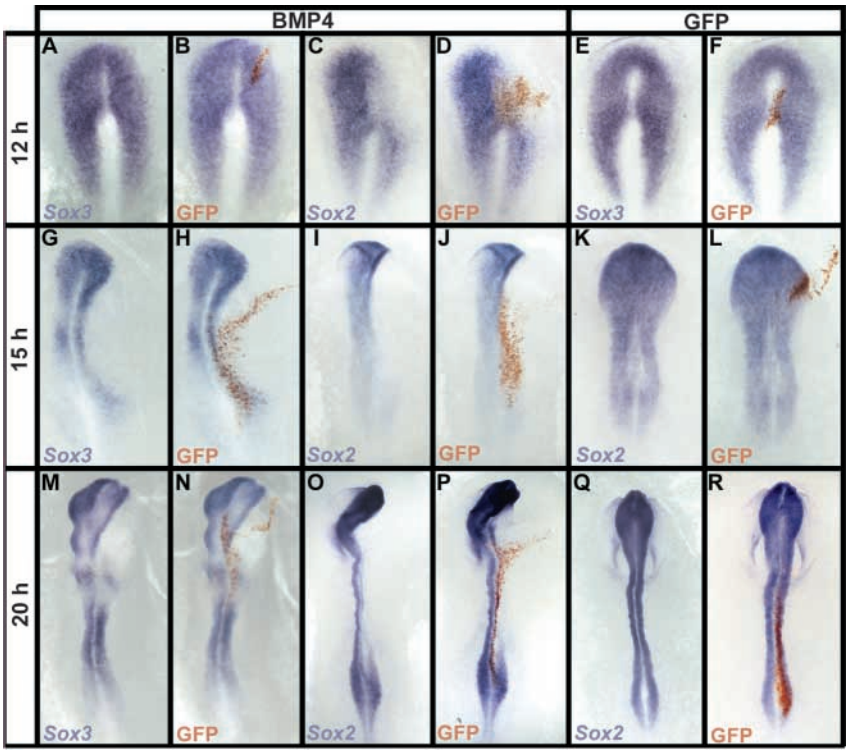

Fig. 3. BMP inhibits late markers of neural induction. *BMP4* was electroporated into the prospective neural plate at stage 3+ and the consequences analysed in time course. After 12 hours and 15 hours of incubation, the early marker *Sox3* is not affected (A,B,G,H), while the later marker *Sox2* is strongly downregulated in the neural plate (C,D,I,J). By 20 hours after electroporation, both *Sox3* (M,N) and *Sox2* (O,P) are downregulated. Neither *Sox3* nor *Sox2* is altered when control GFP is electroporated (E,F,K,L,Q,R).

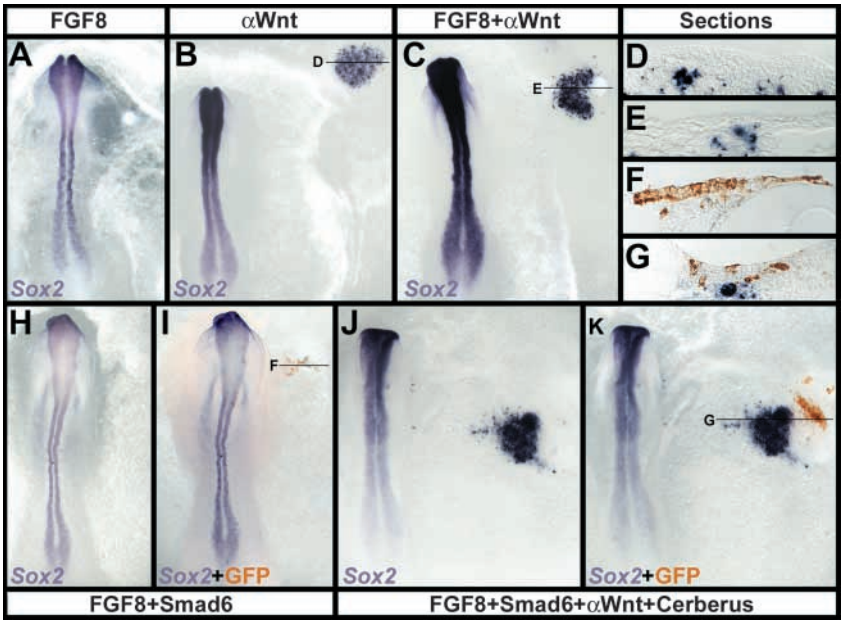

Fig. 4. BMP inhibition does not induce neural tissue even in combination with FGF8 and/or Wnt antagonists. Neither a source of FGF8 protein (A) nor inhibition of Wnt signalling by NFz8+Dkk+ Crescent (αWnt; pellet of transfected cells) (B,D) induces *Sox2* expression in competent area opaca epiblast. The same is seen after misexpression of a combination of FGF8+NFz8+Dkk+Crescent (C,E), FGF8+*Smad6* (F,H,I), or of all of these together (FGF8+NFz8+Dkk+Crescent+Cerberus+*Smad6*) (G,J,K). The histological sections (D-G) show that the epiblast in direct apposition to the source of factors does not express *Sox2*.

where the RNA may be inherited not only by prospective epidermal cells but also by presumptive neural plate or crest cells. To overcome this problem and to generate an assay more directly comparable with those in chick embryos, we injected a lineage tracer together with *cSmad6* mRNA (400 pg-1 ng) into the A4 (most ventral animal) blastomere at the 32-cell stage (Fig. 6A): this is the only blastomere that does not consistently contribute progeny to neural plate or neural crest in intact embryos (Dale and Slack, 1987; Moody, 1987a). Embryos were then grown to the neurula stage and probed with *Sox3* (a definitive neural marker in *Xenopus*). Both in *Smad6*-injected embryos and in *GFP*-injected controls, normal expression of *Sox3* was seen in the neural plate, but no ectopic expression was ever seen in the injected cells, which contributed to the most ventral epidermis (*Smad6*, 0/26, Fig. 6B-E; control, 0/21, Fig. 6F-I). An identical result was obtained using *NCAM* as a neural marker (0/5; not shown).

As differences in competence for mesoderm induction have been reported along the dorsoventral axis of the early frog embryo (Sokol and Melton, 1991), it is conceivable that the A4 blastomere and its descendents are not competent to respond to neural inducing signals, which might account for the above result. To test this, we injected a lineage tracer into the A4 blastomere, grew the embryos to early gastrula stage (stage 10) and then excised the labelled ventral ectoderm cells and grafted them to the dorsal side (adjacent to the organizer) of an unlabelled host embryo, which was then grown to neurula stages and processed for *Sox3* and for the lineage marker (Fig. 5J). The grafted (labelled) descendants of A4 had become incorporated into the neural plate and expressed *Sox3* normally (*n*=12; Fig. 5K,L).

We then tested whether *FGF*s alone or with *Smad6* might induce *Sox3*. Neither *FGF8* (10 pg) nor *FGF4* (0.16 pg) alone induced *brachyury* (FGF8: 0/13; FGF4: 0/12; not shown) or *Sox3* (FGF8, 0/13, Fig. 7D-F; FGF4, 0/13, Fig. 7G-I). In combination, *FGF8+Smad6* (10 pg+1 ng) also failed to induce either marker (*bra*, 0/51; *Sox3*, 0/42), while *FGF4+Smad6* (0.16 pg+ 1ng) induced *Sox3* (45/49; Fig. 7J-L) but not *brachyury* (0/38; Fig. 7A-C) or other mesoderm markers tested (*Chordin*, 0/8; *MyoD*, 0/8; *Goosecoid*, 0/21; *Vent1*, 0/9; not shown).

These results show that in a blastomere that contributes consistently to epidermis, BMP inhibition (through misexpression of Smad6) is not sufficient to cause the injected cells to adopt a neural fate, which argues against the default model. *FGF8* is also not sufficient either alone or in combination with BMP antagonists to induce *Sox3*. However, unlike our findings in chick, the combination of *FGF4+Smad6* induces *Sox3* without inducing five different mesodermal markers tested.

Discussion

BMP signalling in neural induction and the default model

The predominant model for neural induction (the ‘default model’) proposes neural fate as the natural pathway that ectodermal cells would acquire in the absence of any signal. This fate is inhibited in the ectoderm by active BMP signalling. The organizer secretes BMP antagonists, which block BMP signalling in adjacent cells, allowing them to follow their default neural pathway (Hemmati-Brivanlou and Melton, 1997; Weinstein and Hemmati-Brivanlou, 1999; Muñoz-Sanjuán and Brivanlou, 2002). This model is supported by a substantial body of evidence, mainly from *Xenopus* embryos. Dissociated animal cap cells that are subsequently reaggregated develop into neurons (Godsave and Slack, 1989; Grunz and Tacke, 1989; Sato and Sargent, 1989), and this can be blocked by addition of BMP4 protein (Wilson and Hemmati-Brivanlou, 1995). The expression patterns of *BMP4* and its antagonists in *Xenopus* are also consistent with the model: *BMP4* is initially ubiquitous in the ectoderm, and then clears from the neural plate, while many BMP antagonists (including *chordin*, *noggin*, *follistatin*, *DAN* and *cerberus*) are expressed in the organizer, a part thereof, or closely neighbouring tissues (Harland and Gerhart, 1997; Hemmati-Brivanlou and Melton, 1997; Weinstein and Hemmati-Brivanlou, 1999; Muñoz-Sanjuán and Brivanlou, 2002). Misexpression of BMP in the prospective neural plate ventralizes the embryo, as well as suppressing neural markers, while misexpression of BMP antagonists (both natural and artificial, such as dnBMPR) in a one- to four-cell stage embryo expands the neural plate. But most of the evidence in favour of

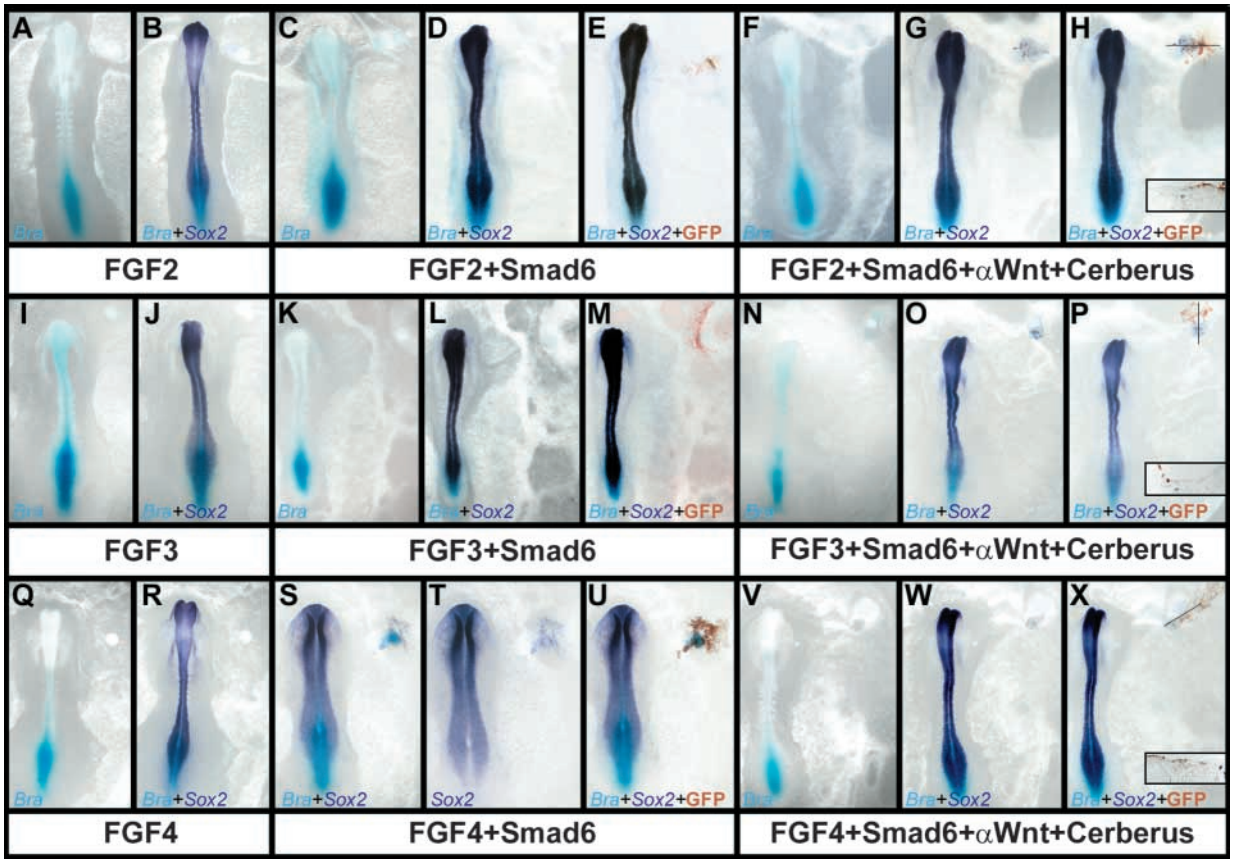

Fig. 5. BMP inhibition in combination with FGF (FGF2, FGF3 or FGF4) and Wnt antagonists does not induce neural tissue directly. (A,B,I,J,Q,R) Beads with FGF2, FGF3 or FGF4 protein cannot induce either *brachyury* (light blue) or *Sox2* (purple). (C-H,K-P) Likewise, FGF2 or FGF3 together with *Smad6* and/or Wnt antagonists does not induce *brachyury* or *Sox2*. (S-U) Misexpression of *Smad6* and FGF4 induces both *brachyury* and *Sox2*; (V-X) addition of Wnt antagonists to the combination inhibits induction of both markers.

the model comes from animal cap experiments, where the early (one- to four-cell stage) embryo is injected animally with an RNA of choice, then grown to blastula or early gastrula stage, when the animal cap is cut and allowed to develop in isolation from the rest of the embryo. Caps obtained from embryos injected with BMP antagonists develop expression of neural markers, while control caps do not. Animal caps from uninjected embryos also express neural markers if they are treated with any of the endogenous BMP antagonists (Noggin, Chordin, Follistatin, Cerberus, etc.) in protein form (Hemmati-Brivanlou et al., 1994; Hemmati-Brivanlou and Melton, 1994; Sasai et al., 1995; Bouwmeester et al., 1996).

Although several objections have been raised to the model and to the interpretation of experiments that led to it (see Streit and Stern, 1999c), this model is generally so dominant that it is described in all current developmental biology textbooks as the accepted mechanism for neural induction.

Conflicting data from chick and other species

The first major objections to the default model as a sufficient explanation for neural induction were raised as a result of observations in the chick (Streit et al., 1998): BMP4, BMP7 and their antagonists Chordin, Noggin and Follistatin are not expressed with the correct spatial and temporal patterns to fit neatly with the proposals of the model, misexpression of Chordin or Noggin in competent epiblast using grafts of secreting cells do not induce neural tissue, and misexpression of BMP4 or BMP7 by the same method in the prospective neural plate does not block neural induction. Furthermore cell dissociation of chick epiblast does not induce neural differentiation but rather muscle (George-Weinstein et al., 1996; George-Weinstein et al., 1997). Consistent with these results in the chick, mouse mutants that lack Chordin, Noggin or both BMP antagonists still have a nervous system, although they lack the most anterior structures (McMahon et al., 1998; Bachiller et al., 2000; Belo et al., 2000; Mukhopadhyay et al., 2001).

However, chick epiblast cells previously exposed to a grafted organizer for at least 5 hours (13 hours are required for full induction) (Gallera and Ivanov, 1964; Gallera, 1971) can respond to Chordin by stabilizing the expression of the early marker *Sox3* (but still do not express the definitive neural marker *Sox2*) (Streit et al., 1998; Streit et al., 2000). This finding led to the hypothesis that signals other than BMP antagonists are required to confer sensitivity to epiblast cells to BMP signalling, and therefore that BMP inhibition may be a relatively downstream step in the induction process, if it is required at all.

But these experiments are open to three major criticisms. First, they cannot address whether it is necessary to inhibit

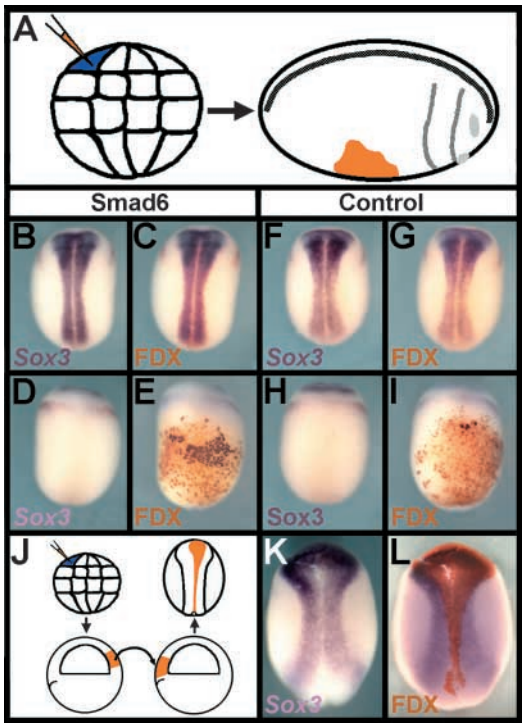

Fig. 6. BMP inhibition by *Smad6* is not sufficient for neural induction in *Xenopus* ventral epidermis. (A-I) *cSmad6* was targeted to the ventral epidermis by injection into the A4 blastomere of 32-cell stage embryos (A). *Smad6* does not activate *Sox3* expression (B-E), compare with *GFP*-injected control embryos (F-I). B,C,F,G are dorsal views; D,E,H,I are ventral views of the embryos in B,C,F,G. C,E,G,I show the embryos in B,D,F,H after staining with anti-fluorescein to reveal the lineage tracer FDX which was co-injected with the mRNA. (J-L) To test that the progeny of the A4 blastomere is competent to respond to neural inducing signals from the organizer, a lineage tracer (FDX) was injected into the A4 blastomere. At late blastula/early gastrula stage, the labelled cells are transplanted into the dorsal side of an unlabelled host embryo, which is grown to early neurula stage (J). (K,L) The transplanted cells (FDX in brown in L) contribute to the neural plate and express *Sox3* (purple).

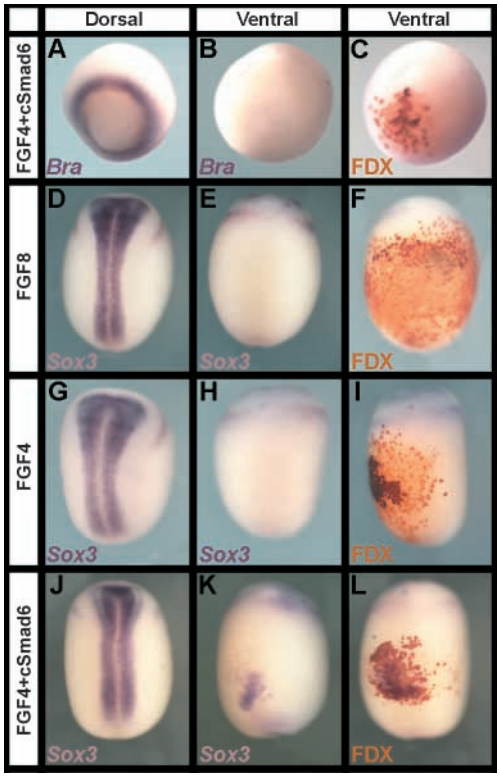

Fig. 7. Effects of BMP inhibition in combination with FGF in *Xenopus*. Embryos were injected into the A4 blastomere at the 32-cell stage. *FGF4* in combination with *cSmad6* does not induce *brachyury* in prospective ventral ectoderm cells at stage 10+ (A-C). Neither *FGF8* nor *FGF4* is able to induce *Sox3* in the ventral epidermis cells of early neurula stage embryos (D-I), while misexpression of *FGF4* together with *Smad6* induces a patch of *Sox3* (J-L). (A,D,G,J) Dorsal views; (B,E,H,K) ventral views of same embryos, which are shown again in C,F,I,L after anti-fluorescein staining.

BMP signalling at all for neural induction to occur. Second, it is possible that misexpression of BMP or its antagonists using a graft of secreting cells does not deliver enough active protein to overcome the endogenous signals. Third, it is possible that any one of the BMP antagonists is not sufficient to inhibit all BMP signalling.

Here we have addressed the first question using electroporation of BMP4 in an expression construct directly into the epiblast; we find that BMP inhibition is indeed necessary for neural plate development. However, although misexpression of BMP4 affects the definitive neural marker *Sox2*, it does not alter the expression of the early marker *Sox3* [which at early stages is not restricted to prospective neural cells but is also expressed in future epidermis and mesoderm cells (see Sheng et al., 2003)], consistent with the idea that BMP inhibition is a relatively late step in a cascade leading to neural induction in the chick.

To address the latter two criticisms, we electroporated the cell-autonomous BMP antagonist Smad6, either alone or together with other BMP antagonists (*Chordin*, Noggin and/or *dnBMPR*). In none of these cases do we see induction of either *Sox3* or *Sox2* in competent epiblast of the area opaca, now strongly suggesting that BMP inhibition is not sufficient for neural induction in the chick.

FGF signalling and neural induction

The finding (see above) that signals from the organizer other than BMP antagonists are required as an upstream step before chick epiblast cells can respond to the antagonists led to a screen for genes that are activated during the initial signalling period. To date, two genes have been described from this screen: *ERNI* (which is induced in just 1 hour) and *Churchill* (induced after 4-5 hours) (Streit et al., 2000; Sheng et al., 2003). In turn, the use of *ERNI* as a marker led to the identification of FGF signalling as both necessary and sufficient to induce all the known early markers (*ERNI*, *Sox3* and *Churchill*) as well as to sensitize cells to Chordin; however, it is not sufficient to induce *Sox2* or a neural plate (Streit et al., 2000; Sheng et al., 2003). It was also shown that this early FGF step takes place during very early stages of development, even before gastrulation begins (Streit et al., 2000; Wilson et al., 2000).

A requirement for FGF signalling has now also been

demonstrated in the ascidian Ciona (Kim and Nishida, 2001; Minokawa et al., 2001; Bertrand et al., 2003; Hudson et al., 2003), where it appears to be the main neural inducing signal. In *Xenopus*, however, the evidence for FGF in neural induction has been controversial (Kengaku and Okamoto, 1995; Lamb and Harland, 1995; Launay et al., 1996; Sasai et al., 1996; Xu et al., 1997; Holowacz and Sokol, 1999; Hongo et al., 1999; Curran and Grainger, 2000; Ribisi et al., 2000; Umbhauer et al., 2000). One of the reasons for this may be that, in *Xenopus*, FGF signalling has mainly been inhibited using a dominant-negative version of the FGF receptor 1 (FGFR1), which may not inhibit all FGF signalling as FGF signals in neural induction appear to be transmitted at least in part by FGFR4 (Hardcastle et al., 2000; Umbhauer et al., 2000).

Recently, it has been shown that signalling by FGF and by other secreted proteins that work through MAP kinase act in part by phosphorylating the linker region of Smad1, rather than the C terminus as does BMP signalling (Pera et al., 2003). A double-phosphorylation mechanism was therefore proposed as a molecular basis for the cooperation between FGF and BMP in neural induction and other embryonic signalling events (Pera et al., 2003). However, a more recent study in mouse by Soriano and colleagues has elegantly demonstrated that mice carrying mutations in these two distinct domains of Smad1 show different and additive phenotypes, and that the mutations cannot complement each other, suggesting that linker and C-terminal phosphorylation of Smad1 (and thus MAPK and BMP signalling) have different functions during early development (Aubin et al., 2004).

Our present experiments demonstrate that in the chick, neither FGF2, FGF3, FGF4 nor FGF8 is a sufficient neural inducer in the absence of *brachyury* expression, even when any of these is combined with Smad6 as a BMP antagonist, consistent with the previous findings that FGF8+Chordin do not induce *Sox2* expression when administered as proteins (Streit et al., 2000). These results suggest that FGFs may only be able to induce definitive neural tissue in cooperation with other signals in addition to BMP antagonists.

A role for Wnt signalling in neural induction?

More recent experiments in the chick, using NFz8 as the Wnt antagonist, explant assays from the area pellucida and an antibody against Sox2, suggested that Wnt inhibition together with FGF can act as a sufficient neural inducer, and FGF3 was suggested as the endogenous factor (Wilson et al., 2001). These experiments were interpreted as indicating that BMP signalling can be inhibited by these treatments through an alternative pathway and that the key event may be downregulation of BMP at a transcriptional level (Bainter et al., 2001; Wilson and Edlund, 2001). In *Xenopus*, however, Wnt signalling seems to promote neural induction (Baker et al., 1999), although it is thought that the conflict is resolved by differential timing of these events: Wnt signalling is required in early (pre-gastrula) stages of development, while inhibition of Wnt may be important for acquisition of neural fate at later stages (Bainter et al., 2001; Wilson and Edlund, 2001).

To test whether inhibition of Wnt signalling can cooperate with BMP antagonism and/or FGF signalling in vivo, we misexpressed combinations of these agents in competent epiblast. Even a combination of FGF (FGF2, FGF3, FGF4 or FGF8), Smad6 and four different Wnt antagonists (alone or in combination) is unable to induce *Sox2* expression in this assay. The difference between our result and those obtained by Wilson et al. (Wilson et al., 2001) are difficult to explain, but it is possible either that isolated explants cultured for 48 hours are somehow sensitized to neural inducing signals, or that the Sox2 antibody might crossreact with Sox3 (which is induced by FGF8). We have attempted to use this same antibody for our experiments with a variety of protocols, but were unable to obtain reliable background-free staining (data not shown). Based on the results of the present experiments, we can only conclude that even a combination of FGF+Smad6+anti-Wnt is insufficient to mimic the effects of a grafted organizer and induce *Sox2* expression, or an ectopic neural plate, in competent epiblast of the area opaca. We suggest that neural induction is a multi-step process that begins very early in development and involves other neural inducing factors, which remain to be identified.

Do different vertebrates use different mechanisms to specify neural fate?

The results of our present experiments in the chick, along with those previously published by ourselves and other groups, still raise the issue of whether different vertebrates might use different molecular pathways to induce the nervous system. We therefore tested whether inhibition of BMP signalling with Smad6 is sufficient for neural induction in *Xenopus*.

Most experiments on neural induction in *Xenopus* have been conducted on animal caps cut from embryos injected at the animal pole at the one- to four-cell stage. It is important to bear in mind that such animal caps almost certainly include some prospective neural plate or neural crest cells (Jacobson and Hirose, 1981; Dale and Slack, 1987; Moody, 1987a; Moody, 1987b; Wetts and Fraser, 1989; Eagleson and Harris, 1990; Saint-Jeannet and Dawid, 1994; Delarue et al., 1997). It is conceivable that prior to their isolation from the embryo, these cells have received some signals that, although not sufficient for neural induction, may represent some early steps in the process. For this reason, we chose to target Smad6 to the most ventral animal blastomere at the 32-cell stage, as this is the only blastomere that does not consistently contribute to neural plate or neural crest, but mainly to the most ventral (belly) epidermis. Using a concentration of *Smad6* mRNA that is sufficient to induce axial duplications when targeted to the marginal zone (400 pg to 1 ng), and also sufficient to induce neural tissue in animal caps (1 ng), we see no ectopic expression of the neural markers *Sox3* (which in *Xenopus* is a definitive neural plate marker) or *NCAM* in the descendants of the injected A4 cell. These findings suggest that, in the *Xenopus* embryo as well as in the chick (Streit et al., 1998; Streit and Stern, 1999a; Streit and Stern, 1999b), BMP inhibition may only be sufficient to deviate the border of the neural plate when the antagonists are targeted to its vicinity, but is insufficient to cause prospective epidermis (cells fated for neither the neural plate nor its border) to acquire neural traits. Therefore animal cap assays are not a good test for whether a candidate molecule has neural inducing activity.

Our chick and *Xenopus* results differ in one respect. Misexpression of *FGF4* (at concentrations that do not induce expression of *brachyury*) with *Smad6* induces *Sox3* in the absence of mesoderm markers in *Xenopus*, but does not elicit a comparable response (*Sox2*) in chick. There are several

possible interpretations for this difference. (1) There may be real differences in the mechanism of neural induction in the two species. We feel that this is unlikely as cross-species grafts of the organizer work very well across all vertebrate classes (Waddington, 1934; Waddington, 1936; Waddington, 1937; Kintner and Dodd, 1991; Blum et al., 1992; Hatta and Takahashi, 1996). (2) The expression patterns of FGF4/eFGF are different in the two species – in chick *FGF4* is expressed in mid-posterior streak but not in the organizer (Streit and Stern, 1999b), while in *Xenopus*, *eFGF* is expressed in a domain including the dorsal lip (Isaacs et al., 1992; Isaacs et al., 1995); different FGFs may therefore fulfill this function in the two species. (3) It is possible that either endoderm or lateral mesoderm (which do not express any of the five markers tested) is induced in *Xenopus*. (4) It is possible that the level of inductive signal provided by Smad6+FGF4 is not sufficient to induce mesoderm, but enough to cause injected cells to produce some products normally secreted by early prospective mesendoderm cells. (5) It is possible that the *brachyury*-expressing cells in the chick are prospective *caudal* neural plate, which has been shown to express this marker in chick (Storey et al., 1998) but not in *Xenopus*. Although this explanation seems the most likely, we have observed that the induced *Sox2* expression is restricted to the Smad6-electroporated cells (marked by GFP), while *brachyury* is also expressed in neighbouring cells; the possibility therefore remains that the *Sox2* induction in this experiment is indirect. Furthermore, this does not explain the finding that in the presence of Wnt antagonists both *brachyury* and *Sox2* expression disappear. Finally, (6) in *Xenopus*, the marker selected for assessing neural plate is *Sox3*, while in chick the definitive marker is *Sox2*. These genes (and the closely related *Sox1*) appear partly to have swapped functions in evolution, even between birds and mammals (Uwanogho et al., 1995; Collignon et al., 1996); it is therefore very likely that the enhancer elements regulating the expression of these two markers differ in the two species. Furthermore, *Sox3* is not an exclusive neural marker in any of the vertebrate classes, and the induced expression seen in *Xenopus* could correspond to a different cell type.

Conclusion: neural induction as a multi-step process

Our experiments provide evidence that BMP inhibition is required for neural induction in the chick, but only as a relatively late step in a molecular cascade. They also strongly suggest that BMP inhibition is not sufficient to cause competent ectodermal cells to acquire neural fates either in chick or in *Xenopus*. In *Xenopus*, FGF synergizes with BMP inhibition to induce neural markers (we cannot yet conclude definitively whether this combination is sufficient). In chick, inhibition of BMP signalling, even together with Wnt antagonists and/or FGF, is not sufficient for neural induction. We propose that neural induction does not occur 'by default' but rather that it involves a succession of signalling events, where some players remain to be identified.

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