Int. J. Dev. Biol. 44: 129-140 (2000)

Effects of follistatin and BMP4 proteins on early dorso-ventral patterning in chick

DAVID J. CONNOLLY, KETAN PATEL, SARAH WITHINGTON and JONATHAN COOKE*

Division of Developmental Neurobiology, National Institute for Medical Research, London, United Kingdom

ABSTRACT In *Xenopus* and zebrafish certain bone morphognetic proteins (BMPs), and proteins that antagonise these by preventing their interaction with receptors, constitute a morphogen system in primary dorso-ventral patterning. This system may be directly involved in the parallel processes, within mesoderm and ectoderm, whereby the boundaries of the dorsal (paraxial) mesoderm and the neural plate are established. The bird blastoderm, amenable to grafting techniques and to direct exposure to specific proteins, has provided an opportunity to explore the phylogenetic conservation of such antagonistic system. We have grafted the gastrular organiser (node) into hosts, testing the effects of prior exposure of either grafted or host tissue to Follistatin (a known antagonist of TGFβ superfamily ligands including BMP4) or to BMP4 protein. Strong, converse effects are seen from the two agents, the most consistent being on the sizes of new dorsalised areas (second neural plates) induced in host epiblast. Follistatin also enhances extension movements due to grafts, though without clear effect upon the rostro-caudal completeness of new patterns. Neural induction in chick epiblast by grafted mouse nodes are also more extensive, after their pre-incubation in Follistatin. Follistatin potentiates other, unknown but distinctive signals coming from the node, being unable to convert other non-inducing pieces of blastoderm into organisers on grafting. Pre-incubation of early blastoderms in BMP4 has such profound effects on normal dorsal axial development that host responsiveness of these blastoderms as hosts to node grafts is difficult to assess. Follistatin has no such overt effect on host development, but greatly enhances the competence of host epiblast to grafts of untreated nodes. Early chick BMP4 and BMP7 expressions are consistent with the proposed roles, though Follistatin is probably an experimental tool only in the present study.

KEY WORDS: *dorsalisation, neural induction, follistatin, BMP4, Hensen's node, pattern formation*

Introduction

During vertebrate gastrulation a restricted region already specified as 'the organiser', which itself gives rise to dorso-anterior mesoderm and endoderm, interacts with its surroundings to establish the axial body plan. There is considerable evidence from work in *Xenopus* and zebrafish that bone morphogenic protein 4 (BMP4) is a major ventralising morphogen in the dorso-ventral aspect of this patterning process (Fainsod *et al.*, 1995; Hemmati-Brivanlou and Thomsen, 1995; Holley *et al.*, 1995; Sasai *et al.*, 1995; Schmidt *et al.*, 1995; Wilson and Hemmati-Brivanlou, 1995; Hammerschmidt *et al.*, 1996). Experimentally, Follistatin protein may act as an antagonising 'dorsaliser' through sequestering certain TGFβ superfamily ligands including BMP4 from their receptors (Hemmati-Brivanlou *et al.*, 1994; Hemmati-Brivanlou and Thomsen, 1995; Wilson and Hemmati-Brivanlou, 1995). The phenotype of *follistatin* null mutant mice shows that while this protein's experimental action may be a clue to normal mechanisms, it is not an indispensable primary dorsaliser in mammal development (Matzuk *et al.*, 1995). Furthermore, at least in chick, the details of its RNA distribution at relevant stages are inappropriate for this exact role (Connolly *et al.*, 1995; Patel *et al.*, 1996). Current candidates for endogenous antagonists of BMP-mediated ventral development in vertebrates are the unrelated secreted proteins Noggin and Chordin (Lamb *et al.*, 1993; Smith *et al.*, 1993; Sasai *et al.,* 1994; Francois and Bier, 1995), and a further molecule structurally related to Follistatin (Towers *et al.*, 1999). All these candidate dorsalisers could function at least in part by interfering with the action of BMP4 or related ligands. Noggin and chordin have recently been shown to compete directly with BMP receptors in binding their ligands (Holley *et al.*, 1996; Piccolo *et al.,* 1996), while work in zebrafish (Hammerschmidt *et al.*, 1996) and in *Drosophila* (Biehs *et al.*, 1996) suggests that the sole function of the dorsalisers may be to antagonise BMP-mediated signalling in this way. Thus a reasonable model

Abbreviations used in this paper: BMP, Bone Morphogenetic Protein; TGFβ, Transforming Growth Factor beta; SHH, Sonic Hedgehog.

***Address for reprints:** National Institute for Medical Research, Laboratory of Embryogenesis, The Ridgeway, Mill Hill, London NW7 1AA, United Kingdom.

0214-6282/2000/$20.00

Printed in Spain
http://www.lg.ehu.es/ijdb

for initial stages, before the boundaries between zones in dorso-ventral pattern are determined, is counter-diffusion and direct interaction of the proteins from opposing 'dorsal' and 'ventral' sources of gene expression which need not be tightly localised. But at least in the case of the known ventraliser BMP4 in *Xenopus* (Hemmati-Brivanlou and Thomsen, 1995) and also BMPs 4 and 7 in chick (Schultheiss *et al.*, 1997), patterning interactions progressively feed back upon the gene activity itself. In *Xenopus* gastrular ectoderm, an initially diffuse and broadly ventrally centred transcription intensifies but becomes sharply excluded from the neural plate, while in chick a precise localisation at the neural/epidermal boundary develops.

The dominant idea for several decades had been that mesoderm ingressed at gastrulation is the primary site of axial pattern formation and passively imposes the outline of that pattern upon ectoderm by 'vertical' inductive signalling. But there is recent evidence from *Xenopus*, and some from chick, for operation of 'planar' signalling. The nascent dorsal-axial mesoderm and presumptive neural region interact before they are superimposed as layers in the gastrulation process, leading to partial pre-specification of the neural territory and even an outline of regionalised gene activities within it (Doniach *et al.,* 1992; Doniach, 1993; Garcia Martinez *et al.*, 1993; Ruiz i Altaba, 1994; Patel *et al.*, 1996). Dorsalisation evidently begins before most of the rearrangements of gastrulation and then continues in parallel within the ectoderm and mesoderm, with pattern then being refined by interactions between the superimposed layers. This is reminiscent of Spemann's original concept after the discovery of the dorsal lip 'organiser' in amphibians, one which he was apparently dissuaded from promulgating by colleagues (Hamburger, 1988). Thus a major component of neural induction may simply be dorsalisation, operating in early gastrula ectoderm. Candidate gene products for major dorsalising or ventralising signals might therefore be expected to function both as mesodermal dorsalisers or ventralisers, and as direct ectodermal neural inducers or inhibitors of neuralisation, in separate experimental assays. BMP4 indeed seems to have both these ventralising actions in *Xenopus* (Fainsod *et al.*, 1995; Sasai *et al.*, 1995; Wilson and Hemmati-Brivanlou, 1995), while Noggin and Chordin proteins display both corresponding dorsalising ones (Lamb *et al.*, 1993; Smith *et al.*, 1993; Sasai *et al.*, 1994). In chick, a strongly conserved *noggin* homologue has an appropriate transcription pattern in the gastrular organiser, but using recombinant Noggin protein supernatants that were highly active in *Xenopus* assays, we have been unable to obtain functional evidence for dorsalising or neuralising activity on the chick blastoderm in biological assays parallel to those of the work to be described here (Connolly *et al.*, 1997). The chick epiblast (ectoderm) may also not respond directly with neural development to Chordin protein alone (Streit *et al.*, 1997), despite the presence of a *chordin* homologue appropriately expressed in the organiser. In exploring possible conservation of the principles of primary dorso-ventral patterning in avian development, therefore, we have used Follistatin protein. It is available in recombinant soluble form, and its reported actions in the *Xenopus* experimental system strongly suggest that ventralising BMPs may be among the TGFβ superfamily proteins that it sequesters. We find that pre-incubation in this protein strongly enhances the new axial induction that follows grafts of Hensen's node, the gastrular organiser, consistent with dorsalising and neuralising action on the host tissues and/or the graft itself. BMP4 protein exhibits converse effects, consistent with ventralising, neural-inhibitory action. Use of the chick-quail marking system, coupled with examination of chick marker gene expression in second axes, has enabled us to deduce that these effects are essentially upon the range of graft influence into surroundings in dorsalisation/neural induction. The mouse node, used as a graft into chick, also organises a neural plate in host epiblast. The inductive capacities of such nodes respond to pre-incubation in Follistatin in a similar manner to those of chick nodes, suggesting a conserved nature for the interactions being studied whatever the identity of the *in vivo* gene products.

We have also incubated whole blastoderms in Follistatin or BMP4 proteins during early to mid-gastrula stages (those where the response to our node grafts occurs), a procedure that could be equivalent to global overexpression of the genes at these stages. BMP4 applied in this way systematically affects axis formation, with attenuation of neural plate and paraxial mesoderm development that is consistent with a normal ventralising role. Follistatin by contrast has no appreciable effects on the body patterns attained in this assay. As perhaps for Noggin and Chordin, regulatory mechanisms could be available in the whole avian embryo to cope with this particular perturbation of dorsal-ventral balance. Interestingly however, Follistatin-treated whole embryos are developmentally in advance of synchronously cultured age-matched controls, 18 h after start of ring-culture. This amounts in fact to a considerable 'rescue effect', from the developmental delay usually incurred by the culture procedure. The potential effects upon patterning mechanisms of global blastoderm exposure to Follistatin are revealed, however, when untreated Hensens' nodes are implanted. There is a strikingly enhanced responsiveness to both neuralising and mesodermal dorsalising signals from such nodes, in contrast to results of equivalent experiments with Noggin- treated blastoderms (Connolly *et al.*, 1997).

We present evidence that BMP4 and BMP7 RNA distributions, during the probable period of normal dorsalisation/neuralisation (and of responses to our node-grafting), are consistent with the proposed ventralising roles (see also Schultheiss *et al.*, 1977). The results are discussed in relation to other recent work on vertebrate primary dorso-ventral patterning, including the early expression of a recently described follistatin -related chick gene (Towers *et al.*, 1999).

Results

Figure 1 shows in diagrammatic form the standard operation. In preliminary experiments we found that use of late stage 3, rather than older hosts, led to more consistent degrees of new patterning caused by grafting (see also Storey *et al.*, 1992, 1995). We also found that pre-incubation of stage 4 chick nodes in Liebovitz/BSS for up to 2 h does not appreciably alter their capacity to induce second axial patterns in such hosts. The cell layer relationships seen, the known normal contributions from the chick node *in situ* (Selleck and Stern, 1991; Inagaki and Schoenwolf, 1993), and particularly the use of sonic Hedgehog as a marker, enable us strongly to infer the graft vs. host origins of the second sets of axial structures developed. We nevertheless carried out a series of quail to chick node grafts to determine directly the graft-host contributions to induced structures, after control graft pre-incubation or pre-incubation in either Follistatin or BMP4. These are illustrated in Figure 2A-D, while Figure 2 E-P illustrates chick-to-chick material. While we tried consistently to place the deep (meso-endodermal) face of grafts against the host basal epiblast surface, the neural inductive ability of implanted nodes, and our ability to deduce the graft- vs. host-derived neural structures, is not affected by the

original germ-layer orientation of graft relative to host (see below).

To clarify description of the experimental effects, we first describe the range of graft-host contributions to new axial structure in our hands with the help of quail-chick grafts. The node self-differentiates into an extending notochord (Fig. 2A), and often a gutter-shaped strip of tissue with the character of ventral neural tube that extends parallel with and closely tied to this notochord (Fig. 2D; see also the midline sHH expression in chick node grafts, Fig. 2M-P). There is also a variable degree of graft contribution to a second set of somite bodies, or converging paraxial mesoderm, flanking the graft-derived notochord. In cases with pronounced somites this contribution is usually small, and to the ventral part of the somite cross-section that is the expected node contribution in the normal axial fate map (Fig. 2A). There has thus been new dorsalisation of host mesoderm by signals from the node. As well as variations in the exact extent of node structures grafted, an intrinsic source of variation in relative somite contribution derives from the degree of contact of individual grafts with the host mesoderm. Such mesoderm is sparsely distributed in peripheral regions at these stages, and significant induction of new mesoderm by a stage 4 node graft into a stage 3 periphery is unlikely (Eyal-Giladi, 1984; Stern *et al.*, 1992).

The clearest and most consistent inductive action, however, is the dorsalisation or neuralisation of host epiblast in the overlying area to form an entirely host-derived second neuraxis (Fig. 2A-C, quail-chick; 2H-K and N,O for earlier-staged chick-chick examples). The graft-derived 'floorplate' neural strip is usually separated from the induction in host epiblast by the notochord or other graft-derived mesoderm, but occasionally when grafts were unintentionally implanted with epiblast facing the host in the culture, the graft- and host-derived neural layers lie adjacent (see Fig. 2O for a clear chick-chick example). There is never graft contribution to the host epiblast layer, i.e. to the new neural tube, in either type of quail-chick case. The cell layer relationships in chick-chick graft-induced axes are as those after the quail-chick grafts. Thus very probably, in the whole series, the new neural plates are entirely and the new paraxial mesoderm (somites) largely host-derived.

Though use of quail node grafts has helped us interpret the main experimental graft series, certain observations are consistent with the idea that our quail nodes are the equivalent of somewhat developmentally 'younger' chick ones. Firstly, in our hands, the neuraxes induced in chick epiblast by quail nodes ranged considerably larger than those caused by chick grafts. Also, significant graft-derived mesenchymal structure was frequently seen anterior to the notochord tip after quail-chick grafts (Fig. 2B), resembling pre-chordal levels of normal early axial structure though we have not characterised it definitively with gene markers. In our main series of chick-chick induced second patterns, such structure was rarely present ahead of the (presumed graft-derived) notochord. There were also massive quail contributions to endoderm around second axes (Fig. 2A-C), whereas such extensive endoderm contributions would not be expected from chick nodes as late as stage 4 (see Stern and Ireland, 1981).

Effects of graft pre-incubation in Follistatin or BMP4 on graft-host interactions

Pre-incubation of nodes in Follistatin on the one hand and BMP4 on the other produces striking and converse effects on the outcome of grafting them in the standard operation. In early experiments we used Follistatin concentrations between 0.1 and 20 μg/ml, and BMP4 concentrations up to 100 ng/ml, and found that although 1 μg/ml Follistatin had some effect, 20 μg/ml had no more effect than 2, while 100 ng/ml BMP4 had little more effect than 50. Figures 2 and 3 therefore show, in sectional views and whole-mount appearance, comparative results of grafting control-incubated nodes and nodes incubated in 2 μg/ml Follistatin or 50 ng/ml BMP4 proteins. In all, 43 such quail-chick grafts were examined (23 control pre-incubated and 10 pre-incubated with each experimental protein), and 146 chick-chick ones (58 control-pre-incubated, 59 Follistatin pre-incubated and 29 BMP4 pre-incubated). Overall, the effects of the experimental agents were seen for quail-chick grafts, but sections of the latter type are shown in Figure 2 to illustrate the fate-mapping and cannot directly be compared with the remaining, chick-chick images.

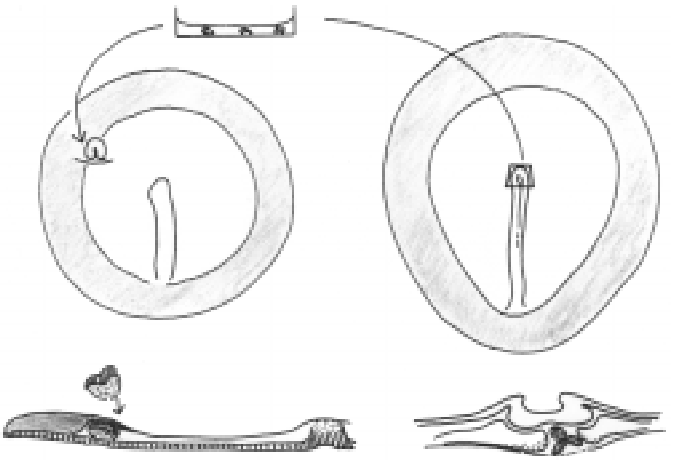

Fig. 1. The node grafting operation and resulting second axis. *Upper row shows the operation in plan view. The donor stage 4 node, cut out as shown from dorsal aspect, is pre-incubated and then placed in direct contact with the underside of the stage 3+ epiblast, through a slit in host hypoblast at region indicated. Lower left; sectional view of the donor node and the newly engrafted site, orientated as at operation with donor epiblast side up (hatched) but host hypoblast side up. Yolky endophyll of area opaca (shaded) normally overlies graft initially. Lower right; sectional view of a second axis at the stages of fixation after operations. Here the image displays the host layers conventionally, neuroectoderm uppermost. Hatching and stipple indicate equivalent layers of host and graft in the left diagram, but stipple indicates typical graft contribution to new structures in right diagram. Graft contributions to somite are probably more variable than those to notochord and inverted neural floorplate 'gutter' (see text).*

The most consistent effects of the proteins are on the cross-sectional size of the induced neuralised area in host epiblast, which is increased by Follistatin (cp. Fig. 2E,F with 2G-J) and diminished by BMP4 treatments (cp. Fig. 2A,B with D; 2K with L). Both the cross-sectional areas and the length of second structures are enhanced for Follistatin-treated nodes, though BMP treatment only reliably diminishes their cross-sections (see below). Thus in terms of the total amount of newly neuralised ectoderm and dorsalised mesoderm, the effects of the proteins on the signaling from implanted nodes are even greater than implied by the neural cross-sectional measurements alone. Figure 4 shows this quantitatively in terms of relative cross-sectional areas, lengths and thus volumes of neuraxial tissue (see figure legend). These were measured for a representative sample of the second axes that could validly be compared in this way because they displayed compara-

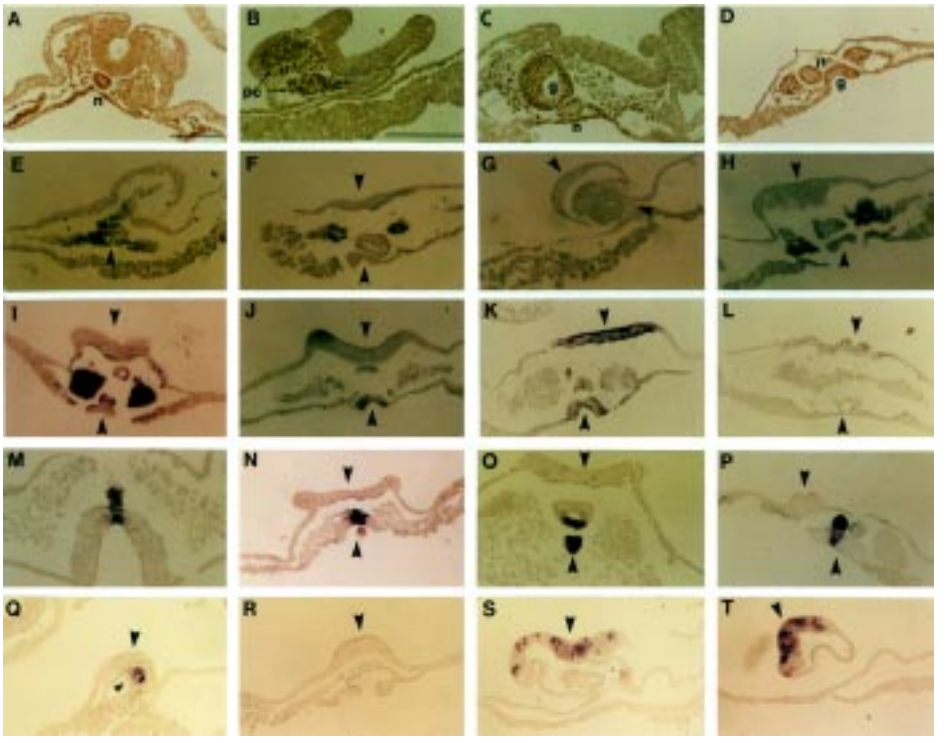

Fig. 2. Sectional views of inductions after node grafts, displayed as in Figure 1 lower right. (A-D) *Quail derivatives shown by HRP immunocytochemistry, at advanced stages of second axis development following quail node grafts into stage 3+ chick hosts. n, notochord; g, gutter of donor presumptive midline neural tube; pc, pre-chordal meso-endoderm.* **(A)** *Control graft at notochordal axial levels. Notochord is graft-derived, but never any of the neuraxis that is in continuity with host epiblast/ectoderm. Ventral and ventro-medial graft cells associated with predominantly host-derived somites are typical, and extensive endodermal and even lateral, vascular endothelial type contributions from quail grafts are frequent.* **(B)** *Contribution to pre-chordal structure at anteriormost end of an axis induced by a control graft. The forebrain-like structure is raised clear of the blastoderm surface as in a normal axis, and the central, non-notochord quail contribution resembles foregut endoderm/pre-chordal plate. Such structure is rarely seen in our chick-chick control graft series.* **(C)** *Follistatin-treated graft; anterior part of induced neural tube fuses with host neuraxis. Note extremely large allocation to self-differentiating neural component within the graft, not seen in control quail grafts or in any of chick grafts. This has not fused with host neuralised layer, even though graft was placed epiblast-to-epiblast with host. Endoderm contributions are also seen from these treated grafts.* **(D)** *BMP4-treated graft. The normal inverted neural 'gutter' node derivative (absent from A) is present . No neural induction in host epiblast or host contribution to small graft-derived somites are seen.* **(E-P)** *Sections of whole-mount preparations probed for gene expression, cut transverse to second axes following stage 4 chick node grafts to stage 3+ chick blastoderms. Developmental stages are younger than for A-D, and each series of sections probed for same gene is from one set of grafting operations. Midlines of induced neural plates dorsally, and of graft-derived axial structures ventrally, are marked on each panel where visible.* **(E,F** *and* **G-J)** *Successive equally spaced levels (posterior to anterior) through axes from control and from Follistatin-treated chick grafts, respectively, probed with follistatin . Second axes typically have a posterior unextended, peg-like structure raised from blastoderm, but the Follistatin case embraces more than twice as many sections as the control due to greater extension (see Fig. 4 middle columns). Note similar sized notochordal and inverted neural, i.e., graft-derived components (F,H-J), but much larger and more strongly follistatin—positive somites and greater neural cross section induced in host tissue by Follistatin-treated graft.* **(K,L)** *Mid levels of axes from control and from BMP4-treated chick grafts, probed with Hox B1. Note much reduced neural cross-section in host epiblast, and reduced expression of the gene in both this and the inverted neural graft component, in the BMP4 case.* **(M)** *Normal axial expression of Sonic Hedgehog (SHH) at mid neurulation stage, shown inverted so that layer relationships are as normally seen in graft derivatives. Notochord and presumptive floorplate (neural midline) express strongly.* **(N,O)** *Two examples of axes induced by Follistatin-treated chick grafts, probed with SHH. Note extensive host-derived neural plates and presumed host-derived paraxial mesodermal tissue. The second neural plates are delayed in developing midline SHH expression relative to their morphology and to the graft-derived floorplate gutter.* **(P)** *An axis induced by a BMP4-treated chick graft, probed with SHH. SHH expression is as strong as in control graft-derived components, but dorsalisation of host tissues, particularly neuralisation, is almost abolished. Small induced neural formation has contracted differentially (toward host axial midline) from the inducing graft at fixation.* **(Q-T)** *Sections as in (E-P), but following grafts of day 71/4 mouse nodes to stage 3+ chick hosts.* **(Q)** *Control graft induction, probed with mouse-specific brachyury probe to show graft-derived component, associated with neural (columnarised) region in host epiblast layer.* **(R)** *Section through structure as in (Q) but probed with chick-specific follistatin probe. Small induced neural area hardly expresses follistatin.* **(S,T)** *Mid- and anteriormost axial levels following a Follistatin-treated graft, probed with chick-specific follistatin probe. Note extensive host-derived neural formation including raised fold anterior to all axial mesodermal structure, strongly follistatin - expressing because more developmentally advanced than that of (R). Bar (see B for images B and C, A for all other images), 50 μ.*

ble geometry, i.e., open plate-staged neuraxes when fixed 18 h after grafting.

There are corresponding effects on the newly dorsalised mesoderm (somite or at least, converging mesoderm) associated with the graft-derived midline structures. The considerable alterations in the extents of this new paraxial mesoderm, caused by the experimental treatments of grafts, cannot derive from diverting the differentiation of graft tissue away from or into the relatively small notochord structures (cp. Fig. 2F with H,I; and K with L). Typical control node grafts cause new paraxial mesoderm which, although small in cross-sectional profile, contains amounts of tissue comparable with that in the clearly node-derived notochord and floorplate 'gutter'. The latter structures are certainly not absent or diminished (-through diversion of their cells into somite) in typical 'Follistatin' node grafts, nor over-sized in 'BMP4' ones. Thus, these mesodermal effects of the experimental agents consist of altered extents of dorsalisation by signalling from grafted into surrounding tissue (cp. also Fig. 2A with D, chick-quail).

Response of mouse nodes to pre-incubation in Follistatin

To explore whether there might be conservation of signalling mechanisms in dorsalisation among amniote embryos (see also Winnier *et al.*, 1995), we made a series of mouse node grafts into stage 3+ chick hosts and ascertained the effect of Follistatin pre-

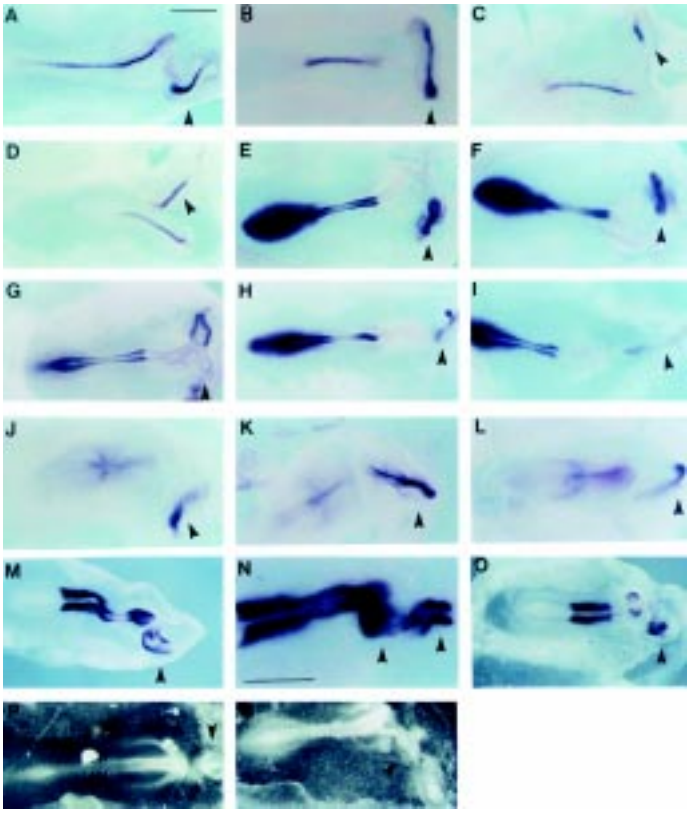

Fig. 3. Whole-mount views of axial inductions 18-24 h after node grafts. *In situ hybridisations with riboprobes for various axial marker genes, viewed with host anterior to right and from ventral surface, except for Hox B1 specimens which are viewed from dorsal, neural aspect. Structures due to graft marked in each case.* **(A)** *Control-incubated graft, SHH probe. Notochord and pre-chordal mesoderm, and for younger axes the regressing node, are marked.* **(B)** *Follistatin-incubated graft, SHH probe, showing the enhanced and more evenly distributed extension movements by graft midline tissue.* (**C,D**) *Two BMP4-incubated grafts, showing how amount of SHH-expressing tissue is often reduced, and its extension movements sometimes inhibited.* **(E,F)** *Control-incubated grafts, Hox B1 probe. In the host axis both posterior mesodermal, and neuraxial expression with a sharp, internal anterior boundary are seen. In second axes neuraxial expression is at earlier stage, without sharp boundary.* **(G)** *Follistatin-incubated graft, Hox B1 probe, showing much more developed second neural tube (anterior downwards) with sharp, internal expression boundary as in the host axis.* **(H,I)** *Two BMP4-incubated grafts, Hox B1 probe, showing greatly reduced extent of newly induced neural plate.* **(J)** *Control-incubated graft, Flik (follistatin-like) gene probe (see text and Patel et al. 1996), showing diffuse expression centred in dorsal-axial, post-gastrulated structure of host and second axial patterns.* **(K,L)** *Two Follistatin-incubated grafts, Flik probe. Stage-dependent intensification of Flik expression shows the relative acceleration of second pattern development, and the enhanced development of neural tube (K) and of extended notochord and somites (L).* **(M)** *Control-incubated,* **(N)** *Follistatin-incubated and* **(O)** *BMP4-incubated grafts, follistatin probe. Intensity and extent of axial follistatin expression again reveal altered extents and developmental schedules of second patterns after treatment with the proteins. Massive second axis fuses with host near panel centre, but shows equally advanced paraxial mesodermal and neural gene expression.* **(P,Q)** *Blastoderms with second axes induced by control and by Follistatin-incubated grafts respectively in the same experiment, photographed live from ventral surface by transmitted light. Much more material is seen to remain unextended posterior to the smaller 'control' second axis (directly below mark), whereas 'Follistatin' example shows massive extended notochord and well-developed posterior zone of tissue recruitment as in host pattern, and foregut tunnel development anteriorly (mark). Bar (see A for all images except higher magnification of N), 500 μ.*

incubation upon such nodes (20 control, 30 Follistatin examples). The second organisations induced by normal day 7.25 mouse nodes are usually smaller than those due to chick nodes but are unmistakable, the main feature being the host-derived neural plate in epiblast, overlying mouse-derived structures as revealed by a mouse-specific *Brachyury* probe (Fig. 2Q). Follistatin pre-incubation enhances the ability for convergent extension of the mouse-derived axial mesoderm, and causes enhanced size and morphogenesis in the newly induced chick neural plate (cp. Fig. 2R with S,T).

Examination of marker gene expression in second axes

Follistatin itself acts as a good marker gene, in revealing the effects of graft pre-incubation in Follistatin protein upon second axis formation. It is expressed widely within the early somite cross-section (Connolly *et al.*, 1995). Thus after Follistatin treatment, grafts often induce sets of better-formed and more massive, intensely *follistatin* -positive somites, within more extended second axes, when compared with control grafts 18 h after operations (Fig. 2G-J; cp. E and F). After BMP4 node treatment the induced paraxial mesodermal as well as neural cross- sections of second patterns are correspondingly reduced compared with controls (Fig. 2D,L,P). Probing for Sonic Hedgehog (SHH) expression as well as that of the *follistatin*-related gene *Flik* (Patel *et al.*, 1996) also reveals how Follistatin often enhances the extension movements that the graft makes and induces in its surroundings (Fig. 3B,K,L; cp. also Fig. 2E,F with G-J).

The effect of BMP4 on the stage 4 node as a graft appears not to be quite the opposite of the Follistatin effect, in that while the degree of new dorsalisation is always diminished, the capacity for graft-driven extension is sometimes not. Second patterns are thus characteristically less massive in cross-section than after control grafts (Fig. 2D cp. with A; and L,P cp. with K), but may still be comparably extended (Fig. 3D,I). We have not systematically compared axes from control, Follistatin- and BMP-treated grafts with marker genes for small anterior axial domains. Figure 2E-G, probed with *Hox B1*, illustrate the more advanced second axial development caused by Follistatin pre-treated graft a set time after grafting into synchronously aged hosts, in that the sharp internal neural expression boundary seen in the experimental induced neuraxis is less apparent in the control cases. But allowing control graft-induced cases to develop further before *B1* probing, as well as probing of second axes with *Krox-20* as a marker of a particular hindbrain level (not shown), fails to reveal any large or systematic effects of the treatments on the degree of anterior completeness of induced axes.

Pre-incubation of host blastoderms in Follistatin and BMP4

We treated a variety of tissue pieces from early chick blastoderms with Follistatin before implanting them into hosts as for node grafts, but never obtained any morphogenesis from these to suggest that the protein itself imbues tissue with organiser properties. The effect is one of augmenting other necessary signals maintained by the node. For both experimental proteins however, the question remains

as to whether the effects are entirely upon processes within the grafts themselves, or are due wholly or in part to the grafted tissue acting as a reservoir, releasing retained factors into surrounding tissue where they alter responsiveness to other node-derived signals. In this particular work we did not achieve significant effects with protein-loaded beads of various kinds, using beads small enough not to interfere mechanically with gastrulation movements themselves. This is unsurprising in view of the maximum loading concentrations of the experimental proteins available to us. To explore this issue further, we therefore pre-incubated whole blastoderms for 2 h in Follistatin, BMP4 or control Liebovitz/BSS, briefly washed them in fresh medium, then returned them to membranes for further development. Normal untreated nodes were then grafted into some of these specimens. Results are illustrated in Figures 5 and 6.

In five experiments, matched sets of 4-8 control pre-incubated blastoderms and 4-8 incubated in 2 μg/ml Follistatin were returned to vitelline membranes in ring culture, and in three of these

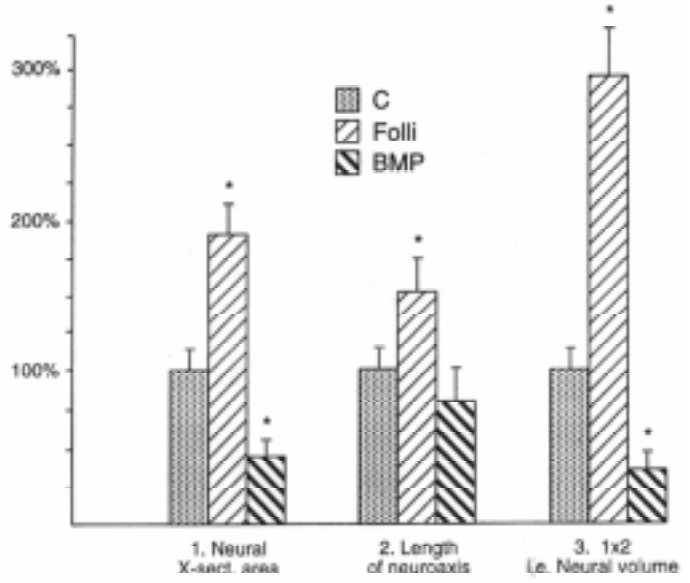

Fig. 4. Quantitative effects, of pre-incubating node grafts in Follistatin or BMP4 proteins, on neural inductions in host epiblast. *Measurements were made from a subset of chick-chick experiments in which graft-induced neuraxes, transversely sectioned at the 18 h timepoint of culture, were clearly anatomically visible as open neural plates (as in. e.g., Fig. 2F, or 2I-L). Photoprints were made at standard magnification from 160 μ (20 section) intervals, and the width, mean depth and area of neural section estimated using a linear scale in approximately 10 μ units (derived from a photomicroscope scale bar). Antero-posterior extent of each neuraxis was also estimated to the nearest 10 μ unit, by counting the serial sections on which it appeared. Relative mean areas (left-hand columns), lengths (middle columns), and volumes (right-hand columns) of neural tissue in the axes (in the appropriate units of 10 μ side-length) were thus computed for the control-grafted (N=11), Follistatin grafted (N=8) and BMP grafted (N=6) samples (two further BMP-grafted second axes showed insufficient neuralisation in host epiblast for measurement). Columns in each set show the sample means and standard errors, from left to right, of control, 'Follistatin' and 'BMP' samples, with the control sample value set at unity (100%). Both differences from control within each column set are significant at <1% level (t-test), except for axial length within the BMP sample, whose large relative standard error reflects the fact that these axes sometimes, but not always, fail to extend normally. The greater relative differences for total neural volume reflect the multiplicative effects of both cross-section and axial extension.*

experiments, untreated stage 4 chick nodes were then grafted to the blastoderms. We failed to detect any effect on the pattern of development in the 'host' axes following such an incubation with Follistatin. However, by around the 10 somite stage, the Follistatin-pre-incubated embryos were significantly developmentally advanced in comparison with their synchronously pre-incubated and cultured controls (cp. Fig. 5A,C). They in fact resembled embryos that had developed continuously *in ovo* throughout the experiment. Thus, although not appreciably altering the normal pattern of development, Follistatin had substantially 'rescued' blastoderms from the delay that is incurred by the explantation and culturing procedures of this work. A truly morphogenetic effect, of their pre-incubation in Follistatin, was however revealed in these blastoderms by the node grafts. Their responsiveness to the standard graft was markedly enhanced, showing a more massive cross-section particularly in the second neural plate, in a way suggesting potentiation of the dorsalisation responses (neural induction) within the host epiblast. New convergences of paraxial mesoderm or somite formation were also more massive and organised in many examples. This is most dramatically shown in a further experiment illustrated in Figure 5, where the hosts were at full stage 4, older than in our main series of chick-chick experiments. In our hands the normal response to grafting at this stage is very circumscribed, consisting of at most a localised neural induction (Fig. 5A,B,H,I), or extension of a thin notochord (E). In this experiment, the responses in Follistatin pre-incubated hosts were not only much more massive and organised (Fig. 5C,D,F,J), but included examples of pre-chordal mesodermal structure beneath anteriormost neural plate (5K), that are not prominently seen in chick node grafts to untreated hosts even at stage 3+. Pre-incubation with Follistatin had in effect enhanced, or extended the normal duration, of competence of the host epiblast and mesoblast to dorsalisation processes. Streit *et al.* (1997) have recently reported a comparable phenomenon after incubation in Hepatocyte Growth Factor/Scatter factor.

In six experiments, matched sets of 6-8 whole blastoderms were similarly incubated in BMP4 (50 ng/ml). Normal subsequent development only occurred if exposure to the protein was delayed until late in stage 4. Diminution of responsiveness to grafts was not clearly demonstrable by this stage, when such responsiveness is in any case localised at best (see Fig. 5). In progressively younger material than this, any altered responsiveness to grafts could not reliably be studied due to pronounced effects of the protein on 'host' development, illustrated in Figure 6. Dependent on precise stage of treatment, there is delay in completion of streak elongation and node formation, then head-process emergence and headfold formation. The posterior end of the shortening streak often remains massive, as if material normally fated to elongate and/or to move out of it has failed to do so. In more mildly affected cases there is finally shortening, and diminution in cross-section, of the neuraxis, with slender thin-walled somites. The next grade of severity involves abnormal flattening and failure to elongate of the neural plate and tube. The most severe cases have no recognisable axial structure within a mass of tissue lying ahead of the abnormally thin, cell-poor streak-like region. Effects are much more severe when treatment is before the half-length streak stage, perhaps corresponding in organisation to the amphibian early gastrula. Sensitivity diminishes sharply during stage 4 (mid-gastrula), with effects being limited to production of thin and cell-poor somite bodies and neural tubes of reduced cross section. Full analysis of this disruption due to BMP4 will require extensive use of gene probes and is underway.

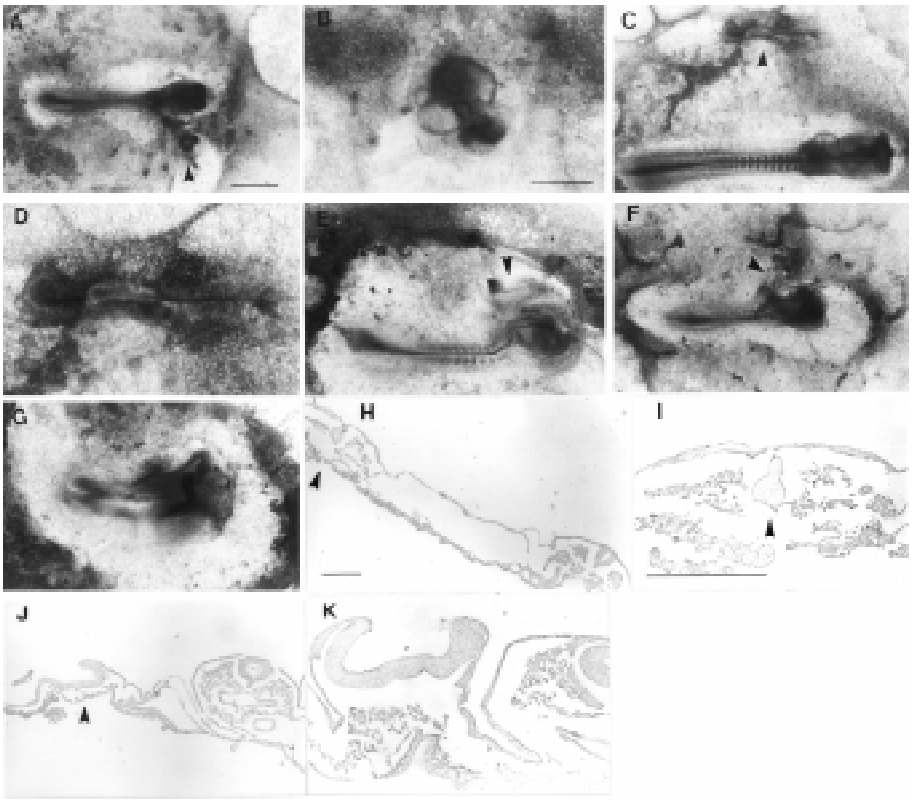

Fig. 5. Results of host blastoderm pre-incubation in Follistatin before node-grafting. *Hosts for these operations were late stage 4, thus of diminished normal responsiveness as compared with those illustrated in Figures 2 and 3.* **(A-G)** *Live specimens in ring culture from ventral aspect.* **(A)** *Eighteen hours after grafting into a control blastoderm, even with the graft relatively near the host axis, response has been minimal with no extension movements.* **(B)** *High power view of a further control graft; local neural formation and unextended axial mesoderm.* **(C)** *Eighteen hours after grafting into a blastoderm pre-incubated for two hours in Follistatin, 2 μg/ml. Graft remains at a peripheral position, and an extended and organised second axis has formed. Note more advanced development in the host axis, originally developmentally synchronous with that of (A).* **(D)** *High power view of graft site in (C); extended notochord, prominent paraxial converging mesoderm, a neuraxis and foregut tunnel (to left) are apparent.* **(E)** *A control case where even though proximity of the graft has disturbed the extension of the host axis, new axis formation at graft site is minimal (notochord and little neural induction).* **(F)** *An experimental case where the graft, comparable in positioning to that of (E), has caused a second axis including rows of large somites and a neural tube, running into the host anteriorly.* **(G)** *Extreme experimental case where there has been partitioning of the field into two parallel axes where essentially equal-sized notochords underlie an abnormal, partially double neural plate with delayed development. Such profound duplication never results from grafting into control blastoderms.* ***(H-K)*** *Sections through host and parallel second axes.* **(H)** *Result of grafting to peripheral site in control blastoderm, showing host axis (lower right) and graft site (upper left).* **(I)** *High power view of graft site from (H). The inverted graft-derived components are marked, the amount of (probably host-derived) converging mesoderm is small, and the neuralisation response in host epiblast, if any, is limited to slight epithelial thickening.* **(J)** *Low power sectional view comparable to that of (H), for a Follistatin-treated grafted blastoderm. The massive neural cross-section induced by the graft (marked at left) is comparable to that of the host axis (right).* **(K)** *High power view of a second Follistatin-treated grafted specimen, in which the anterior regions of the massive second axis lie closer to the host. Abundant mesenchymal mesoderm underlying this neural morphology ahead of notochord levels (not shown) is as at prechordal levels of normal whole axes, and is seldom induced by stage 4 chick node grafts even in our control stage 3+ blastoderms. Bar, 1 mm for A,C,E,F and G (see A), 500 μ for B,D (see B), H,J (see H) and I,K (see I).*

Expression of BMP4 and BMP7 genes in chick mid-gastrula stages

Figure 7 shows whole-mount *in situ* hybridisations to *BMP4* (A-C) and *BMP7* (D-F) mRNAs in normal chick blastoderms at stages 4 (A,D) to 4+ (C,F), i.e. in the developmental period when events within ectoderm are presumably setting the extents of final host responses to node grafts made at stage 3+ (see also Schultheiss *et al.*, 1997). It can be seen that progressive clearance of expression from the presumptive neural region (dotted line on stage 4 images) is often the more extensive, but is also more variable, for *BMP7*. By stage 4+, while the edge of *BMP7* expression in ectoderm rather closely defines an area corresponding to the fate map position of the entire plate, *BMP4* is still quite extensively expressed within the more peripheral and posterior parts of this area.

Discussion

We have exposed the avian equivalent of Spemann's organiser, Hensen's node, to either Follistatin or BMP4, and followed the ability of this structure to induce a second axis when grafted. We find pronounced reinforcing and attenuating effects, respectively, of these proteins on the processes of new mesodermal dorsalisation and especially neuralisation in host ectoderm. Additionally, we have explored the effects of these factors on normal whole blastoderms, and in the case of Follistatin, on the responsiveness of such blastoderms to untreated node grafts. Follistatin, the dorsalisation enhancer, does not itself cause non-node tissue to acquire node-like organiser properties, but its potentiating effect also operates on mouse nodes implanted into chick. We propose that even though Follistatin might not be involved in the normal developmental mechanism at this point, the experiments are manipulating mechanisms of dorso-ventral patterning that are evolutionarily conserved among vertebrates.

The case for a component of planar signalling in neural induction in *Xenopus* is now well-documented, and for chick also, evidence is accumulating for early influences spreading from the anterior streak into epiblast to establish the neural plate, before the mesodermal head process has spread beneath the ectoderm (e.g., Alvarez and Schoenwolf, 1991; Patel *et al.*, 1996, Streit *et al.*, 1997). Foregut endoderm, already emerging from the streak before the latter attains full length, is probably not a potent neural inducer (Storey *et al.*, 1995), so these early signals probably are transmitted within the epiblast plane from the node region through-

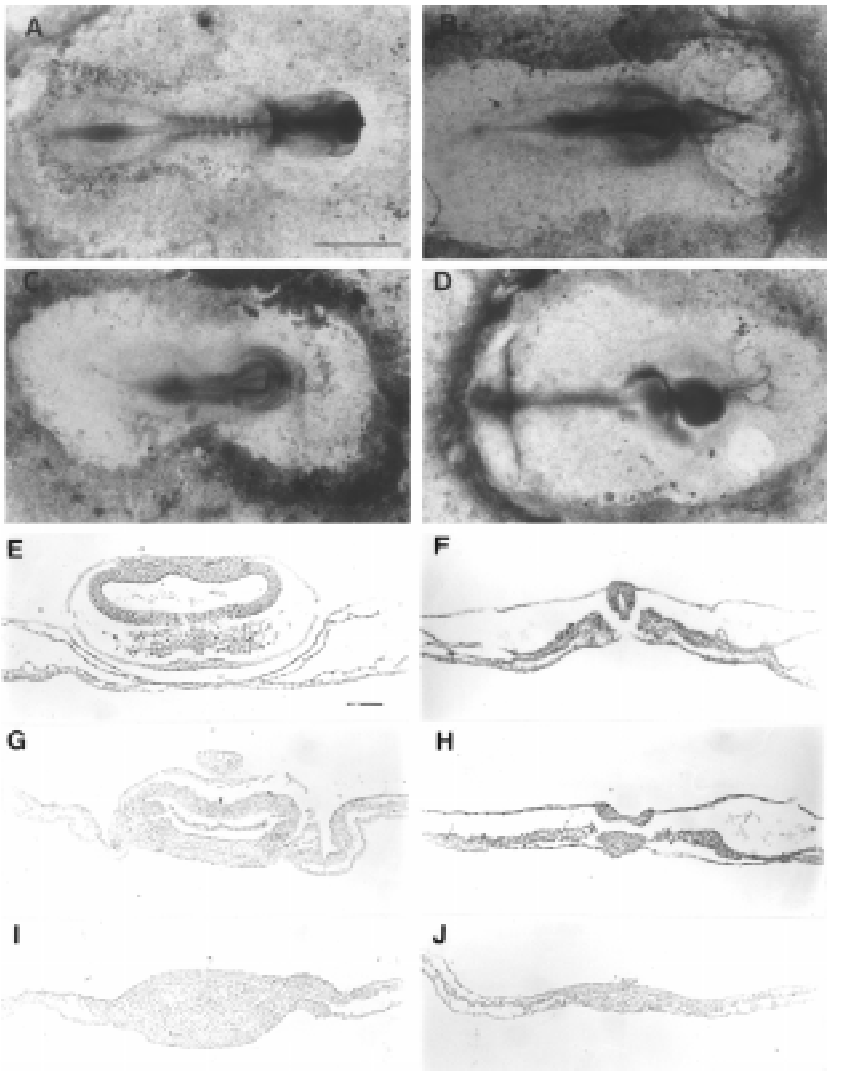

Fig. 6. Results of blastoderm incubation in BMP4 for 2 h during stages 3-4. (A-D) *Live blastoderms in ring culture seen from ventral surface, anterior to right.* **(A)** *Typical example, 20 h after replacement to incubate on the vitelline membrane following pre-incubation in control medium at stage 3+-4 (see Materials and Methods). Cardiac and neural development are normal for an 8 somite embryo.* **(B)** *Embryo 20 h after pre-incubation in 50 ng/ml BMP4 at stage 4. Neurulation and axial mesodermal morphogenesis are greatly delayed and the shortened neuraxis will never extend beyond the foregut pocket.* **(C)** *Synchronous example after BMP4 incubation at stage 3+, showing more severe delay, further reduction in neurulation schedule and mesodermal convergence, and failure to maintain normal recruitment of tissue from the posterior streak region into the axis.* **(D)** *After BMP4 incubation earlier during stage 3, no organised extension of an axis occurs, while an abnormal mass of undifferentiated cells remains along where the streak should lie, concentrated at the posterior end.* **(E-J)** *Transverse sections.* **(E)** *Normal embryo, cultured through the control procedure as in (A), at forebrain (optic) level.* **(F)** *Embryo of (E), just posterior to last-segmented somite.* **(G)** *Embryo of (B), near anterior end of neuraxis at level of foregut opening.* **(H)** *Posterior axial level – approximately equivalent to (F) for the normal embryo – in a moderately affected 'BMP4' specimen. Large cross-section of notochord probably due to its lack of extension, but note abnormally small paraxial mesodermal and neural cross-sections.* **(I)** *Section through large unorganised anterior mass of the specimen of (D). The layer structure of the embryo appears to have broken down at this level, although it resembles a 'gastrulated' rather than pre-gastrular region.* **(J)** *Posterior, streak-like region of the specimen of (D). This entire region is without a groove, as seen only in posteriormost normal streak regions that give rise entirely to mesenchymal extra-embryonic mesoderm. Bar, 1 mm for A-D (see A), 100 μ for E-J (see E).*

out streak elongation. Following the grafts described here, the tissue layer relationships show that all induction occurs without any anatomical integration of inducing node cells into the responding epiblast, and thus any possible passage of 'planar' signals. The neural-inductive signals being enhanced or attenuated by the present experiments can therefore pass between cells of different layers, whether or not it they operate in this way normally. In fact, comparison of Figures 2M and N (of sections probed with *SHH*) illustrates a regular feature of these second neuraxes, that probably relates to this modification of normal neural induction. Lacking the strip of *SHH*-expressing cells at their midlines, directly derived from the epiblast component of the normal node (Selleck and Stern, 1991), the second neural plates are retarded in medio-lateral organisation including floorplate-associated expression of *SHH*, when compared with equivalently developed normal neural plates.

There is little evidence for anteriormost, pre-chordal levels of structure in most of our chick-chick induced second axes, and though we have not made a detailed study with gene markers for such axial levels, we see no evidence for strong effects of the experimental proteins upon the rostro-caudal completeness of induced second patterns. On this evidence BMP4 or related ligands, and the *in vivo* equivalent of Follistatin's counteracting effects, would appear to be selectively involved in setting up the medio-lateral or dorso-ventral aspect of body pattern. De Robertis and Sasai (1996) have recently reviewed the evidence that this is the case, and that such a system is conserved in functioning (with reversal of 'dorsal' and 'ventral' labels where appropriate) between different metazoan phyla.

A largely independent system, for instance involving retinoids as intercellular signals and the responses to them of *Hox* and other homeobox genes, could be involved in fundamental antero-posterior axial patterning (e.g., Durston *et al.*, 1989; Sharpe, 1991; Kessel, 1992; Simeone *et al.*, 1992a,b; Deschamps and Wijgerde, 1993; Kappen and Ruddle, 1993). But there is a certain interaction between these dimensions of pattern at the outset. In amphibian embryos, for instance, it has been observed that the more extensive second axes are in terms of tissue size, the more anteriorly complete they tend to be. It is thus interesting that in the most massive graft-induced second axes we have seen, some of those developed after Follistatin pre-treatment of full stage 4 host blastoderms, pre-chordal structure is clearly present (Fig. 5K).

In *Xenopus*, neuralisation and dorsalisation are essentially processes of establishing boundaries between zones for different commitments, within sheets of tissue that are moving and undergoing cell division but not at this stage truly growing. Correspondingly the effects of experimental interference with the BMP signalling pathway, of *BMP* over-expression or of over-expression of agents that might sequester BMP ligands, are essentially to alter the normal gastrular fate-map by shifting these boundaries. In *Xenopus, BMP4* expression appears to retreat during dorsalisation, from a near ubiquitous distribution in early gastrula meso- and ectoderm. It becomes sharply excluded from the dorsal axial territories of these layers only by early neurula stages (e.g., Fainsod *et al.,* 1995; Sasai *et al.*, 1995; Schmidt *et al.*, 1995; Wilson and Hemmati-Brivanlou, 1995). Correspondingly, the effects of global *BMP4* over-expression are interpretable solely as alteration of the fate map by the progressive shifting of boundaries to eliminate the territories normally resulting from dorsalisation (Dale *et al.*, 1992; Jones *et al.*, 1992; Fainsod *et al., 1995*; Hemmati-Brivanlou and Thomsen, 1995). In zebrafish, where this patterning might also be largely independent of true growth, the early *BMP4* expression evolves similarly (Hammerschmidt *et al.*, 1996). In mammal development, where body plan establishment occurs within a rapidly growing tissue, the *BMP4* null mutant phenotype appears not so much a fate map alteration as an extreme interference with proper ventro-posterior growth and differentiation (Winnier *et al.*, 1995). The effects seen on global exposure of early gastrula chick to BMP4 protein (Fig. 6), while generally in keeping with a normal ventralising role for the gene, may not correspond in detail with the equivalent disturbances in either the *Xenopus*/zebrafish or the mammal type of development. Thorough study with gene markers etc. will be required, further to understand them. This raises the possibility that while the early developmental function of such *BMPs* is in a sense conserved in evolution, amniote and anamniote vertebrate developments may deploy this function somewhat differently. The gastrular expression patterns of chick *BMP4* and *BMP7* RNAs, however, are indeed consistent with the idea that dorsalisation involves progressive retreat of the transcription domains of ventralising signals, in some competitive process (Fig. 7). They are particularly striking in relation to demarcation of the neural plate boundary in the epiblast at relatively late stages (see also Schultheiss *et al.*, 1997), but earlier within stage 4, when other work reveals establishment of a region of 'pre-neural' competence in epiblast (e.g., Streit *et al.*, 1997), *BMP4* RNA expression, especially, is only in the course of retreating from this region, rather than marking its outer boundary. Extrapolation from RNA expression patterns to probable protein patterns further suggests that normal interactions between ventralising BMPs, their receptors and putative sequestering proteins may extend well into the period during which interactions between our grafts and surroundings are occurring.

The effectiveness of Follistatin after pre-incubation of host blastoderms as well as of grafts suggests that the effects operate at least partly in the host tissue surround, and not only within graft tissue, but do not prove this. Conversely, Follistatin pre-incubated hosts could act as depots for the protein which then diffuses into grafts, to exert potentiating effects there e.g., by counteracting ventralising ligands. If both experimentally applied proteins simulate the actions of an antagonistic pair that are endogenous to the embryo, then changes in both the signalling capacity of grafts and the effective

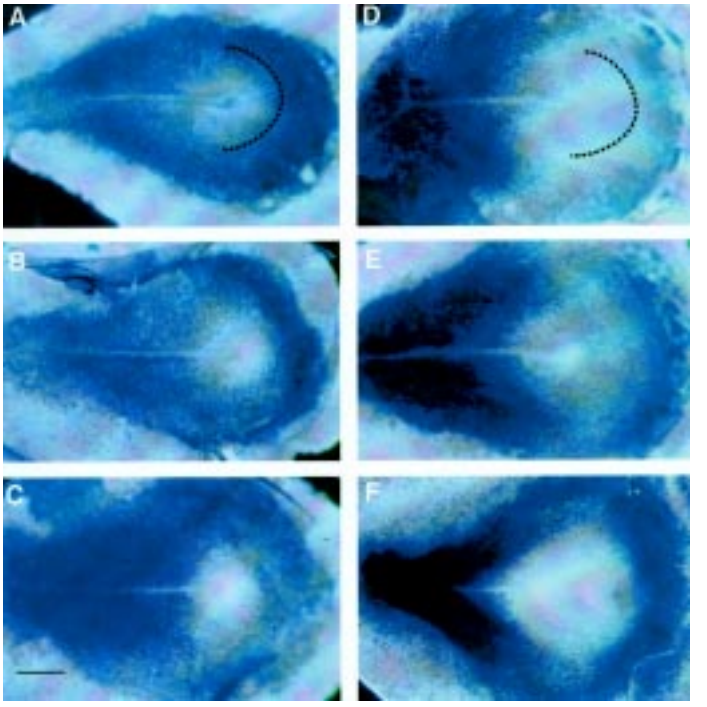

Fig. 7. BMP4 and BMP7 expression at mid-late gastrulation in chick. *Panels* **(A-C** *and* **D-F)** *show dorsal views of whole-mount digoxygenin in situ hybridisations, for BMP4 and BMP7 respectively, in developmental age series from mid- to very late stage 4. Anterior is to right, and the best estimate for the boundary of the more anterior regions of the presumptive neural plate, around Hensen's node, is indicated for mid stage 4 in A and D (dotted line – see also Smith and Schoenwolf, 1997). NB., by late stage 4, BMP7 RNA distribution includes a small anterior midline area, in emerging prechordal mesoderm and endoderm, distinctive to this gene (F). Bar, 1 mm.*

responsiveness of surroundings might be expected from experimental treatment of either component. The endogenous genes might be inter-related such that each protein's presence tends to enhance its own gene's activation and/or repress the other one locally. This could occur via autocatalytic self-activation for *BMP4,* and also cross-negative feedback onto transcription of the antagoniser protein's gene, both triggered by binding of BMP4 to its receptor(s). *In vivo*, some developmental BMP-related ligands are probably not secreted to diffuse freely, but rather associated with cell surface and/or matrix in some way that greatly reduces effective lateral diffusion (Kessler and Melton, 1995). In such a way, the system would have enhanced capacity for creating and sustaining a 'gradient' or 'morphogen landscape' with respect to the interacting genes on the required small spatial scale and over the required time.

Our findings are consistent with what has recently been reported in *Xenopus* following *follistatin* and *BMP4* gene over-expression, as well as interference with BMP4 signalling by dominant-negative receptor and ligand experiments (Dale *et al.*, 1992; Jones *et al.*, 1992; Hemmati-Brivanlou *et al.*, 1994; Fainsod *et al.*, 1995; Hawley *et al.*, 1995; Hemmati-Brivanlou and Thomsen, 1995; Holley *et al.*, 1995; Sasai *et al.*, 1995; Schmidt *et al.*, 1995; Wilson and Hemmati-Brivanlou 1995; DeRobertis and Sasai, 1996; Xu *et al.*, 1996). Thus Follistatin is proposed to induce neural tissue experimentally by interfering with the operation of a paracrine TGFβ superfamily

signalling pathway, for which BMP4 is currently the leading candidate ligand. It is proposed that sufficient operation of this pathway serves to keep ectoderm (equivalent to chick epiblast) from its 'default' pathway of neural differentiation, in order to give rise to epidermis. Cultured disaggregated *Xenopus* ectoderm has a tendency to develop neural differentiation markers, a tendency from which it can be saved by BMP4 protein though not by activin, the ligand originally associated with Follistatin in a functionally antagonistic pair (Nakamura *et al.*, 1991). A current hypothesis is that the endogenous Follistatin-like 'dorsaliser' interferes with BMP4 function by sequestering the protein from interaction with its receptor(s). Whereas there is circumstantial evidence that Follistatin protein acts in sequestering true BMPs in certain parts of its later developmental expression pattern (K. Patel unpublished work), there is now direct evidence for this action by the proteins Chordin and Noggin, two strong candidates for the endogenous initial dorsalisers/ neuralisers (Holley *et al.*, 1996; Piccolo *et al.*, 1996). It has been proposed that this system operates in parallel within ectoderm and mesoderm, with an important aspect of neural induction being, in effect, ectodermal dorsalisation, while mesodermal dorsalisation specifies tissue near the notochord as somitogenic. Endogenous molecules acting like Follistatin may be major dorsalising signals, and such signals may control themselves by autocatalytic effects on tissue, but our results suggest that the whole process is only stably initiated as a downstream response to genetic specification of tissue as 'organiser'. Otherwise any early blastoderm tissue pre-incubated in Follistatin should by itself exert at least some of the effect of a node when subsequently grafted. We were unable to obtain any such effects.

The *follistatin* gene itself has a mouse null-mutant phenotype that is inconsistent with the role of primary dorsaliser (Matzuk *et al.*, 1995), and shows a transcription pattern in the chick gastrula/ neurula that is inappropriate in detail for this role. Chick *follistatin*, though transcribed in the general organiser region at mid-gastrulation, soon down-regulates in this region, which remains inductively active when grafted. The RNA is also sharply bounded and graded from lateral to medial within the neural plate. Such a distribution is consistent with a role where the protein interacts with opposing gene products in controlling more local patterning within the neural plate. We have recently contrasted this transcription pattern with that of another gene *Flik* (Follistatin-like; Patel *et al.*, 1996), cloned by homology with a structurally follistatin-related gene from mouse (Shibanuma *et al.*, 1993). *Flik* shows long-lasting, mid-dorsally centred but widely graded RNA distribution in both epiblast and mesoderm throughout gastrulation, and is specifically focused in the emerging head process, chordamesoderm and (transiently) neural floorplate. It's protein must therefore be considered a further candidate for an endogenous functional equivalent of the experimentally effective Follistatin. Anti-*Flik* interference experiments have further supported the idea that Flick protein is an endogenous antagonist of ventralising signals (Towers *et al.*, 1999). Work with heterologously expressed, soluble Flick protein is underway.

The real possibility exists of parallel function in primary dorsalisation/neural induction by several genes, with at least partial functional redundancy (see Harland, 1995 for review). Thus *noggin* has many credentials for an endogenous dorsaliser/ neuraliser from *Xenopus* work , but we saw no evidence for 'over-expression' effects using active Noggin protein supernatants, in recent chick experiments of both the types reported here with Follistatin (Connolly *et al.,* 1997). Any protein active *in vivo* may require presentation to cells in a special way not simulated by crude incubation, though soluble Noggin is highly active in *Xenopus* dorsalisation and neural induction. A further 'leading candidate', in view of its endogenous transcription pattern and experimental over-expression effects in *Xenopus*, is *chordin* (Sasai *et al.*, 1994; De Robertis and Sasai, 1996). It will be fascinating in future work to explore the degree of redundancy, parallelism or specialisation of function for all these proteins in vertebrate dorso-ventral specification and neural induction.

Materials and Methods

Operations

Fertile egg yolks for ring culture and surgery were handled in Pannett and Compton chick saline. Hensen's nodes were excised with tungsten needles from stage 4 embryos (Hamburger and Hamilton, 1951), as square grafts containing both deep and superficial layers. These were immediately incubated in shallow (1 mm) layers of 1 part Liebovitz air-buffered TCM with glutamine (Gibco): 1 part Hank's BSS with 1/10 mM $CaMg^{++}$ ions: 1 mg/ml BSA (Liebovitz /BSS). An equivalently sized region from the most similar-looking stage of quail embryos was used for lineage-tracing grafts. The node regions from the distal tips of day 7.25 mouse egg cylinders were similarly incubated but in a DMEM/rat serum -based medium adapted for culture of such egg cylinders (Beddington, 1987). Control nodes were incubated for 2 h in plastic dishes at 38.5°C in Liebovitz/BSS alone, while experimental nodes were similarly incubated but with inclusions of either Follistatin protein (a gift of National Hormone and Pituitary Repository, USA), or BMP4 protein (Genetics Institute, Boston, USA, and a gift from J.C. Smith, NIMR). For concentrations, see Results. In other experiments whole chick blastoderms at stages from early 3 (half-length streak) to 4+ were removed from their vitelline membranes, and similarly incubated in control Liebovitz/BSS or with addition of these agents, before being replaced on the membranes in ring culture (New, 1955; Cooke and Isaac, 1998).

For grafting operations, groups of host chick blastoderms in ring cultures were kept at 20-22°C, with enough albumen medium beneath the flat vitelline membranes to act as a cushion, and a very shallow overlying layer of the above TCM: BSS medium. Nodes were implanted beneath or in place of hypoblast or yolky endophyll layer, in the region of the border between the areas pellucida and opaca, opposite or just anterior to the host anterior streak tip. We endeavoured to place the original deep-lying, meso-endodermal face of nodes onto the underside of host epiblast that faced upwards in the culture, thus inverting the layer relationships in graft relative to host (see Fig. 1). Additionally, we orientated the grafted nodes so that their extension movements in later development would be most likely to cause axial structures distinct from those of the host. Immediately after operations the membrane was domed by filling the space beneath with further albumen medium, the overlying space drained of fluid to pin the graft in position, and the culture incubated at 38.5°C in humidified air. 'Albumen medium' is 8 parts thin egg abumen, 1 part Liebovitz TCM, 1 part Pannett and Compton Saline, with 200 µg/ml Gentamycin as antibiotic. We have found these small refinements of the original ring-culturing technique to give optimal development of younger blastoderms, after the delays incurred in groups of grafting operations, incubating off the membrane etc.

In situ hybridisation

Node-grafted specimens were removed from the vitelline membranes after 18-24 h of culture, quickly washed in PBS and fixed in fresh 4% phosphate-buffered paraformaldehyde. Whole-mount *in situ* hybridisation was performed according to Nieto *et al.* (1995). The chick Krox 20 probe was a 250bp PCR fragment; the chick Hox B1 probe was a 2.0 Kb fragment, size-reduced to 500bp average by alkaline hydrolysis; the chick Sonic hedgehog probe was a 1.5 Kb fragment and the mouse Brachyury probe was a 300bp fragment. Whole-mount *in situ* specimens for sectioning were

postfixed, after washing and photography, for 30 min in 1% glutaraldehyde, 2% buffered formalin. They were then embedded in the third change of 58°C fibrewax, after dehydration in two quick changes each of absolute methanol and absolute isopropanol and clearing in tetrahydronaphthalene (20 min). Specimens were orientated to cut the second axial pattern as nearly transversely as possible, and sectioned at 8 microns. Slides were dewaxed in Histoclear for 5 min only at room temperature, re-hydrated to 70% ethanol and counterstained for 45 sec with alcoholic eosin, then dehydrated again finishing with 30 min in absolute ethanol. Individual slides were cleared for 30 sec only in Histoclear (to conserve *in situ* signal) before mounting in XAM. Viewing was by brightfield with closing of the condenser diaphragm, to optimise signal color against visible structure.

Chick-quail lineage labelling

Chick blastoderms, cultured after grafting of quail rather than chick nodes, were washed and fixed for 1 h at room temperature in Carnoy's fluid (alcohol/acetic acid/formalin), then transferred to 4% phosphate buffered formalin on a rocker in the cold overnight. They were then washed in PBS for 4 h and dehydrated through an alcohol series (10 min each step), transiting from ethanol to butanol via mixtures at the 80% stage and finishing with 30 min in absolute butanol. After clearing in toluene (10 min) they were embedded in a third change of wax and sectioned as above. Slides were cleared rapidly in toluene and rehydrated in an ethanol series (5 min per step) to PBS for 20 min. Immunocytochemistry was performed in PBT (PBS including 1 mM Ca Mg++, 0.05% Tween 20 and 1 mg/ml BSA. Slides were incubated at room temperature in a wet chamber, successively in 1) 5% normal goat serum in PBT, 30 min; 2) a 1:2 dilution of QCPN quail-specific monoclonal supernatant in PBT (Developmental Studies Hybridoma Bank, Bethesda, MD 20892, USA), 2 h; 3) four changes of PBT wash with stirring over 1 h; 4) 1:400 HRP-conjugated affinity-purified goat anti-mouse Ig (H+L chain), 2 h followed by 4 changes of PBT with stirring over 1 h; 0.25 mg/ml Di-amino Benzidine in PBS, freshly thawed, 10 min, and the same DAB solution, re-added immediately after mixing in 0.05% H_2O_2. Reaction at the site of quail cells was monitored under the dissecting microscope, and the reaction stopped by replacement with excess PBS before background colour developed (2-5 min); distilled water, 5 min; Carazzi Haematoxylin (a progressively staining Haematoxylin) 2 min; distilled water 2 min; running tapwater, with final addition of a few drops per litre of ammonia solution to ensure alkaline pH. for at least 5 min. Rapid dehydration was through a complete ethanol series, followed by clearing in Histoclear and mounting in XAM.

Acknowledgments

Follistatin protein was kindly supplied by the National Hormone and Pituitary Repository, and BMP4 protein by Genetics Institute, Boston, USA. QCPN quail-specific monoclonal supernatant was supplied by Developmental Studies Hybridoma Bank, Bethesda, MD 20892, USA. We thank Jane Dodd for the Shh probe, Vicky Prince and Andrew Lumsden for the Chox B1 probe, Philippa Frances-West for the BMP4 probe, Angela Nieto for the cKrox 20 probe and Dave Wilkinson for the mouse Brachyury probe.

References

ALVAREZ, I.S. and SCHOENWOLF, G.C. (1991). Patterns of neuroepithelial rearrangement during avian neurulation are established independently of notochordal inductive interactions. *Dev. Biol. 143:* 78-92.

BEDDINGTON, R.S.V. (1987). A Practical Approach. In *Mammalian Development* (Ed. M. Mark). IRL Press.

BIEHS, B., FRANCOIS, V. and BIER, E. (1996). The *Drosophila* short gastrulation gene prevents Dpp from autoactivating and suppressing neurogenesis in the neurectoderm. *Genes Dev. 10:* 2922-2934.

CONNOLLY, D.J., PATEL, K. and COOKE, J. (1997). Chick noggin is expressed in the organiser and neural plate during axial development, but offers no evidence of early essential functions. *Int. J. Dev. Biol. 41:* 389-396.

CONNOLLY, D.J., PATEL, K., SELEIRO, E.A., WILKINSON, D.G. and COOKE, J. (1995). Cloning, sequencing, and expressional analysis of the chick homologue of follistatin. *Dev. Genet. 17:* 65-77.

COOKE, J. and ISAAC, A. (1998). Gene interference using Antisense oligonucleotides in the culture whole chick embryo. In *Molecular Embryology; Methods and Protocols.* (Eds. C. Sharpe and I. Mason). Humana Press, New Jersey.

DALE, L., HOWES, G. and PRICE, B.M.J. (1992). BMP-4-a ventralising factor in early *Xenopus* development. *Development 115:* 573-585.

DE ROBERTIS, E.M. and SASAI, Y. (1996). A common plan for dorso-ventral patterning in bilateria. *Nature 380:* 37-41.

DESCHAMPS, J. and WIJGERDE, M. (1993). Two phases in the establishment of HOX expression domains. *Dev. Biol. 156:* 473-480.

DONIACH, T. (1993). Planar and vertical induction of anteroposterior pattern during the development of the amphibian central nervous system. *J. Neurobiol. 24:* 1256-1275.

DONIACH, T., PHILLIPS, C.R. and GERHART, J.C. (1992). Planar induction of anteroposterior pattern in the developing central nervous system of *Xenopus laevis*. *Science 257:* 542-545.

DURSTON, A.J., TIMMERMANS, J.P.M., HAGE, W.J., HENDRICKS, H.F.J., DE VRIES, N.J., HEIDEVELD, M. and NIEUWKOOP, P.D. (1989). Retinoic acid causes ananteroposterior transformation in the developing central nervous system. *Nature 340:* 140-147.

EYAL-GILADI, H. (1984). The gradual establishment of cell commitments during early stages of chick development. *Cell Differ. 14:* 245-255.

FAINSOD, A., STEINBEISSER, H. and DE ROBERTIS, E. (1995). On the function of BMP-4 in patterning the marginal zone of the Xenop*us* embryo. *EMBO J. 13:* 5015-5025.

FRANCOIS, V. and BIER, E. (1995). *Xenopus* chordin and *Drosophila* short gastrulation genes encode homologous proteins functioning in dorsal-ventral axis formation. *Cell 80:* 19-20.

GARCIA-MARTINEZ, V., ALVAREZ, I.S. and SCHOENWOLF, G.C. (1993). Locations of the ectodermal and non-ectodermal subdivisions of the epiblast at stages 3 and 4 of avian gastrulation and neurulation. *J. Exp. Zool. 267:* 431-446.

HAMBURGER, V. (1988). *The Heritage of Experimental Embryology.* Oxford University Press. Oxford-New York.

HAMBURGER, V. and HAMILTON, H.L. (1951). A series of normal stages in the development of the chick. *J. Morphol. 88:* 49-92.

HAMMERSCHMIDT, M., SERBEDZIJA, G.N. and MCMAHON, A. (1996). Genetic analysis of dorso-ventral pattern formation in the zebrafish: requirement of a BMP-like ventralising activity and its dorsal repressor. *Genes Dev. 10:* 2452-2461.

HARLAND, R.M. (1995). Neural induction in *Xenopus*. *Curr. Opin. Genet. Dev. 4:* 544-549.

HAWLEY, S.H.B., WUNNENBERG-STAPLETON, K., HASHIMOTO, C., LAURENT, M.N., WATANABE, T. BLUMBERG, B.W. and CHO, K.W.Y. (1995). Disruption of BMP signalling in embryonic Xeno*pus* ectoderm leads to direct neural induction. *Genes Dev. 9:* 2923-2935.

HEMMATI-BRIVANLOU, A. and THOMSEN, G.H. (1995). Ventral mesoderm patterning in Xenopus embryos: Expression patterns and activities of BMP-2 and BMP-4. *Dev. Genet. 17:* 78-89.

HEMMATI-BRIVANLOU, A., KELLY, O.G. and MELTON, D.A. (1994). Follistatin, an antagonist of activin, is expressed in the Spemann organiser and displays direct neuralising activity. *Cell 77:* 283-295.

HOLLEY, S.A., JACKSON, P.D., SASAI, Y., LU, B., DE ROBERTIS, E.M., HOFFMAN, F.M. and FERGUSON, E.L. (1995). A conserved system for dorso-ventral patterning in insects and vertebrates involving *sog* and *chordin*. *Nature 376:* 249-253.

HOLLEY, S.A., NEUL, J.L., ATTISANO, L., WRANA, J.L., SASAI, Y., O'CONNOR, M.B., DE ROBERTIS, E.M. and FERGUSON, E.L. (1996). The *Xenopus* dorsalising factor noggin ventralises *Drosophila* embryos by preventing DPP from activating its receptor. *Cell 86:* 607-617.

INAGAKI, T. and SCHOENWOLF, G.C. (1993). Axis development in avian embryos: the ability of Hensen's node to self-differentiate, as analysed with heterochronic grafting experiments. *Anat. Embryol. 188:* 1-11.

JONES, C.M., LYONS, K.M., LAPAN, P.M., WRIGHT, C.V.E. and HOGAN, B.L.M.

(1992). DVR-4 (Bone Morphogenic Protein 4) as a posterior and ventralising factor in *Xenopus* mesoderm induction. *Development 115:* 639-647.

KAPPEN, C. and RUDDLE, F.H. (1993). Evolution of a regulatory gene family: Hom Hox genes. *Curr. Opin. Genet. Dev. 3:* 931-938.

KESSEL, M. (1992). Respecification of vertebral identities by retinoic acid. *Development 115:* 487-501.

KESSLER, D.S. and MELTON, D.A. (1995). Induction of dorsal mesoderm by soluble, mature Vg1 protein. *Development 121:* 2155-2164.

LAMB, T.M., KNECHT, A.K., SMITH, W.C., STACHEL, S.E., ECONOMIDES, A.A., STAHL, N., YANCOPOULOS, G.D. and HARLAND, R.M. (1993). Neural induction by the secreted polypeptide Noggin. *Science 262:* 713-718.

MATZUK, M.M., LU, N., VOGEL, H., SELLHEYER, K., ROOP, R. and BRADLEY, A. (1995). Multiple defects and perinatal death in mice deficient in follistatin. *Nature 374:* 360-363.

NAKAMURA, T., SUGINO, K., TITANI, K. and SUGINO, H. (1991). Follistatin, an activin-binding protein, associates with heparan sulphate chains of proteoglycans of follicular granulosa cells. *J. Biol. Chem. 266:* 19432-19437.

NEW, D.A.T. (1955). A new technique for the cultivation of the chick embryo *in vitro*. *J. Embryol. Exp. Morphol. 3:* 326-331.

NIETO, M.A., PATEL, K. and WILKINSON, D.G. (1995). In situ hybridisation of chick embryos in whole-mount and tissue sections. In *Methods in Avian Embryology*. (Ed. M. Bronner-Fraser), San Diego, Academic Press.

PATEL, K., CONNOLLY, D.J., AMTHOR, H., NOSE, K. and COOKE, J. (1996) Cloning and early dorsal-axial expression of Flik, a chick follistatin-related gene; evidence for involvement in dorsalisation/neural induction. *Dev. Biol. 178:* 327-342.

PICCOLO, S., SASAI, Y., LU, B. and DE ROBERTIS, E.M. (1996). Dorso-ventral patterning in *Xenopus*: Inhibition of ventral signalling by direct binding of chordin to BMP4. *Cell 86:* 589-598.

RUIZ I ALTABA, A. (1994). Pattern formation in the vertebrate neural plate. *Trends Neurosci. 17:* 233-243.

SASAI, Y., LU, B., STEINBEISSER, H. and DE ROBERTIS, E.M. (1995). Regulation of neural induction by Chd and BMP4 antagonistic patterning signals in *Xenopus*. *Nature 376:* 333-336.

SASAI, Y., LU, B., STEINBEISSER, H., GEISSERT, D., GONT, L.K. and DE ROBERTIS, E.M. (1994). *Xenopus* chordin: a novel dorsalising factor activated by organiser-specific homeobox genes. *Cell 79:* 779-790.

SCHMIDT, J.E., SUSUKI, A., UENO, N. and KIMELMAN, D. (1995). Localised BMP-4 mediates dorsal/ventral patterning in the early *Xenopus* embryo. *Dev. Biol. 169:* 37-50.

SCHULTHEISS, T.M., BURCH, B.E. and LASSAR, A.B. (1997). A role for bone morphogenetic proteins in the induction of cardiac myogenesis. *Genes Dev. 11:* 451-462.

SELLECK, M.A. and STERN, C.D. (1991). Fate mapping and cell lineage analysis of Hensen's node in the chick embryo. *Development 112:* 615-626.

SHARPE, C.R. (1991). Retinoic acid can mimic endogenous signals involved in transformation of the *Xenopus* nervous system. *Neuron 7:* 239-247.

SHIBANUMA, M., MASHIMO, J., MITA, A., KUROKI, T. and NOSE, K. (1993). Cloning from a mouse osteoblastic cell line of a set of a set of transforming-growth-factor B1-regulated genes, one of which seems to encode a follistatin-related polypeptide. *Eur. J. Biochem. 217:* 13-19.

SIMEONE, A., ACAMPORA, D., GULISANO, M., STORNAIUOLO, A. and BONCINELLI, E. (1992a). Nested expression domains of four homeobox genes in developing rostral forebrain. *Nature 358:* 687-690.

SIMEONE, A., GULISANO, M., ACAMPORA, D., STORNAIUOLO, A., RAMBALDI, M. and BONCINELLI, E. (1992b). Two vertebrate homeobox genes related to the *Drosophila* empty spiracles gene are expressed in the embryonic cerebral cortex. *EMBO-J. 11:* 2541-2550.

SMITH, J.L. and SCHOENWOLF, G.C. (1997). Neurulation: coming to closure. *Trends Genet. 20:* 510-517.

SMITH, W.C., KNECHT, A.K., WU, M. and HARLAND, R.M. (1993). Secreted noggin protein mimics the Spemann organiser in dorsalising Xenopus mesoderm. *Nature 361:* 547-549.

STERN, C.D. and IRELAND, G.W. (1981). An integrated experimental study of endoderm formation in avian embryos. *Anat. Embryol. 163:* 245-263.

STERN, C.D., HATADA, Y., SELLECK, M.A. and STOREY, K.G. (1992). Relationships between mesoderm induction and the embryonic axes in chick and frog embryos. *Development (Suppl):* 151-156.

STOREY, K.G., CROSSLEY, J.M., DEROBERTIS, E.M., NORRIS, W.E. and STERN, C.D. (1992). Neural induction and regionalisation in the chick embryo. *Development 114:* 729-741.

STOREY, K.G., SELLECK, M.A.J. and STERN, C.D. (1995). Neural induction and regionalisation by different subpopulations of cells in Hensen's node. *Development 121:* 417-427.

STREIT, A., SOCKANATHAN, S. PEREZ, L., REX, M., SCOTTING, P.J., SHARPE, P.T., LOVELL-BADGE, R. and STERN, C.D. (1997). Prolonging the period of competence for neural induction: HGF/SF,L5 and Sox 2. *Development 124:* 1191-1202.

TOWERS, P., PATEL, K., WITHINGTON, S., ISAAC, A. and COOKE, J. (1999). Flick, a chick follistatin-related gene, functions in gastrula dorsalisation/neural induction and in subsequent maintenance of midline sonic hedgehog signalling. *Dev. Biol. 214:* 298-317.

WILSON, P.A. and HEMMATI-BRIVANLOU, A. (1995). Induction of epidermis and inhibition of neural fate by BMP-4. *Nature 376:* 331-333.

WINNIER, G., BLESSING, M., LABOWSKY, P.A. and HOGAN, B.L.M. (1995). BMP4 is required for mesoderm formation and patterning in the mouse. *Genes Dev. 9:* 2105-2116.

XU, R.H., KIM, J., ZHAN, S., SREDNI, D. and KUNG, H.F. (1996). A dominant negative BMP-4 receptor causes neuralisation in Xenopus ectoderm. *Biochem. Biophys. Res. Commun. 212:* 212-219.