FGF signaling is essential for the early events in the development of the chick nervous system and mesoderm

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ABSTRACT Fibroblast growth factor (FGF) belongs to a family of polypeptides with diverse biological functions. In the present study we have assessed the role of FGF signaling in the development of nervous system and mesodermal tissues in chick embryo. Treatment of *in vitro* cultured embryos with exogenous, human recombinant FGF led to abnormalities in neural induction and development, notochord formation and somitogenesis as studied by gross morphology and histology. Overall growth and development was also adversely affected as seen from the measurement of body axis length. Further, treatment of embryos with FGF resulted in differential modulation of expression of two genes important in normal development as studied by whole mount *in situ* hybridization using DIG-labeled riboprobes. The expression of *Brachyury,* which is necessary for mesoderm formation, was down-regulated in FGF-treated embryos. The expression of *noggin,* the product which participates in the patterning of the chick neural tube was, on the other hand, up-regulated within 2 h. We also studied development of neural and mesodermal tissues in conditions where FGF signaling was defective. This was achieved by culturing the embryos in the presence of suramin. In the presence of low doses of suramin (100-150 nmole/culture), abnormalities were detected mainly in the mesodermal structures while at higher doses (200-400 nmole/culture), the nervous system too was found to be abnormal in a large proportion of embryos. Treatment of chick embryos with suramin (200 nmole/culture) also modulated the expression of *Brachyury* and *noggin* within a 2 h period. The results show that FGF signaling plays an important role in the molecular events leading to the development of nervous system and mesodermal tissues in the chick embryo.

KEY WORDS: *FGF signaling, nervous system, mesoderm, Brachyury, noggin, chick embryo*

Introduction

Fibroblast growth factor (FGF) is a family of several polypeptides which participate in diverse biological phenomena such as embryonic development, tissue repair, cardiovascular diseases, angiogenesis, neuronal cell survival and cancer. FGF is one of the mesoderm inducing factors in vertebrates and is present during the early embryonic development (for reviews, see Smith, 1989; Asashima, 1994; Ghaskadbi, 1996). In *Xenopus*, the presence of embryonic FGF (eFGF) is essential for morphogenetic movements leading to the development of a normal embryonic axis (Pownall and Slack, 1995). In chick, b-FGF is present right from Eyal-Giladi stage XI (Mitrani *et al.,* 1990). It induces posterior neural tissue in the early gastrula stage ectoderm in *Xenopus* and chick (Lamb and Harland, 1995; Alvarez *et al.,* 1998; Storey *et al.,* 1998).

Two of the important genes in the molecular cascade of events leading to the formation of mesodermal and neural tissues in vertebrates are *Brachyury* and *noggin*. *Brachyury* (T) is a pan-

mesodermal marker gene and is activated as an early response to the axis-inducing signals *in vivo.* Brachyury protein binds to DNA and may influence transcriptional activity of genes required for mesoderm formation, and for the differentiation and function of chordamesoderm (Kispert and Herrmann, 1993). All the cells in the primitive streak do not express the same levels of Brachyury. Depending on the amount of Brachyury they express, the invaginating cells are assigned to one of the 3 germ layers; cells expressing highest levels of Brachyury are most likely to populate mesoderm (Wilson and Beddington, 1997). *Noggin,* which codes for a secreted molecule, is first expressed in the organizer region and later in the notochord in *Xenopus* embryos (Smith and Harland, 1992). It functions as one of

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the neural inducers in the amphibian embryos. In chick, unlike *Xenopus, noggin* participates in the patterning of the neural tube rather than in neural induction (Connolly *et al.,* 1997).

Suramin, a polysulphonated compound, has been used to block the endogenous FGF action in vertebrate embryos (Grunz, 1992, 1999; Mitrani *et al.,* 1990; Oschwald *et al.,* 1993), since it interacts with growth factor receptors (Moscatelli and Quarto, 1989). This property of suramin to block action of growth factors has been exploited to study the role of FGF in mesoderm induction in the chick (Mitrani *et al.,* 1990).

Present study was initiated to assess the role of FGF signaling in the early development of nervous system and mesodermal tissues in chick embryo. Effects of excess bFGF on the early embryonic development and expression of *Brachyury* and *noggin* in chick embryo explants cultured *in vitro* were studied for this purpose. The effects of defective FGF signaling, experimentally created by the use of suramin, were also studied.

Results

Effects of FGF on chick embryos at gross and tissue levels

Development of chick embryos in the presence of either PC saline or BSA in New's culture was normal in over 95% explants for the entire period of 22 h (Fig. 1A). The control embryo (Fig. 2A) showed a well developed nervous system with optic vesicles, looped heart and 10 pairs of somites. In the embryos treated with 10 ng FGF at HH stage 4 for 22 h, about 60% developed abnormally (Fig. 1A). The abnormalities were detected in the developing nervous system, heart and somites. In most of the abnormal embryos of the FGF treated group, the nervous system was deformed in the anterior region. The deformations were in the form of open neural tube (Fig. 2 B-D, F), bending of both the neural folds on one side of the body axis (Fig. 2 C,D), incomplete fusion of the neural folds along the body axis (Fig. 2 C-F) or a hole in the forebrain (not shown). A few treated embryos were highly abnormal showing extremely poor differentiation in various regions of the embryo (Fig. 2F). Abnormalities observed in the somites included mismatched and diffused somites (Fig. 2 E,F). In a small proportion of embryos, the development of the heart was inhibited by FGF treatment as judged from either a complete absence of the median heart tube or absence of looping of heart (not shown). The most commonly affected was the nervous system (in over 80%

embryos) followed by somites (in over 50% embryos) and heart (in less than 20% embryos).

For the morphometric analysis at the end of 22 h incubation, PC saline- 47 embryos, BSA- 57 embryos and FGF-treated 61 embryos, which reached approximately the same stage of development (HH stage 8+-9+) were selected. Mean length of the body axis of PC saline- and BSA-treated embryos was 4.32±0.12 and 4.26±0.1 mm, respectively (Fig. 1B). The mean body axis length of FGF-treated embryos was 3.93±0.1 mm (Fig. 1B). Thus chick embryos treated with FGF at HH stage 4 for 22 h suffered a significant reduction in the length of the body axis indicating inhibition of axis elongation.

Interestingly, the same dose of exogenous FGF did not interfere with the normal development of the embryo when treated at HH stage 5 (Fig. 1C). The head process stage (HH stage 5), thus, appeared relatively much less susceptible to interference in development from exogenous FGF.

FGF-induced abnormalities in chick embryos at the cellular level were revealed in the study of thin sections of embryos from various groups. The control embryos exhibited distinct neural tube, notochord and foregut (Fig. 3 A-C). In FGF-treated embryos, there occurred abnormal intermingling of neuroectodermal and notochordal cells (Fig. 3 D,E). Notochord formation was improper as seen from its indistinct shape (Fig. 3F). In severely affected cases no identifiable notochord was formed. In some cases there was thickening of neural folds on the dorsal side (Fig. 3 E,G,H), failure of the neural folds to fuse in the brain region (Fig. 3F) or abnormal extension of neuroectodermal cells on the ventral side of the neural tube (Fig. 3G). Abnormal thickening of foregut and heart mesoderm was also detected in some cases (Fig. 3 H,I). The control embryos exhibited proper organization and compaction of paraxial mesodermal cells leading to formation of normal somites (Fig. 3J). In some of the FGF treated embryos, the organization and compaction of paraxial mesodermal cells was affected leading to formation of diffused somites (Fig. 3K).

Effects of suramin on chick embryonic development

Treatment with suramin brought about abnormal development in the chick embryos when administered at HH stage 4. Controls were normal with properly formed nervous system, optic vesicles, looped heart and 10 pairs of somites (Fig. 4A). At doses of 100, 150 and 200 nmole/culture, suramin induced abnormalities in developing somites
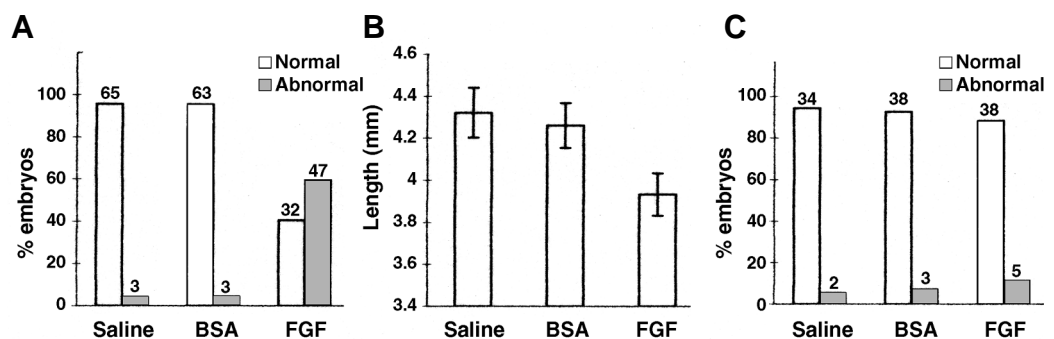

Fig. 1. Effect of exogenous basic FGF on the development of chick embryos cultured *in vitro* for 22 h in the presence of 10 ng FGF/culture. (A) *Effects of FGF on chick embryos explanted at HH stage 4. Note abnormal development in over 60% of FGF-treated embryos. Development of control embryos, in the presence of either PC saline or BSA is normal in over 95% of the cases. Numbers over bars indicate the actual number of embryos.* **(B)** *Effects of FGF on the body axis length of chick embryos. Chick embryos explanted at HH stage 4 were treated with 10 ng FGF/culture for 22 h. Body axis length was measured with a micrometer scale. Mean length is given with 95% confidence limits (vertical bars). Note the significant reduction of body axis length in FGF-treated embryos as compared to controls.* **(C)** *Effects of FGF on chick embryos explanted at HH stage 5. Chick embryos were explanted at HH stage 5 and treated with 10 ng FGF for 22 h in New's cultures. Note that the development of embryos treated with FGF is comparable to that of embryos developing in the presence of PC saline and BSA (controls). Numbers over bars indicate the actual number of embryos.*
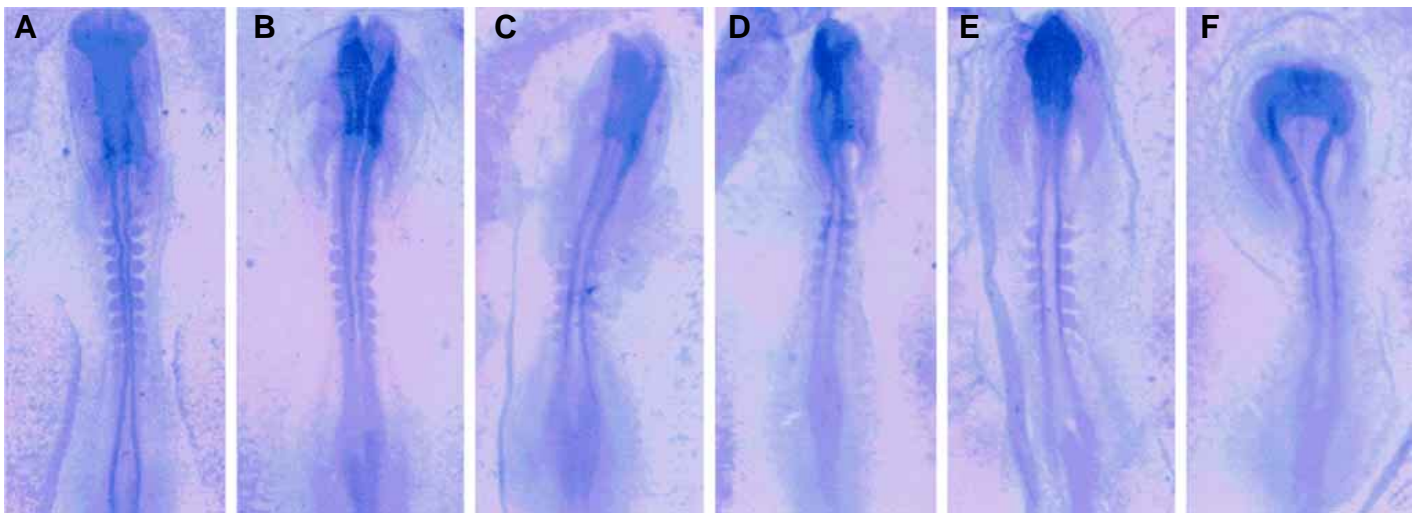
Fig. 2. Effects of FGF on chick embryonic development. (A) *Control chick embryo explanted at HH stage 4 and cultured in the presence of 100 ng BSA for 22 h. Note well developed nervous system with optic vesicles, looped heart and 10 pairs of somites.* **(B-F)** *Chick embryos explanted at HH stage 4 and treated with 10 ng FGF for 22 h in New's culture. Note various abnormalities, such as incomplete closure of neural tube (B-D,F), bending of neural folds on one side of the embryo (C,D), incomplete fusion of neural folds along the anteroposterior axis (C-F) and mismatched (F) or diffused (E,F) somites.*

and nervous system in over 60% of the treated embryos. More than 75% of the affected embryos showed dispersed somites and in some cases loose mesenchymal cells were seen in head fold region and body axis (Fig. 4 B,C). At 200 nmoles concentration of suramin, about 30% of the affected embryos showed complete inhibition of somitogenesis and cardiogenesis, while neurogenesis was grossly abnormal and retarded (Fig. 4D). In other cases, neural tube closure was incomplete and forebrain had a hole (Fig. 4B). It was observed that somite abnormalities were consistently seen at all 3 doses of suramin while neural abnormalities also became prevalent at 200 nmoles suramin treatment. At higher dose than this, i.e., 400 nmoles, suramin almost completely inhibited mesodermal and neural differentiation. These studies indicate that experimental interference with FGF signaling results in defective development of neural and mesodermal structures in the early chick embryo.

Effects of FGF and suramin on expression of Brachyury

Modulation of gene expression by FGF and suramin was studied after 2 h of treatment by whole mount *in situ* hybridization using DIG-labeled riboprobes. In the control (PC saline or BSA) embryos, *Brachyury* expression was found all along the primitive streak (Fig. 5A). In transverse sections, *Brachyury* expression was detected in the cells invaginating through the primitive streak and in the mesodermal cells that had traversed through the primitive streak and placed adjacent to it (Fig. 5 B-D). Few endodermal cells were also found to express *Brachyury* (Fig. 5B). In bFGF and suramin treated embryos, the spatial distribution of *Brachyury* transcripts was identical to the control (see fig. 5 F-H for FGF and K-M for suramin treated embryos). However, there was a significant reduction in the staining intensity in bFGF treated embryos, indicating down-regulation of *Brachyury* expression (Fig. 5 E-H) and this effect of FGF was seen in over 70% of the treated embryos (26/36 embryos).

In about 65% of the embryos after suramin treatment, there was a hint of slight up-regulation of *Brachyury* expression, all along the streak as the staining intensity indicated (Fig. 5 I-L). Approximately, in 30% of the treated embryos, *Brachyury* was not expressed in the posterior two third region of the primitive streak (Fig. 5J). The cells

invaginating through the primitive streak in this region failed to express *Brachyury* (Fig. 5M). In the presence of defective FGF signaling, *Brachyury* expression was modulated in more than 78% of the suramin treated embryos (18/23 embryos).

In the presence of excess FGF and in absence of active FGF signaling, the development of the primitive streak was apparently normal as indicated by proper organization of different cell layers (see Fig. 5 B-D; F-H and K-M).

Effects of FGF and suramin on expression of Noggin

Noggin was found to be expressed at low levels in the anterior two third portion of the primitive streak in the control embryos (Fig. 6A). In the Hensen's node region, *noggin* was expressed in the cells located within the primitive streak (Fig. 6B) while it was barely detectable in the postnodal regions (Fig. 6C).

In FGF-treated embryos, significantly enhanced expression all along the primitive streak was observed. The enhancement in *noggin* expression was especially noticeable in the Hensen's node (Fig. 6 D,E). *Noggin* expression in the posterior part of the primitive streak was also clearly observed in the FGF treated embryos (Fig. 6F). In addition, in more than 80% of FGF-treated embryos, *noggin* expression was detected in the prospective neural plate region (Fig. 6E). In the normal course of development, *noggin* expression is detectable in the prospective neural plate from around HH stage 5 (Connolly *et al.,* 1997). Thus the expression pattern indicates that exogenous FGF not only up-regulates *noggin* in the primitive streak and especially Hensen's node but also induces *noggin* expression in at least some cells of the prospective neural plate. FGF was capable of altering the expression of *noggin* in about 90% of embryos (40/42 FGF treated embryos).

In suramin treated embryos, more than 50% of the embryos exhibited enhanced expression of *noggin* all around the primitive streak (Fig. 6G). In about 65% of the treated embryos, *noggin* expression was enhanced specifically on the lateral sides of the Hensen's node (Fig. 6G). As compared to the *noggin* expression observed in the Hensen's node region of the saline control embryos (Fig. 6B), *noggin* expression in the cells invaginating through the
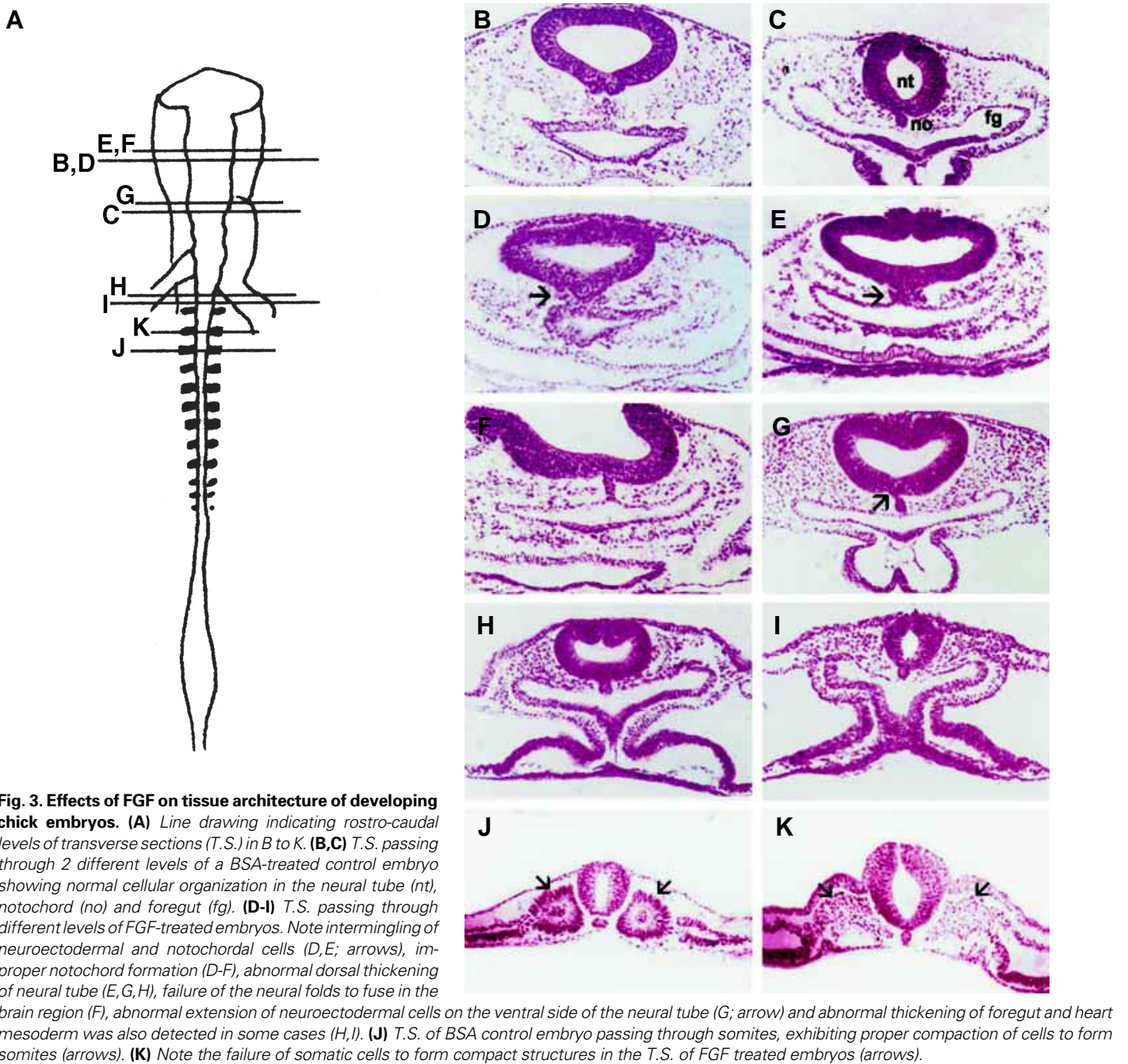
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Fig. 3. Effects of FGF on tissue architecture of developing chick embryos. (A) *Line drawing indicating rostro-caudal levels of transverse sections (T.S.) in B to K.* **(B,C)** *T.S. passing through 2 different levels of a BSA-treated control embryo showing normal cellular organization in the neural tube (nt), notochord (no) and foregut (fg).* **(D-I)** *T.S. passing through different levels of FGF-treated embryos. Note intermingling of neuroectodermal and notochordal cells (D,E; arrows), improper notochord formation (D-F), abnormal dorsal thickening of neural tube (E,G,H), failure of the neural folds to fuse in the brain region (F), abnormal extension of neuroectodermal cells on the ventral side of the neural tube (G; arrow) and abnormal thickening of foregut and heart mesoderm was also detected in some cases (H,I).* **(J)** *T.S. of BSA control embryo passing through somites, exhibiting proper compaction of cells to form somites (arrows).* **(K)** *Note the failure of somatic cells to form compact structures in the T.S. of FGF treated embryos (arrows).*

primitive groove was not modulated to a grater extent in the suramin treated embryos (Fig. 6H) whereas in the cells located on the lateral sides of the node, *noggin* expression was noticeably enhanced (Fig. 6H). The enhanced expression of *noggin* was detected throughout the primitive streak (Fig. 6I). Thus *noggin* expression was modulated within 2 h, in around 76% of the suramin treated embryos (19/25 embryos).

The *in situ* RNA localization studies showed that FGF and suramin can modulate the expression of developmentally crucial genes within 2 h in cultured stage 4 embryos. Further, the development of the primitive streak is not hampered in both FGF and suramin treated embryos, at least within 2 h.

Discussion

Maternal FGF is present in all parts of chick blastoderm right from stage XI onwards and FGF transcripts are detected at the marginal zone at full hypoblast stage. bFGF is distributed evenly in the epiblast, hypoblast and marginal zone of chick blastula (Mitrani *et al.,* 1990). Our studies indicate that exogenously added bFGF leads to abnormal morphogenesis specifically in chick embryos treated at HH stage 4 (Hamburger and Hamilton, 1951). Since normal distribution of growth and differentiation factors and their interactions with each other are apparently crucial for morphogenesis of the embryo (see Gilbert, 2000), exogenous bFGF may have
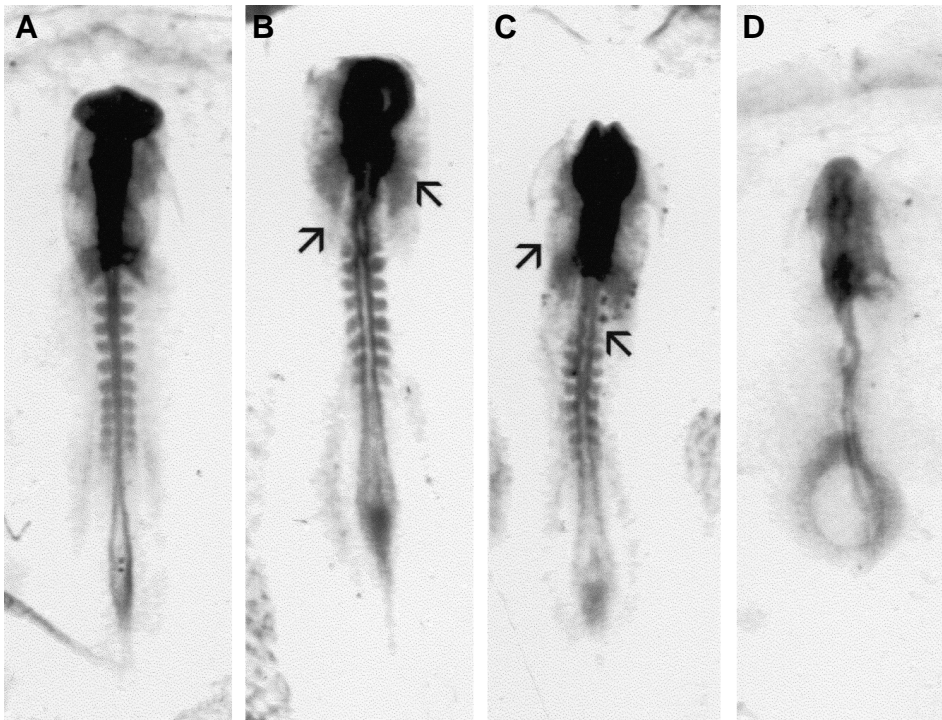
Fig. 4. Effects of suramin on chick embryonic development. (A) *Control chick embryo explanted at HH stage 4 and cultured in the presence of PC saline for 22 h. A well developed nervous system with optic vesicles, looped heart and 10 pairs of somites can be seen. (B-D) Chick embryos explanted at HH stage 4 and treated with 100* **(B)**, *150* **(C)** *and 200* **(D)** *nmoles suramin for 22 h in New's culture. Dispersed somites and loose mesenchymal cells (arrows) in the head fold region and body axis (B,C) and complete inhibition of formation of somites and heart (D) are the most common abnormalities in these embryos. Abnormalities in the nervous system included a major gap appearing as a hole in the forebrain and incomplete closure of the neural tube (B).*

interfered with the in-built distribution of FGF molecules, leading to abnormal morphogenesis. The abnormalities were mostly concentrated in the developing neural and mesodermal structures. Studies at cellular level suggested improper floor plate induction in embryos treated with exogenous bFGF. These results are in agreement with earlier reports wherein neural induction in chick embryos by beads soaked in bFGF has been shown to occur (Rodriguze-Gallordo *et al.,* 1997; Alvarez *et al.,* 1998). In fact a recent report states that FGF signaling is an essential component in the early neural induction process in chick and presence of FGF molecules in the organizer confers the latter the ability to induce neural tissue. However FGF signaling alone is not sufficient to induce the complete nervous system (Streit *et al.,* 2000). We also find that notochord formation was hampered in bFGF treated embryos, as seen from its indistinct shape. Further in these embryos, developing heart was less affected than somites as observed at gross morphology level. In histological studies however, abnormal thickening of heart mesoderm and gut endoderm was clearly evident. Thus our results confirm the role of FGF in ventral mesoderm formation in addition to development of axial structures (Mitrani *et al.,* 1990).

We find that exogenously added bFGF leads to down-regulation of *Brachyury* expression. Lesser amounts of Brachyury (T) are known to lead to reduced amounts of mesoderm and defects in formation and differentiation of notochord (Wilson and Beddington, 1997; Conlon and Smith, 1999). This correlates well with the improper formation of notochord observed by us. It has indeed been suggested (Kispert and Herrmann, 1993) that absence of sufficient T to maintain the notochord leads to reduced body axis and defective axial organization of embryonic structures. This too correlates very well with the present data. Interference with Brachyury function leads to inhibition of convergent extension during *Xenopus* gastrulation (Conlon and Smith, 1999). However

we find that the morphogenetic movements are not affected within 2 h after FGF treatment, even though *Brachyury* expression is modulated within the same treatment period. Shortening of body axis and the improper formation of the notochord observed by us could have resulted from reduced amount of axial mesoderm formed in presence of exogenous bFGF.

Chick *noggin* is expressed in the Hensen's node and neural plate during axial development and later in the neural folds and somites; it is involved in the patterning of the neural tube rather than neural induction (Connolly *et al.,* 1997). We find that *noggin* expression is enhanced in the Hensen's node and is induced *de novo* in the prospective neural plate region in chick within 2 h of FGF treatment at HH stage 4. This could be one of the reasons for the development of abnormal nervous system. *Noggin* is also involved in avian somite patterning (Hirsinger *et al.,* 1997). Elevated levels of *noggin* in FGF-treated embryos may be a cause of abnormal somitogenesis observed in the present study. Thus, differential modulation of *Brachyury* and *noggin,* two of the important genes in the formation of mesodermal structures and neural development, by FGF indicate the latter's role in the regulation of neural and mesodermal development. A recent report also indicates a possible role of FGF in determining the boundaries and positions of somites (Dubrulle *et al.,* 2001).

To confirm the role of FGF signaling in the early embryonic development of chick, we used suramin to block the endogenous FGF signaling. In the dose range used in the present study (100 to 200 nmole/culture), the development of mesoderm, especially somitogenesis, was affected to a great extent. As the dose of suramin was increased to 200 nmole/culture and above, embryos developed with abnormal nervous tissue in addition to abnormal mesodermal structures. Thus, mesodermal abnormalities were induced by relatively low doses of suramin while at increasing doses, nervous system abnormalities also became apparent.
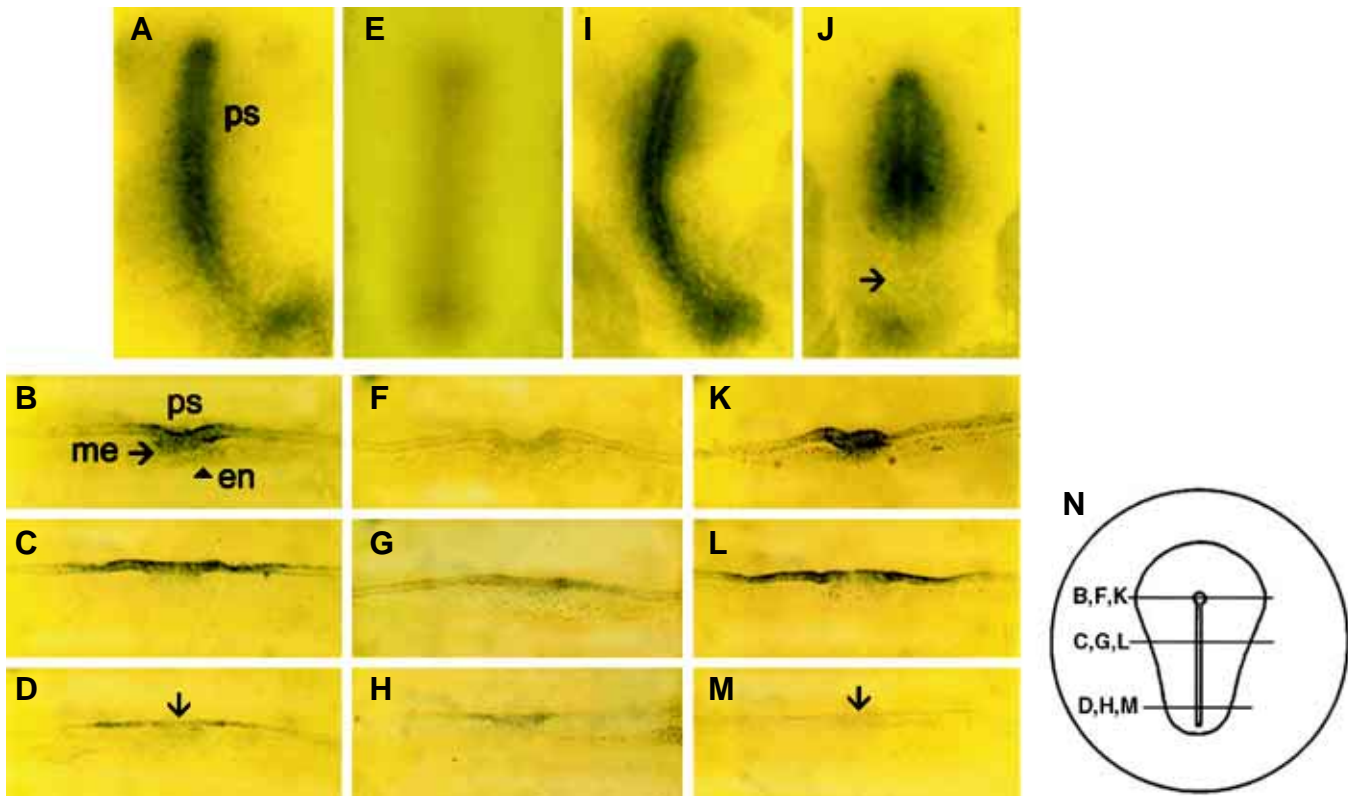
Fig. 5. Effects of FGF and suramin on *Brachyury* expression in chick embryo. *Chick embryos were explanted as before and cultured either in the presence of 10 ng FGF or 200 nmole suramin/culture for 2 h. The embryos were processed for whole mount* in situ *hybridization using DIG-labeled riboprobes. After whole mount* in situ *hybridization, these embryos were transverse sectioned to study the tissue specific distribution of the transcripts.* **(A-D)** *Expression of* Brachyury *in a control chick embryo. (A) Note staining along the entire primitive streak (ps). (B-D) T.S. of control embryo, passing through 3 different levels, shows the presence of* Brachyury *transcripts in the invaginating cells through the primitive streak (ps) and in the newly formed mesoderm cells (me). Note* Brachyury *expression in few endoderm (en) cells (B; arrowhead). The presence of* Brachyury *transcripts in the cells invaginating through the primitive streak and mesodermal cells is clearly indicated by the staining intensity in the postnodal regions of the control embryo (C, arrow in D).* **(E-H)** *Expression of* Brachyury *in FGF-treated embryos. Note the reduction in staining intensity along the entire streak (E). Tissue specific distribution of* Brachyury *transcripts in an FGF-treated embryo is identical to that observed in control embryos. A reduction in staining intensity is clearly seen in sections (F-H).* **(I,J)** *Differentially modulated expression of* Brachyury *in suramin treated embryos. A slightly enhanced staining intensity is seen in about 65% of treated embryos (I). Note the complete absence of staining in the posterior one third region of the primitive streak, observed in around 30% of embryos treated with suramin (J; arrow).* **(K-M)** *Spatial distribution of* Brachyury *transcripts in suramin-treated embryos. Note the identical pattern of distribution of* Brachyury *transcripts as compared to control and FGF-treated embryos; only the intensity of staining is slightly higher (K,L). The absence of* Brachyury *transcripts in the posterior one third region of the primitive streak is distinctly indicated by the absence of staining in cells invaginating through the primitive streak and in the mesodermal cells (M; arrow).* **(N)** *Line drawing indicating the levels of sections in control, FGF and suramin treated embryos.*

Since suramin interferes with FGF signaling in the chick embryo (Mitrani *et al.,* 1990), these results further support our contention that FGF signaling is essential in both mesodermal and neural development. The development of the primitive streak and gastrulation movements are not disturbed by defective FGF signaling, at least in 2 h of treatment period. We also find that expression of *Brachyury* is modulated differentially within 2 h in the embryos treated with suramin, which seems to have resulted in abnormal morphogenesis. The results indicate that FGF signaling governs the morphogenetic movements, partially by regulating *Brachyury* expression in chick embryo. Further, we find that defective FGF signaling results in enhanced expression of *noggin* all along the primitive streak, especially on the lateral sides of the Hensen's node. Cells invaginating through the lateral sides of the node contribute to the medial somites and to some extent to notochord while cells invaginating through the node participate in notochord, head mesoderm and neural floor plate formation (Selleck and

Stern, 1991; Schoenwolf and Smith, 1999). *Noggin* is involved in avian somite patterning; it is proposed to be a key molecule in promoting medial somite patterning (Hirsinger *et al.,* 1997). The enhanced expression of *noggin* after suramin treatment, would have led to the improper formation of the neural tube, diffused somites and loose head mesenchymal cells, observed by us.

It is interesting to note that *noggin* expression was enhanced both in the presence of exogenous FGF as well as in the absence of proper FGF signaling. However, the pattern of enhancement in the two cases is quite different. How *noggin* expression is differentially enhanced in the two cases is not clearly understood at this moment. *Noggin* is expressed at several different times during development. The spatial and temporal expression of *noggin* is thus governed by different mechanisms, depending on the role and site of expression (Hirsinger *et al.,* 1997; Sela-Donenfeld and Kalcheim, 2000). Therefore the enhanced expression of *noggin* observed by us, in the two cases, could be a result of two different
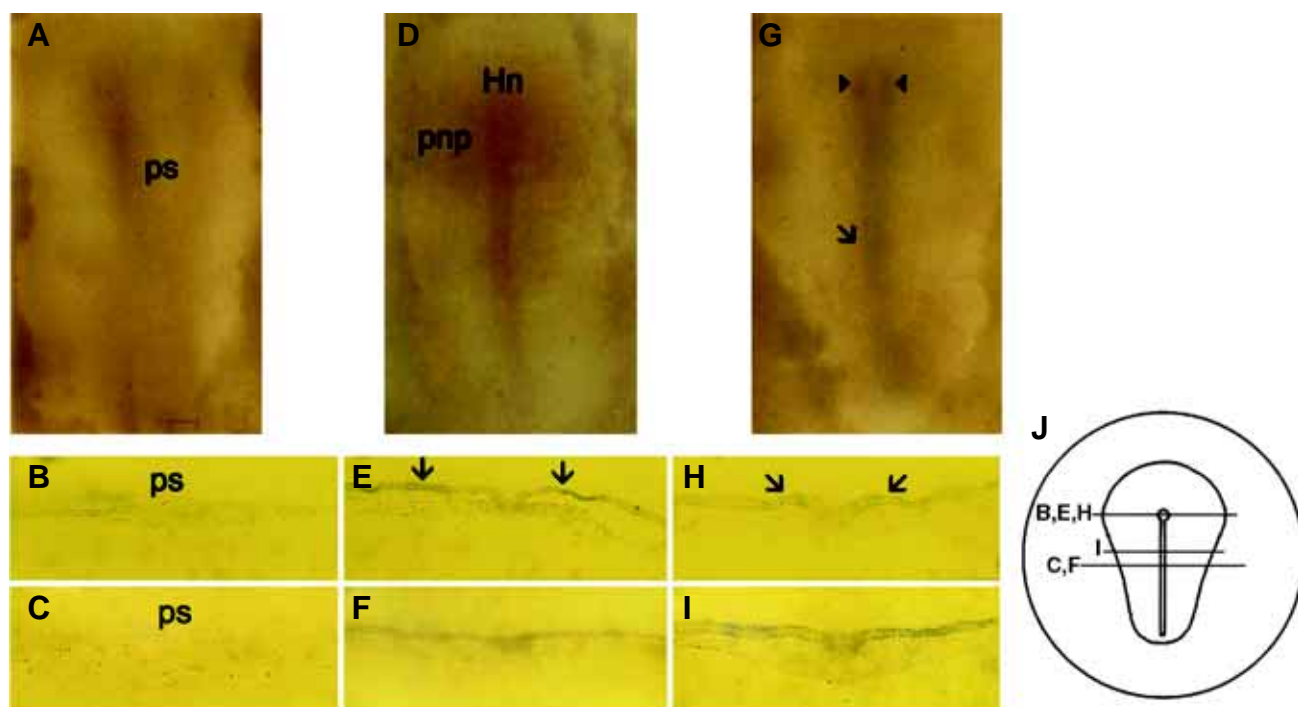
Fig. 6. Effects of bFGF and suramin on *noggin* expression. (A-C) Noggin *expression in a control chick embryo. Note staining in the anterior two third region of the primitive streak (ps, A). The T.S. passing through Hensen's node region indicates the presence of* noggin *transcripts in barely detectable amounts in the cells located within the primitive streak (ps, B). Expression of* noggin *is below detection levels in the postnodal regions of the streak (ps) in control embryos (C).* **(D-F)** *Expression of* noggin *in FGF-treated embryos. (D) Enhanced expression in the anterior two third of the primitive streak is obvious. Note increased expression in the cells of Hensen's node (Hn) and ectopic expression of* noggin *in the ectodermal cells located in the prospective neural plate area (pnp in D, arrows in E). In the postnodal sections, enhanced* noggin *expression is clearly seen (F).* **(G-I)** *Expression of* noggin *in suramin treated embryos. (G) Enhanced staining all along the primitive streak is seen (arrow). Note more intense staining on the lateral sides of Hensen's node (arrowheads in G, arrows in H). Note the presence of detectable levels of* noggin *transcripts in the cells of the postnodal region (I).* **(J)** *Line drawing indicating levels of sections in control, FGF and suramin treated embryos.*

mechanisms, as the sites of enhanced expressions are quite different.

In conclusion, exogenous bFGF brings about developmental abnormalities, especially in the developing mesoderm and neural tissue, in the chick embryo, apparently by interfering with the endogenous distribution pattern of FGFs. This effect of FGF is at least partially mediated through rapid modulation of expression of the two important genes, *Brachyury* and *noggin*. How FGF brings about this effect is not yet known but in the case of *Brachyury*, it could possibly be through specific repression modules in the promoter (Lerchner *et al.,* 2000). Inhibition of endogenous FGF signaling by using suramin leads to abnormal morphogenesis of the chick embryo. Defective FGF signaling leads to alterations in the expression of *Brachyury* and *noggin*. These results demonstrate the essential role of FGF signaling in the development of nervous system and mesodermal tissue in the chick embryo.

Materials and Methods

Materials

Freshly laid White Leghorn chicken eggs were obtained either from Institute of Veterinary Biological Products, Pune or Central Hatchery, Pune. Human recombinant bFGF (Sigma, St. Louise, USA) was dissolved in phosphate buffered saline (PBS) containing 0.1% bovine serum albumin (BSA). The stock solution of 0.1 µg/µl concentration was aliquoted and stored at -70°C. Stock solution (10 mM) of suramin (Sigma, St. Louise, USA) was prepared in autoclaved distilled water and stored at -20°C. Eco RI and Xba I were from Bangalore Genei, Bangalore, India. All reagents for *in situ* hybridization, such as, digoxigenin (DIG) RNA labeling kit and relevant DIG detection reagents were procured from Roche Molecular Biologicals, Mannheim, Germany.

In vitro *culture of chick embryo explants*

The eggs were incubated at 37.5°C for appropriate duration of time to obtain the desired stages of development. Chick embryos were explanted and cultured *in vitro* using New's single ring technique (New, 1955). The embryo was dissected in Pannet-Compton saline (PC saline, pH 7.4; New, 1966). The yolk ball was freed of adhering albumin and the vitelline membrane was cut equatorially keeping the blastoderm in the center. The vitelline membrane was carefully peeled off along with the blastoderm and a glass ring was placed on the membrane in such a way that the blastoderm lies in the center. Extra vitelline membrane was stretched and folded over the ring to give proper tension to the blastoderm. Thin albumin was placed below the ring to provide nutrition. In this technique the embryo is cultured with its ventral side up. The embryos were staged on the basis of standard morphological criteria (Hamburger and Hamilton, 1951). Chick embryos of Hamburger-Hamilton (HH) stage 4 (gastrula stage) and HH stage 5 (head process stage) were used in the present study.

Treatment of embryos

Embryos were treated as described before (Ghaskadbi and Mulherkar, 1984; Patwardhan *et al.,* 1996). One hundred microlitres of PC saline containing the desired concentration of the test chemical was carefully

placed on the embryo, inside the ring. The embryos were left at room temperature for 30 min to allow proper diffusion of the test chemical and then incubated at 37.5°C for the desired duration of time. The different treatments were carried out as follows:

To study effects of exogenous FGF, the cultures were randomly divided into 3 groups. Groups treated with PC saline alone and 100 ng BSA/culture served as controls. The third group of embryos was treated with 10 ng bFGF/culture. The embryos were treated with FGF for 22 h for gross morphology and histology and for 2 h for the study of expression of *Brachyury* and *noggin.*

To study the effects of defective FGF signaling on the early embryonic development, embryos were treated with different concentrations (100, 150, 200 and 400 nmole/culture) of suramin for 22 h. Embryos incubated with PC saline served as controls. Embryos were treated with 200 nmoles of suramin/culture for 2 h to study expression of *Brachyury* and *noggin.*

All experiments were carried out at least in triplicate with appropriate controls.

Gross morphology and histology

The embryos were carefully studied at the end of the treatment and abnormalities, if any, were recorded. To get a quantitative estimation of the overall effect of bFGF on the embryonic development, anteroposterior length of the body axis of live embryos was measured with a micrometer scale. The embryos were then fixed in ethanol:acetic acid (3:1) at room temperature overnight. Next day, they were washed with 70% ethanol several times to remove all traces of acetic acid, stained with hematoxylin and eosin and mounted in DPX. The whole mounts were studied to record the development of different organs and systems and abnormalities therein, if any. Representative specimens were photographed using transmitted light.

For histology, several embryos were fixed overnight at room temperature in Bouin's fixative, washed thoroughly with distilled water till complete disappearance of the yellow color of picric acid, dehydrated in graded series of ethanol, cleared in benzene and embedded in paraffin wax with ceresin of M.P. 60°C (Merck, Mumbai, India). Embryos were transverse sectioned (5-7 μm thickness) with a Bright (UK) rotary retracting microtome. The sections were dewaxed and stained with hematoxylin and eosin.

Study of gene expression by whole mount *in situ hybridization*

Chicken *Brachyury* (Smith *et al.,* 1991) and *Xenopus noggin* (Smith and Harland, 1992) cDNAs cloned in pBS and pGEM5Zf(-) vectors respectively, were a kind gift from Prof. J. C. Smith, Cambridge. The plasmid *pCBRA9* was linearized with Xba I and transcribed using T3 RNA polymerase in the presence of DIG labeled UTPs to get antisense transcript of 350 bases. Likewise, DIG labeled antisense transcripts of *X-noggin* (463 bases) were generated by using T7 RNA polymerase from the plasmid *pnoggin5.5* linearized with Eco RI.

Whole mount *in situ* hybridization was performed as per Nieto *et al.* (1996) described briefly as follows. HH stage 4 chick embryos cultured in the presence of either PC saline, 100 ng BSA/culture or 10 ng FGF/culture for 2 h were fixed in 4% paraformaldehyde in PBS, pH 7.4 overnight at 4°C. The embryos were thoroughly washed in PBT (1X PBS with 0.1% Triton X-100), dehydrated in graded series of PBT:methanol and stored at -20°C in 100% methanol till further use. Prior to hybridization, the embryos were rehydrated, permeabilized (2 μg/ml proteinase K for 20 min at room temperature), refixed in 4% paraformaldehyde in PBT, pH 7.4 (20 min at 4°C). The embryos were stabilized in prehybridization buffer at 58°C for a minimum of 2 h. The embryos were then hybridized at the same temperature overnight and the unbound probe was washed off during low stringency (2XSSC, 0.1% CHAPS, 58°C, 3 washes of 20 min each) and high stringency (0.2XSSC, 0.1% CHAPS, 58°C, 3 washes of 20 min each) washes. After extensive washes with KTBT (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM KCl, 1% Triton X-100), embryos were blocked in 20% fetal calf serum in KTBT (4°C, 3h) and incubated in 1:2000 dilution of anti-DIG antibody in the blocking solution (4°C, overnight). To remove excess antibody, embryos were extensively washed with KTBT (5 washes of 1 h each), equilibrated with NTMT (100 mM Tris-HCl, pH 9.5, 50 mM MgCl$_2$, 100 mM NaCl, 0.1% Triton X-100; 2 washes of 15 min each). Embryos were then incubated in dark with NBT and BCIP. The color reaction was terminated with KTBT washes. The embryos were photographed with a combination of transmitted and incident lights.

Study of tissue specific distribution of transcripts: Brachyury *and* noggin

The colour product was fixed overnight in 4% paraformaldehyde at 4°C. Embryos were washed thoroughly with 1X PBS, gradually dehydrated to methanol (50% methanol and 100% methanol, 10 min in each grade), washed with isopropanol (10 min) and then cleared in benzene (7-10 min). Embryos were transferred to wax:benzene (1:1) mixture at room temperature (3 washes of 20 min each). The embryos were then transferred to molten wax (3 washes for 20 min each, 60°C). Embryos were embedded in paraffin wax with ceresin of M.P. 60°C (Merck, Mumbai, India). Embryos were transverse sectioned at the thickness of 15 μm with a Bright (UK) rotary retracting microtome. The sections were mounted on glass slides and spread. These were dewaxed in benzene, mounted in DPX and photographed.

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