

Mesoderm patterning and somite formation during node regression: differential effects of chordin and noggin

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Abstract

In *Xenopus*, one of the properties defining Spemann's organizer is its ability to dorsalise the mesoderm. When placed adjacent to prospective lateral/ventral mesoderm (blood, mesenchyme), the organizer causes these cells to adopt a more axial/dorsal fate (muscle). It seems likely that a similar property patterns the primitive streak of higher vertebrate embryos, but this has not yet been demonstrated clearly. Using quail/chick chimaeras and a panel of molecular markers, we show that Hensen's node (the amniote organizer) can induce posterior primitive streak (prospective lateral plate) to form somites (but not notochord) at the early neurula stage. We tested two BMP antagonists, noggin and chordin (both of which are expressed in the organizer), for their ability to generate somites and intermediate mesoderm from posterior streak, and find that noggin, but not chordin, can do this. Conversely, earlier in development, chordin can induce an ectopic primitive streak much more effectively than noggin, while neither BMP antagonist can induce neural tissue from extraembryonic epiblast. Neurulation is accompanied by regression of the node, which brings the prospective somite territory into a region expressing BMP-2, -4 and -7. One function of noggin at this stage may be to protect the prospective somite cells from the inhibitory action of BMPs. Our results suggest that the two BMP antagonists, noggin and chordin, may serve different functions during early stages of amniote development. © 1999 Elsevier Science Ireland Ltd. All rights reserved.

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1. Introduction

Gastrulation in amniote embryos involves the formation of a slit-like structure, the primitive streak, which acts as a passageway for cells that will contribute to mesoderm and endoderm, and which elongates to its maximum length as the head process (the cranial portion of the notochord) emerges from Hensen's node (the cranial tip of the streak) (reviewed by Bellairs, 1986). Almost at the same time, all three germ layers at the rostral end of the head process begin to fold ventrally to form the head fold (defining stage 6 of Hamburger and Hamilton, 1951) and the streak starts to shorten, which is accompanied by caudal migration of Hensen's node ('regression') (Spratt, 1947; Spratt and Codon, 1947; Vakaet, 1970; Stern and Bellairs, 1984). Eventually, the primitive streak and node become condensed to form a mass of cells in the tail bud of the 3–4 day embryo (see Catala et al., 1995, 1996; Knezevic et al., 1998).

Throughout gastrulation, precursors of the most dorsal, or axial, mesoderm reside near the anterior (cranial) tip of the streak, and the most lateral/ventral (including extraembryonic) prospective tissues reside posteriorly (Spratt and Codon, 1947; Spratt, 1955, 1957; Nicolet, 1965, 1967, 1970a, 1971; Rosenquist, 1966, 1970, 1983; Schoenwolf et al., 1992; Psychoyos and Stern, 1996). As the head process starts to emerge at stages 4⁺–5, this fate map begins to become more compressed: precursors of axial and paraxial mesoderm move anteriorly within the streak. After this time no notochord precursors can be found caudal to Hensen's node and the somite precursors are confined to the most anterior third of the streak (Psychoyos and Stern, 1996).

In amphibians, mesoderm patterning appears to be controlled by graded levels of several secreted factors. Activin/Vg-1 like members of the TGF β superfamily can induce mesoderm from ectodermal explants, highest concentrations giving rise to notochord and somites (muscle) and lower concentrations to muscle and ventral mesoderm (Green and Smith, 1990). Factors belonging to the bone morphogenetic protein (BMP) subgroup of the TGF β superfamily have a different activity: they promote ventral mesoderm

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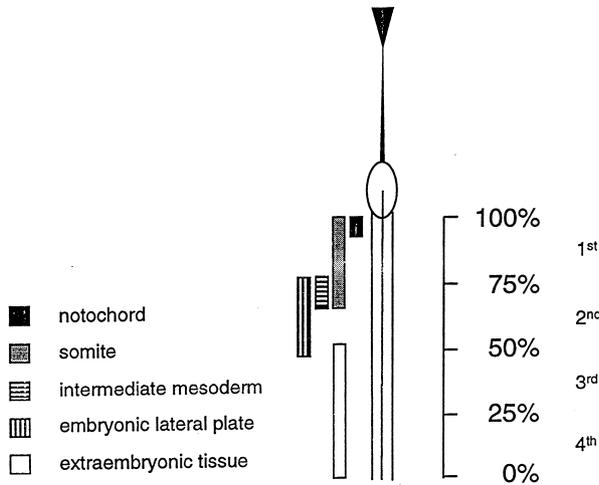


Fig. 1. Diagram summarising the regions of primitive streak used for grafting. The diagram shows a stage 5 primitive streak, the head process and triangular prechordal mesoderm. On the left, the fates of mesoderm derived from different positions of the axis of the streak are shown as vertical bars (based on the fate maps of Schoenwolf et al., 1992; Psychoyos and Stern, 1996; Rosenquist, 1966). On the right, the scale indicates the position and nomenclature for the four pieces (1st, 2nd quarters, etc.) of the streak that were used for grafting.

(mesenchyme, mesothelium, blood) at the expense of dorsal cell types (Dale et al., 1992; Jones et al., 1992; Fainsod et al., 1994; Hemmati-Brivanlou and Thomsen, 1995; Harland and Gerhart, 1997). Other factors can cooperate with these. For example, Wnt-related signals have been shown to act synergistically with BMPs (e.g. Hoppler and Moon, 1998; Phillips et al., 1999) and with Vg1/activin (e.g. Sokol and Melton, 1992; Watabe et al., 1995; Cui et al., 1996; Crease et al., 1998). It has been widely believed that mesoderm induction and its subdivision into dorsal and ventral derivatives occurs mostly before gastrulation in amphibians, largely because animal cap explants lose their responsiveness to activin just before the first visible signs of gastrulation (see Harland and Gerhart, 1997 for discussion). However, in the chick, activin responsiveness continues at least into the mid-primitive streak stage, when different concentrations of activin applied to extraembryonic epiblast can still generate different types of mesoderm (Stern et al., 1995).

During early neurulation, *BMP-2*, *-4* and *-7* are expressed in a domain that overlaps the posterior part of the primitive streak (Watanabe and Le Douarin, 1996; Schultheiss et al., 1997; Tonegawa et al., 1997; Streit et al., 1998), perhaps accounting for why this region gives rise only to ventral and extraembryonic mesoderm. However, node regression brings the prospective somite territory to lie within this putative 'somite-inhibiting' domain (Pourquié et al., 1996; Tonegawa et al., 1997; Andréé et al., 1998). Since the node expresses several BMP inhibitors (Connolly et al., 1995; 1997; Levin, 1998; Streit et al., 1998; Tonegawa and Takahashi, 1998; Streit and Stern, 1999), it is conceivable that the backwards migration of the node serves a protective

function upon the prospective somite territory as the latter gradually becomes surrounded by BMPs. Several classical observations appear to support this hypothesis: reversal of short anterior pieces of the primitive streak leads to restoration of the normal dorsoventral pattern (Waddington, 1932; Abercrombie and Waddington, 1937; Abercrombie, 1950; Abercrombie and Bellairs, 1954; Spratt, 1955; Bellairs, 1963). Placing prospective notochord (including Hensen's node in some of the experiments) adjacent to posterior primitive streak allows the posterior streak to give rise to somites, despite its normal lateral/extraembryonic fate (Nicolet, 1970b). Other experiments suggest that Hensen's node (Hornbruch et al., 1979) and noggin (Tonegawa and Takahashi, 1998) can generate somites from lateral plate cells that have already emerged from the primitive streak, but it is unclear whether the node also contributes to pattern the streak itself.

Here we investigate whether BMP and its inhibition by chordin or noggin can modulate the ability of the primitive streak to give rise to axial structures at early neurulation (head process) stages. We first confirm, using molecular markers, the observation of Nicolet (1970b) that Hensen's node can 'dorsalise' posterior primitive streak explants, changing their fate from lateral/extraembryonic to somite mesoderm. *BMP4* can 'ventralise' the node at this stage, inducing lateral mesoderm from a region fated to give rise to axial mesoderm. Surprisingly, we find that two of the BMP inhibitors expressed by the node and its derivatives at this stage differ in their ability to induce somite formation in posterior primitive streak: chordin is unable to induce, while noggin is a strong inducer. By contrast, chordin can induce a primitive streak (including the organiser) if misexpressed at or before early gastrulation stages, while noggin does this much less efficiently. Our results suggest that the expression of noggin may serve to ensure the continued production of somites during node regression, and that dorsoventral mesoderm patterning is still taking place after gastrulation.

2. Results

2.1. Hensen's node 'dorsalises' the posterior primitive streak

To investigate whether signals from Hensen's node can pattern the primitive streak, we combined a chick stage 5 node with the posterior 1/3 of the primitive streak from a stage 5 quail embryo (which normally only gives rise to extraembryonic mesoderm; Psychoyos and Stern 1996 and Fig. 1) and grafted them together into the area opaca of a stage 5-6 chick host embryo (which can no longer respond to grafts of Hensen's node; Streit et al., 1997). We assayed for the expression of mesodermal markers (*HNF3 β* and *chordin* for axial mesoderm, *paraxis* for somites, *Sim1* for lateral somite and intermediate mesoderm, *Pax2* for inter-

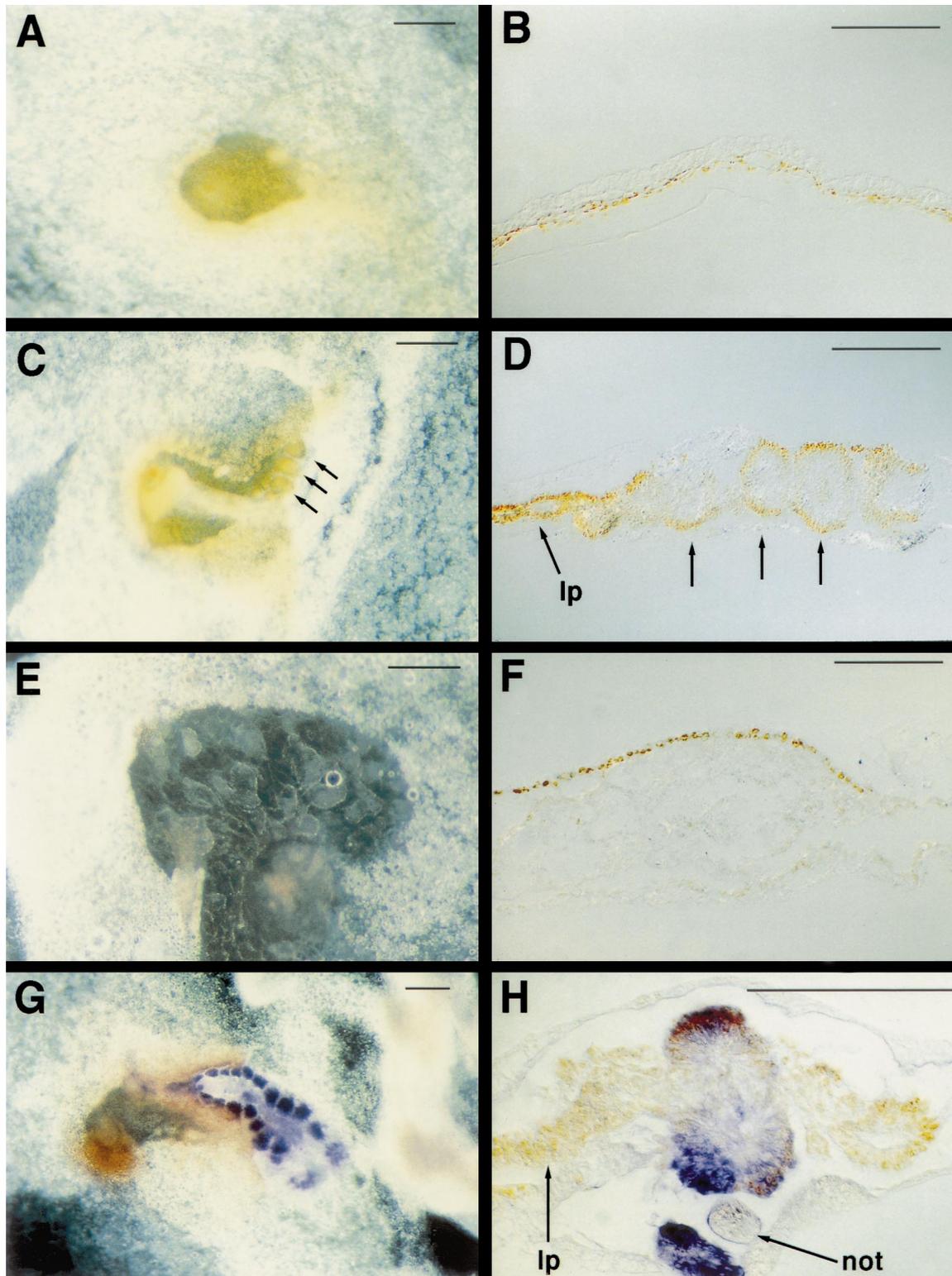


Fig. 2. Hensen's node dorsalis ventral mesoderm. A quail stage 5 posterior primitive streak was explanted into the area opaca of a chick host either alone (A,B,E,F) or together with a chick node of the same stage (C,D,G,H). After 24 h culture, the embryos were processed for whole mount immunocytochemistry with quail specific antibody QCPN (A–D) or with QCPN in combination with in situ hybridisation with *paraxis* (E–H). Quail staining appears in brown; *paraxis* expression in blue. Posterior streak grafts alone spread as a sheet and form blood island-like structures (A,B,E,F), which do not express *paraxis* (E,F). In the presence of the node, posterior primitive streak cells form somites (C,D; arrows) which express *paraxis* (G,H) and lateral plate mesoderm (lp in D and H), but do not contribute to notochord (not in H). Scale bars, (A–H) 200 μ m.

mediate mesoderm, *Tbx6L* for primitive streak mesoderm

and *BMP4* for lateral plate) in quail cells by combining in

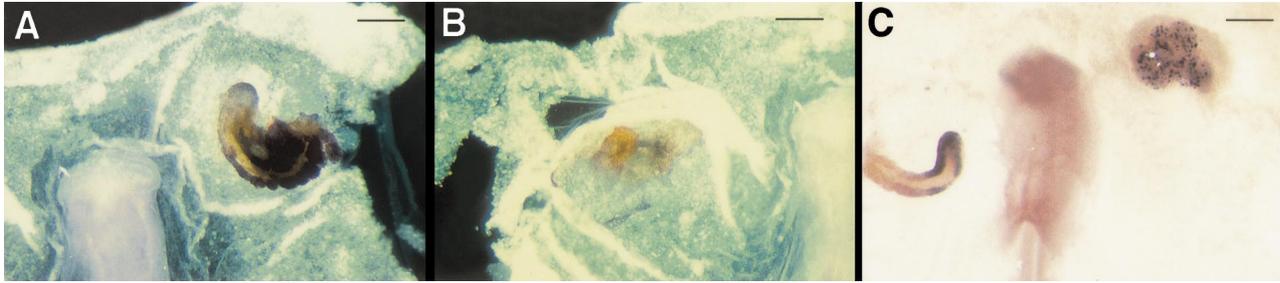


Fig. 3. BMP-4 ventralises node-derived axial mesoderm. A quail Hensen's node was transplanted into the area opaca in the presence of mock- (A; left in C) or BMP-4 transfected (B; right in C) COS-cells. In the control, the node elongates, expresses *Tbx6L* (blue) after 8 h culture (left in C) and differentiates into somites expressing *paraxis* (blue) and notochord after 24 h (A). In the presence of BMP-4, the node remains as a clump and does not express *Tbx6L* after 8 h (right in C; note: dotted blue staining results from trapping in transfected cells); after 24 h the node tissue has not formed somites and is *paraxis* negative (B). Quail tissue was visualised using QCPN antibody and appears in brown. Scale bars, (A–C) 200 μ m.

situ hybridisation for these markers with immunohistochemistry with antibody QCPN, which recognises a perinuclear antigen in all quail cells.

When quail posterior primitive streak is explanted alone into the area opaca of a chick host and cultured for 24 h, it does not express any of the above markers except in a few cases when it retains *BMP4* expression. The quail cells spread as a sheet in the area opaca of the host, and often form blood islands (Fig. 2A,B,E,F). Therefore these explants develop according to their fate in the normal embryo. In contrast, when explanted together with a chick stage 5 node, the quail primitive streak forms somite-like structures that express *paraxis* (14/15; Fig. 2C,D,G,H). In histological sections, about half of these also show a contribution of quail cells to lateral plate that extends laterally from the ectopic somites (Fig. 2D,H). Although these combinations do form a notochord (which expresses *chordin*, not shown), no quail cells are seen in the notochord (Fig. 2H).

These results confirm the finding of Nicolet (1970b) that Hensen's node can alter the fate of posterior primitive streak from extraembryonic mesoderm/blood islands to somites. However, the node-derived signals do not induce adjacent primitive streak cells to become notochord.

2.2. *BMP4* 'ventralises' Hensen's node

The posterior part of the primitive streak at stage 4-6 lies within a region that expresses *BMP2*, *-4* and *-7* (Watanabe and Le Douarin, 1996; Schultheiss et al., 1997; Streit et al., 1998), while Hensen's node, at its anterior end, expresses the BMP inhibitors *chordin* (Streit et al., 1998), *noggin* (Connolly et al., 1997; Streit and Stern, 1999) and *follistatin* (Connolly et al., 1995; Levin 1998). Since BMPs have been shown to have ventralising activity in *Xenopus* (Dale et al., 1992; Fainsod et al., 1994; Jones et al., 1992, 1996; Suzuki et al., 1994; Steinbeisser et al., 1995), these patterns suggest that BMPs around the posterior primitive streak may function to prevent it from giving rise to dorsal/axial mesoderm (notochord, somites, intermediate mesoderm). To test whether *BMP4* can ventralise primitive streak/node meso-

derm, we explanted a quail stage 5 Hensen's node (which contains cells fated to form notochord and somites; Psychoyos and Stern, 1996) together with a pellet of either *BMP4*- or mock-transfected COS cells into the area opaca of a stage 5-6 chick host.

After 24 h of incubation, the nodes grafted together with control cells expressed the axial mesoderm marker *HNF3 β* (4/5) and the somite marker *paraxis* (5/6) and formed a notochord and somites (Fig. 3A). In contrast, nodes grafted with *BMP4*-transfected cells only expressed *HNF3 β* in 1/5 and *paraxis* in 1/7 cases, and the node cells spread as a sheet and developed into blood island-like structures (Fig. 3B). We also examined the expression of the primitive streak mesoderm marker *Tbx6L* in the same experiment after 8 h of incubation (Fig. 3C). When nodes were explanted with control cells, *Tbx6L* expression was seen in a strong patch surrounding one side of the node graft, which had formed an elongated structure (5/5; Fig. 3C, left). With *BMP4*-transfected cells, *Tbx6L* expression was greatly reduced, the grafts were never elongated and remained as a clump (Fig. 3C, right), or had spread to form blood island-like structures ($n = 4$).

These results show that *BMP4* has 'ventralising' activity on chick primitive streak mesoderm: it causes the organiser region to change its fate from axial mesoderm (notochord and somites) to lateral/extraembryonic mesoderm, even at the 'early neurula' (head process) stage.

2.3. *Noggin*, but not *chordin*, mimics 'dorsalisation' of the primitive streak by Hensen's node

If the posterior primitive streak is normally repressed from contributing to axial mesoderm by the action of *BMP4*, then inhibition of this activity by *chordin* or *noggin* should be sufficient to obtain more dorsal structures from posterior primitive streak explants. To test this, we explanted the posterior third of a quail primitive streak together with *chordin* or *noggin* secreting cells into the area opaca of stage 5-7 chick host embryos.

When posterior streak was transplanted with mock-transfected COS cells, no expression of the somite marker *para-*

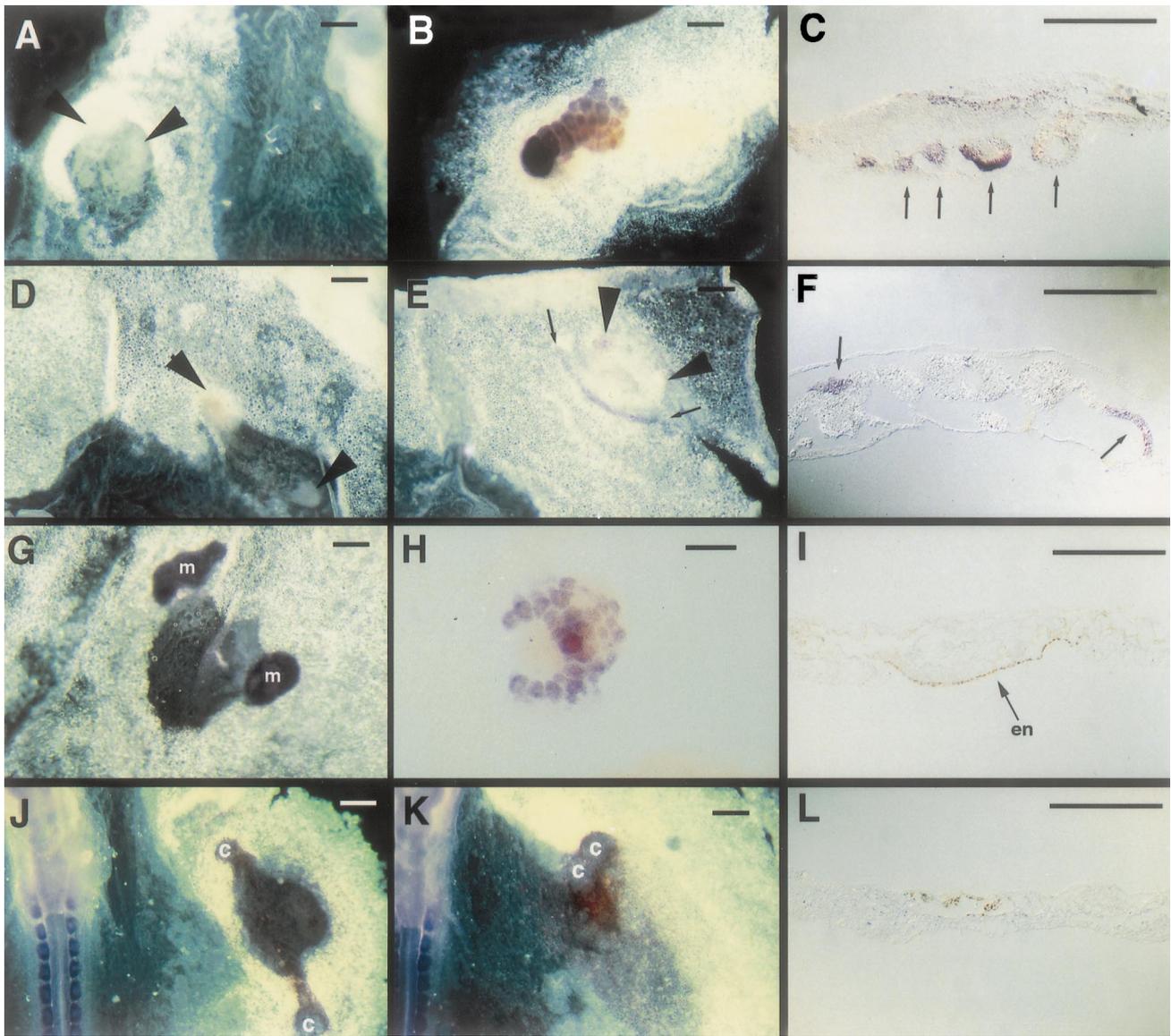


Fig. 4. Noggin, but not chordin, dorsalises primitive streak mesoderm. When noggin-secreting CHO cells (B,C,E,F) or *noggin*-transfected COS cells (H) are grafted with a quail posterior primitive streak explant, the quail tissue forms somites that express *paraxis* (B,C (arrows),H) and intermediate mesoderm that expresses *Pax2* (arrows in E and F; arrowheads in E indicate location of cells). When quail posterior streak is explanted with chordin-transfected COS cells (c in J, K), the explant becomes condensed (K) or forms a structure like beads on a string (J). No *paraxis* expression is observed (J,K). Sections (I,L) show that quail cells exposed to chordin contribute to endoderm (en in I) and mesenchyme. In the presence of control CHO cells (arrowheads in A and D) or mock-transfected COS cells (m in G), neither *paraxis* (A,G) nor *Pax2* (D) is expressed, and the graft spreads and forms blood island-like structures (G). Quail staining is visualised brown and *paraxis* or *Pax2* expression in blue. Scale bar, (A–L) 200 μ m.

axis (0/12; Fig. 4A) or the notochord marker *cNot1* (0/8) was seen after 24 h. Similar results were obtained with posterior streak explanted with chordin-transfected COS cells (2–3 pellets of 1000 cells each): the explants did not express *paraxis* (0/12; Fig. 4J,L), *cNot1* (0/8) or the lateral somite/intermediate mesoderm marker *Sim1* (0/7), but almost half (6/15) showed weak expression of *BMP4*, which at this stage can be considered a marker of lateral plate mesoderm (Tonegawa et al., 1997). However, about 50% of the quail explants had a more condensed appearance when co-transplanted with chordin cells (Fig. 4J,K) than with control cells (Fig. 4G). In histological section, the quail cells appeared to

have given rise to endoderm and mesenchyme (Fig. 4I). We also tested whether chordin can induce more anterior primitive streak explants (second quarter or first quarter excluding the node; see Fig. 1) to express the node/notochord marker *HNF3 β* no expression was seen (0/23).

When this experiment was done with noggin-secreting cells, different results were obtained. *Paraxis* expression (Fig. 4A–C) was seen when the posterior quarter (7/7), third quarter (16/17) or second quarter (8/8) of the primitive streak was explanted with noggin-secreting cells, but not with control CHO cells (0/12). *Paraxis* expression was always associated with somite-like structures, which were

derived entirely from the quail explant (Fig. 4B,C). These ectopic somites were of similar morphology and size as the somites in the host axis. Noggin also sometimes induced expression of *Pax2* from the third (7/21) and posterior (7/21) quarters of the primitive streak (Fig. 4E,F). The intermediate mesoderm expressing *Pax2* always lay just lateral to the ectopic somites (Fig. 4F). None of the primitive streak explants with noggin produced notochord, assessed by the expression of *HNF3 β* after either 10 or 24 h culture ($n = 29$).

To confirm that the different effects of chordin and noggin are not due to some peculiarity of the different type of secreting cells or to the species of origin of the factor, we repeated this experiment using COS cells (1–3 pellets of 1000 cells each) transiently transfected with chick *noggin*. The results were identical as obtained with the stable cell line: *paraxis* expression was seen in 10/10 explants of the posterior third of the primitive streak (Fig. 4H); 6/10 had morphologically visible somites (Fig. 4H), while in the remainder (4/10) *paraxis* expression was in a structure that resembled segmental plate mesoderm.

These experiments show that noggin, but not chordin, can mimic the activity of Hensen's node on primitive streak explants. Both noggin and the node can elicit the formation of somites, but not notochord, from any part of the primitive streak.

2.4. Chordin is more effective than noggin in induction of an ectopic primitive streak

To assess whether noggin, like chordin (Streit et al., 1998) is sufficient to initiate primitive streak formation, grafts of noggin-secreting CHO or COS cells, or *chordin* transfected COS cells or the appropriate controls were transplanted anteriorly (180° from the posterior marginal zone) into chick host embryos at stage XII-3. Embryos were grown until stage 4-8 and the expression of markers for mesoderm (*brachyury*) or organiser (*cNot-1*) assessed by whole mount in situ hybridisation.

As reported previously (Streit et al., 1998), misexpression of chordin at the anterior edge of the area pellucida (just inside the marginal zone) results in the formation of a structure resembling a short primitive streak, expressing *brachyury* (18/23), which sometimes terminates in a node-like structure; in 4/4 such cases analysed, this structure expressed the organiser marker *cNot-1*. These ectopic structures could still be generated in host embryos that had already initiated the formation of their own primitive streak, but not when chordin expressing cells were grafted to the anterior marginal zone (just outside the area pellucida), at any stage. By contrast, no ectopic primitive streaks were seen when noggin-secreting CHO cells were transplanted to the anterior area pellucida of stage XII-XIII (0/28) or of stage 2–3 (0/10) embryos (data not shown). Only occasionally (4/28) in pre-streak embryos, noggin caused the formation of a small button-like structure that

expressed *brachyury* very weakly. These structures did not resemble a primitive streak and never generated any axial derivatives. Similar results were obtained with grafts of COS cells transfected with chick *noggin* (0/8 forming an ectopic streak). Noggin-secreting cells were also not capable of initiating primitive streak formation when grafted at 90° from the posterior margin.

These results show that chordin can initiate the formation of an ectopic primitive streak and organiser when misexpressed in the area pellucida up to the early primitive streak stage, but noggin is ineffective.

2.5. Neither chordin nor noggin is sufficient for neural induction

We previously reported that chordin does not induce ectopic neural tissue when misexpressed in the area opaca or non-neural portion of the area pellucida of embryos at stages 3–4 (Streit et al., 1998). We now tested whether noggin might do this. As with chordin, we did not observe induction of the early pan-neural markers *Sox-2* (0/8) or *Sox-3* (0/11) or the formation of a neural plate-like structure adjacent to the grafted cells either in the area opaca or in embryonic non-neural epiblast.

Taken together, our results reveal that the BMP inhibitors noggin and chordin have different effects in the early chick embryo. Chordin, but not noggin, can initiate ectopic primitive streak formation. Noggin, but not chordin, can dorsalise mesoderm, as assessed by the formation of ectopic somites from posterior primitive streak. Finally, neither noggin nor chordin can induce neural tissue from non-neural epiblast.

3. Discussion

3.1. Different roles for different BMP inhibitors during early development

In *Xenopus*, compelling evidence implicates BMPs and their inhibition by noggin, chordin and/or follistatin in at least three separate processes during early development (reviewed by Harland and Gerhart, 1997). First, BMP inhibitors can dorsalise the embryo before gastrulation. Evidence for this includes the finding that injection of *noggin*, *chordin* or *follistatin* mRNA can rescue the formation of a blastopore and subsequent axial development in UV-ventralised embryos. Second, these inhibitors can mimic the ability of Spemann's organiser to dorsalise mesoderm from the marginal zone: DNA encoding *noggin*, *chordin* or *follistatin* injected ventrally, as well as noggin and chordin protein administered to lateral/ventral mesoderm from the gastrula stage, can generate muscle from cells fated to give rise only to lateral plate mesoderm. Third, all three inhibitors can elicit expression of neural markers when injected as RNA or DNA, or when added as protein (under certain conditions) to animal cap explants from the gastrula stage.

All of these effects have been ascribed to inhibition of BMP activity, and particularly of BMP4. However, several findings suggest that the situation in the embryo may be more complex than this. For example, *noggin* binds BMP4 with a K_D of approximately 20 pM, an affinity about 10 times greater than chordin and at least 100 times greater than follistatin for the same ligand (Wilson and Hemmati-Brivanlou, 1995; Piccolo et al., 1996; Zimmerman et al., 1996; Harland and Gerhart, 1997). Despite this, the concentration of protein required to dorsalise mesoderm is identical for chordin and *noggin* (about 1 nM), but neuralisation requires 10 times more *noggin* (about 10 nM) than chordin (1 nM) (Harland and Gerhart, 1997). These results suggest that BMP4 may not be the only ventralising molecule present in the embryo, and that neuralisation of the ectoderm and dorsalisation of the mesoderm may be mediated in part by inhibition of different TGF- β superfamily members.

Our results reveal further differences between *noggin* and chordin in the chick embryo: *noggin* can cause posterior primitive streak to develop somites ('dorsalisation of the mesoderm'), but it cannot elicit neuralisation of the epiblast and is ineffective at causing the formation of an ectopic primitive streak ('dorsalisation of the embryo'). In contrast, chordin is effective at generating a second primitive streak, but can neither generate somites from posterior streak nor neuralise epiblast. These differences are unlikely to be due to differences in the amount of factor produced by the grafted cells. An amount of chordin sufficient to induce an ectopic primitive streak cannot induce somites, while conversely, an amount of *noggin* that can induce somites cannot induce a primitive streak. Even a much larger number of chordin-producing cells cannot induce somites, and the same is true for *noggin* and streak formation. These results could imply that the distinct effects of the BMP antagonists are due to different BMPs being involved in primitive streak initiation and mesoderm patterning.

In the chick, BMP-2 and -4 protein can suppress somite formation and even causes somites that have already formed to lose their epithelial structure (Pourquié et al., 1996; Tonegawa et al., 1997; Andréé et al., 1998). BMP-4 can also interfere with node functions (including the formation of axial structures and the expression of organiser markers) and with the initiation of primitive streak formation (Streit et al., 1998). Therefore, as in *Xenopus*, BMP-4 can act as an inhibitor of dorsal/axial development both before and after gastrulation.

Noggin, *chordin* and *follistatin* are all expressed in the frog organiser during gastrulation. However, in other species the three inhibitors have distinct spatial and temporal expression patterns. In the zebrafish *chordin* mRNA is expressed very early before gastrulation (Schulte-Merker et al., 1997), but *noggin* and *follistatin* are not expressed in the organiser (Bauer et al., 1998). In the chick, *chordin* is again expressed very early, and the level of *noggin* and *follistatin* mRNA in the node is only barely detectable, if at all, during gastrulation (Amthor et al., 1996;

Connolly et al., 1997; Levin, 1998; Streit and Stern, 1999). *Noggin* becomes noticeably upregulated in the node at the beginning of neurulation (stage 5), when the node begins to regress. Therefore, these three BMP antagonists may have diverged in their functions during evolution.

The idea of different roles for these BMP antagonists is also supported by loss-of-function studies. *Noggin*-null mouse embryos develop with severe somite abnormalities but an essentially normal early axis and neural tube (McMahon et al., 1998). *Follistatin*-deficient mouse embryos are essentially normal during early development (Matzuk et al., 1995). A *chordin* loss of function mutation, [*chor*]dino, has been isolated in zebrafish (Hammerschmidt et al., 1996; Schulte-Merker et al., 1997). Homozygous *chordino* embryos have severely affected dorsoventral patterning, but do not fail to form the organiser (embryonic shield), as might have been expected. Rather, they seem to fail in maintaining the organiser and its derivatives. This suggests that other BMP antagonists expressed during early development remain to be discovered, at least in the zebrafish.

3.2. Relationship between induction and dorsoventral patterning of the mesoderm

In amphibians, the prospective mesoderm is induced from cells in the marginal zone by signals from the vegetal pole (Nieuwkoop, 1969; Green and Smith, 1990; reviewed by Harland and Gerhart, 1997). This process is probably complete before the beginning of gastrulation, because older animal caps recombined with vegetal cells can no longer respond to signals from the latter, and because the vegetal cells themselves lose the ability to induce mesoderm even from younger caps. The mesoderm induced by the dorsal part of the vegetal pole in the frog has dorsal character (organiser, notochord, etc.), while that induced by the ventral part is ventrolateral (blood, mesenchyme). Since high concentrations of activin-like molecules induce dorsal mesoderm, while lower concentrations induce intermediate and ventrolateral mesoderm (Green and Smith, 1990), it has often been assumed that mesoderm induction and some dorsoventral patterning of the marginal zone occur at the same time, and therefore that both processes are complete by the beginning of gastrulation (e.g. Green and Smith, 1990). However, grafts of the organiser to the ventral side of a gastrula-stage embryo can dorsalise the mesoderm, and it is now becoming increasingly clear that the initial induction of mesoderm and establishment of its dorsoventral pattern are two separable processes (see Harland and Gerhart, 1997 for discussion).

In amniotes there is as yet no process that is clearly analogous to mesoderm induction: although the epiblast does give rise to the mesoderm during gastrulation, it has not been definitively established whether this is a result of induction, and if so, which tissue secretes the inducing signal(s). One common assumption is that formation of the primitive streak is the amniote equivalent of this

process. In addition, as in the frog, peptide factors including activin (Stern et al., 1995), Vg1 (Shah et al., 1997) and FGF (Mitrani et al., 1990; Storey et al., 1998) have all been shown to induce mesoderm *in vivo* and *in vitro*. In the case of activin, explants of extraembryonic epiblast can respond in a concentration-dependent way, with higher concentrations of activin generating more dorsal mesoderm (Stern et al., 1995). Unlike the frog, however, epiblast obtained from advanced gastrulation stages can still respond. These results suggest that competence of mesoderm induction to activin-related molecules may continue in the chick after gastrulation has started. In agreement with this conclusion, single cells within the node of late primitive streak stage embryos sometimes contribute progeny to both mesoderm and ectoderm (Selleck and Stern, 1991). However, ectopic Vg1 can no longer induce a second primitive streak after the appearance of the normal streak (Shah et al., 1997), suggesting that initiation of the primitive streak and generic 'mesoderm induction' are different events.

It has been shown that BMP activity can convert already formed somites to lateral plate (Pourquié et al., 1996; Tonegawa et al., 1997; Andrée et al., 1998). Here we show that the BMP antagonist noggin can convert prospective lateral plate mesoderm in the posterior streak to somites, as late as early neurulation (stage 5). However, neither chordin nor noggin are able to induce expression of node or notochord markers from more posterior regions of the streak. It is therefore possible that another of the inhibitors may fulfill this role; based on their expression patterns, the most likely candidates are follistatin and Flik, a follistatin/TSC36-related protein (Amthor et al., 1996; Patel et al., 1996). However, the former binds BMP4 with low affinity and the latter has not yet been shown to bind any members of the family. An alternative hypothesis is that noggin and/or chordin protect the organiser during regression (as suggested by the phenotype of the [*chor*]dino mutant; Hammerschmidt et al., 1996), yet are not sufficient to induce cells that are not fated to contribute to axial mesoderm to acquire this fate. A final, and most likely possibility is that the competence of cells to become organiser has been lost by stage 5. This is supported by our finding that even the normal node is unable to generate notochord from other regions of the primitive streak at this stage.

Taken together, these results suggest not only that primitive streak formation can be separated from mesoderm induction, but also that dorsoventral patterning (at least for somites) continues at much later stages of development, even perhaps at tailbud stages (Catala et al., 1995, 1996; Knezevic et al., 1998).

3.3. Node regression and the induction and maintenance of somite fate

Have amniotes evolved a prolonged period for generating and patterning the mesoderm? It was once assumed that in anuran amphibians and teleosts, the blastopore and immedi-

ate regions contributed few if any cells to the paraxial mesoderm after the early neurula stage (e.g. Elsdale and Davidson, 1983). In contrast, in amniotes, it has long been recognised that the primitive streak contains somite precursors for several days after gastrulation, and the production of these cells continues from the remnants of the primitive streak in the tail bud region (Packard, 1980; Selleck and Stern, 1991; Tam and Tan, 1992; Catala et al., 1995, 1996; Nicolas et al., 1996; Psychoyos and Stern, 1996; Knezevic et al., 1998). More recent research in amphibians (Gont et al., 1993; Beck and Slack, 1998) and teleosts (Kanki and Ho, 1997), however, has revealed a situation very similar to that of amniotes. It now appears that, in all vertebrate classes, cells derived from the organiser region become incorporated into the tail bud during neurulation, from where somite formation continues.

During regression of the primitive streak, the prospective somite-forming territory in the streak becomes progressively surrounded by *BMP2*, *-4* and *-7* expressing cells, and the fate map of the streak becomes progressively more compressed (Spratt and Codon, 1947; Spratt, 1957; Nicolet, 1965, 1967, 1970a; Rosenquist, 1966; Schoenwolf et al., 1992; Catala et al., 1995, 1996). We therefore propose that from stage 5, when the expression of the BMP inhibitor noggin in the node and its immediate derivative, the notochord, becomes upregulated, noggin may function to protect the prospective somite territory, and perhaps even the organiser itself, from the surrounding BMPs during regression. The expression of BMPs and their inhibitors in the tail bud and organiser region suggests that a similar process takes place in amphibians and teleosts (e.g. Smith and Harland, 1992; Fainsod et al., 1994; Sasai et al., 1994; Nikaido et al., 1997; Beck and Slack, 1998), and even in protochordates (Panopoulou et al., 1998).

Grafts of Hensen's node have an autonomous tendency to move as they lay down notochord. Experiments like those of Nicolet (1970b) and Hornbruch et al. (1979) cannot rule out that the movement itself is important for somite formation, as suggested by Lipton and Jacobson (1974). Likewise, when a pellet of noggin expressing cells is placed into the lateral plate but close to the axis of the host embryo (Tonegawa and Takahashi, 1998), the graft region continues to elongate because it is caught up in the movements of the host axis. In our experiments, implantation of a pellet of noggin expressing cells adjacent to posterior primitive streak but away from the host axis allows somites to form in the *absence* of massive cell movements, which accounts for the 'bunches of grapes' arrangement of the induced somites. This result argues that it is not the regression movements themselves, but rather the inhibition of BMP activity which triggers somite formation from prospective lateral plate mesoderm.

In conclusion, our findings provide a direct assay in the chick embryo for a process analogous to what has been described as 'mesoderm dorsalisation' in amphibians. We confirm the finding of Nicolet (1970b) that the organiser can

induce somite formation from regions of the streak not normally fated to do so, and show that misexpression of noggin can mimic this activity. Our findings suggest that during node regression the progressive inhibition of BMPs that results from the movement of the noggin-expressing node to more posterior regions triggers somite formation in the mesoderm. We also show that noggin and chordin have distinct effects during early chick development, chordin being primarily involved in axis formation and noggin in somite formation and maintenance.

4. Materials and methods

4.1. Embryos and transplants

Fertile hens' eggs (White Leghorn; Spafas, MA) and quails' eggs (Karasoulas, CA) were incubated at 38°C for 2–28 h to give embryos between stages XII (Eyal-Giladi and Kochav, 1976) and 7 (Hamburger and Hamilton, 1951). Chick host embryos were explanted and maintained in New culture (New, 1955), modified as described by Stern and Ireland (1981), for 8–24 h. Quail or chick donor embryos were immersed in Pannett-Compton saline (Pannett and Compton, 1924), and a piece of primitive streak and/or Hensen's node was excised and transplanted into the inner margin of the area opaca of a chick host as described previously (Storey et al., 1992). Selection of the regions of primitive streak for grafting was guided by the fate maps of Psychoyos and Stern (1996): the primitive streak was subdivided into thirds or quarters (Fig. 1). At stage 5, the region fated to contribute to somites is confined to the anterior one-third of the streak.

Pellets of transfected COS cells (1000 cells per pellet; see below), or aggregates of noggin-secreting CHO cells (B3) or control CHO cells (see below), were grafted into different regions of chick host embryos as indicated in the individual experiments.

4.2. cDNA clones

BMP-4 (Liem et al., 1995) and *chordin* (Streit et al., 1998) were cloned into pMT-23 to allow their transfection and expression in COS cells and contained sequences encoding the myc- or HA-epitopes. Full-length chick *noggin* cDNA was a kind gift of Karel Liem and Tom Jessell and was subcloned into pCDNA3 for expression in COS cells. *Sox-2* and *Sox-3* plasmids were kindly provided by Drs. R. Lovell-Badge and P. Scotting (Uwanogho et al., 1995; Collignon et al., 1996); chick *brachyury* was a gift from Jim Smith (Kispert et al., 1995), *HNF3 β* from Ariel Ruiz i Altaba (Ruiz i Altaba et al., 1995), *cNot-1* from Michael Kessel (Stein and Kessel, 1995), *Pax-2* from Martyn Goulding and Domingos Henrique (Burrill et al., 1997), *Paraxis* from Eric Olson (Sosic et al., 1997), *Sim1* from Olivier Pourquié (Pourquié et al., 1995) and *Tbx6L* from Susan Mackem (Knezevic et al., 1997).

4.3. Cells and transfection

COS-1 cells were grown in DMEM containing 10% newborn calf serum. Transfection with *BMP-4*, *chordin* and *noggin* was performed using lipofectamine (Gibco BRL). Twenty-four hours after transfection, pellets containing 1000 cells were generated by setting up hanging drop cultures. The pellets were transplanted into embryos 48 h after transfection. The secretion of the factors by transfected COS cells was confirmed in Western blots from conditioned medium as described previously (Streit et al., 1998). As controls in experiments with transfected COS cells we used mock-transfected cells.

A stable cell line secreting noggin has been described previously (Lamb et al., 1993); as controls for these cells we used the parent CHO cell line. Both were a kind gift of Richard Harland. CHO cells do not spontaneously form aggregates in hanging drop culture. To produce aggregates suitable for grafting, a suspension of cells was centrifuged lightly (200 \times g, 5 min) in an Eppendorf tube, the resulting pellet loosened with a steel needle and removed with a Gilson micropipette. It was then cut into suitably sized pieces for grafting (about 100–150 μ m) using fine mounted steel pins.

4.4. Immunocytochemistry and in situ hybridisation

To visualise quail tissue we used the monoclonal antibody QCPN (Developmental Studies Hybridoma Bank, maintained by the Department of Pharmacology and Molecular Sciences, The Johns Hopkins University School of Medicine, Baltimore, MD 21205 and the Department of Biological Sciences, University of Iowa, Iowa City 52242, under contract N01-HD-2-3144 from NICHD). The staining was performed as described before (Streit et al., 1997; 1998). Whole mount in situ hybridisation using DIG-labeled RNA-probes was performed as described by Stern et al., 1998.

In some experiments, the antibody staining was performed before in situ hybridisation. In these cases 1M LiCl was included in all antibody and wash solutions during the immunostaining portion of the protocol, as described previously (Stern et al., 1998).

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