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

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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 dorsalisied 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: dorsalisisation, 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 indispensible 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 (Bienh 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.

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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-qual 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 Hensen's 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 2E-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 darsalisation 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 darsalisation 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 neuraxises 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.

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-
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-
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, overlaying 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 HoxB1, 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 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 explanation 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.
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 Schulteis 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 neuronalisation 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-
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* or zebrafish or the mammalian 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 counteringact 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 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 antagonist 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 somiticogenic. 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 areas pellucida and opaca, opposite or just anterior to the host anterior streak tip. We endeavoured to place the original deep-lying, mesodermal node onto the underside of host epiblast that faced the streak tip. We endeavoured to place the original deep-lying, mesendodermal 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 the 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, 1996).

For grafting operations, groups of host chick blastoderm 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 yolk endoderm 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, mesendodermal 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 albumen, 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 blastoderm, 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 tetrahydroanaphthacline (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 bath 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-amine Benzidine in PBS, freshly thawed, 10 min, and the same DAB solution, re-added immediately after mixing in 0.05% H2O2. 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.

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