Fibroblast growth factor regulates early mesoderm and neural development in chick embryo through its action on brachyury, goosecoid, ERNI and noggin

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We have analysed the molecular mechanism of action of fibroblast growth factor (FGF) in the development of mesodermal and neural structures in chick embryo. Experimentally altered levels of FGF signalling were found to differentially modulate expression of brachyury, goosecoid, ERNI and noggin, genes implicated in mesodermal and neural development. These effects became evident within 2 h and persisted for 6 h post-treatment, with either exogenous FGF or FGF inhibitor. Significantly, changes in gene expression correlate well with the abnormal mesodermal and neural phenotypes observed after 22 h. Thus FGF seems to regulate the development of mesodermal and neural structures in early chick embryo through its action on specific genes.

Keywords: Chick embryo, developmental gene expression, fibroblast growth factor signalling, mesoderm, nervous system.

Our earlier work has demonstrated that appropriate levels of fibroblast growth factor (FGF) signalling are essential for mesodermal and neural development in chick1. Expression of brachyury, essential for induction and differentiation of mesoderm2 and noggin, involved in the patterning of the nervous system3 and somites3, was differentially modulated within 2 h in embryos with altered levels of FGF signalling. This suggested involvement of brachyury and noggin in the early molecular events elicited by FGF1. Changes in the expression of brachyury and noggin, however, account only partially for the array of abnormalities seen in FGF- and suramin-treated embryos1.

Some of the spectacular developmental outcomes of altered FGF signalling were irregular notochord formation and shortening of body axis1. Goosecoid, an organizer-specific homeobox gene, is important in regulating gastrulation movements, specification of dorsal mesoderm and formation of body axis5,6. ERNI (Early Response to Neural Induction), a pre-neural marker, represents cells that receive the initial signals for neural induction7. FGF signalling is necessary for the acquisition of neural fate in cultured explants8 and in whole chick embryos7. In the light of these observations, we have studied the expression of goosecoid and ERNI in chick-embryo explants with altered FGF signalling. Further, along with goosecoid and ERNI, we have extended studies on brachyury and noggin expression to examine if the effects of altered FGF signalling persist for longer duration. The data reveal that action of FGF is mediated through four developmentally crucial genes; brachyury, goosecoid, ERNI and noggin. This conclusion supports our contention that FGF signalling is required for the development of neural and mesodermal structures in chick embryo.

Materials and methods

Freshly laid White Leghorn chicken eggs were obtained from a local hatchery. Suramin and human recombinant bFGF were procured from Sigma (USA).

In vitro culture and treatment of chick embryos

Eggs were incubated for 18 h at 37.5°C to obtain Hamburger Hamilton (HH) stage 4 embryos9, cultured in vitro by New’s single ring technique10 and treated with either PC saline, BSA (100 ng/culture), 6 mM bFGF or 2 mM suramin, as described1. Embryos treated with either PC saline11 or PC saline containing BSA served as control. At the end of 2, 4 or 6 h of incubation at 37.5°C, the embryos were either fixed in 4% paraformaldehyde for whole mount in situ hybridization or homogenized in TRIzol reagent (Gibco BRL) for RNA extraction12.

Study of gene expression

DIG-labelled UTPs (Roche Molecular Biologicals, Germany) were used to generate the anti-sense riboprobes. cGsc plasmid (kind gift from Dr J. C. Izpisua-Belmonte,
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California) was linearized with NolI and transcribed using T3 RNA polymerase to get gooseoid probe of 2 kb size. A 2.1 kb cERNI probe was generated by linearizing cERNI 2 Sub plasmid (kind gift from Prof. C. D. Stern, London) with KpnI and transcribing with T3 polymerase. cBRA9 plasmid (kind gift from Prof. J. C. Smith, Cambridge, UK) was linearized with XbaI and transcribed using T3 RNA polymerase to generate 350 bases probe. Plasmid cNog (kind gift from Dr J. Cooke, London) was linearized with SacII and transcribed with T7 RNA polymerase to get a 1.3 kb probe.

Whole-mount in situ hybridization was performed according to Nieto et al., with a few modifications. Hybridization was carried out at 58°C for brachyury, 65°C for gooseoid and noggin, and 70°C for ERNI probes. The tissue-specific distribution of the transcripts was studied as described earlier. Northern hybridization was carried out according to Karandikar and Ghaskadbi. Hybridization temperature was 65°C for brachyury, 70°C for gooseoid and noggin, and 74°C for ERNI. The blots were scanned on a gel documentation system (Herolab, Germany). The staining intensity of the signals obtained in the control and treated groups was compared using E.A.S.Y.Win32 software (Herolab, Wiesloch, Germany) to estimate relative abundance of transcripts in different tracks. Further, the intensities of 28S and 18S RNA bands from the control and treated groups were compared to ensure loading of equal amounts of total RNA from each group. Any bias in the loading amount was normalized against the intensity of these bands obtained after hybridization in the corresponding track. All the experiments were carried out at least three times.

Results

Effects of FGF and suramin on gooseoid expression

In control (PC saline- and BSA-treated) embryos, gooseoid expression was seen predominantly on the lateral sides of the Hensen’s node (Figure 1a) in ectodermal, mesodermal and endodermal cells (Figure 1b). The post-nodal region of the primitive streak was faintly stained (Figure 1a) and barely detectable in transverse sections (T.S.; Figure 1c). FGF-treated embryos showed reduced staining intensity on the lateral sides of the Hensen’s node, suggesting down-regulation of gooseoid in over 77% (27/35) embryos (Figure 1d). The spatial distribution of gooseoid transcripts (Figure 1d–f) was however comparable to controls (Figure 1a–c). In more than 80% (30/37) of suramin-treated embryos, a slight upregulation of gooseoid was seen, especially on the lateral sides of the Hensen’s node (Figure 1g) with transcript distribution pattern (Figure 1h and i) matching the controls (Figure 1b and c).

By the end of 4 h of treatment with either saline, FGF or suramin, around 50% of the embryos treated at stage 4 attained stage 4’. Control embryos showed gooseoid expression at the tip of the Hensen’s node, in the newly formed head process, neural plate and at very low levels in the primitive streak (Figure 1j). Down-regulated expression of gooseoid in the neural plate, head process and primitive streak was observed after 4 h in about 70% (21/30) of FGF-treated embryos (Figure 1k). All (28/28) suramin-treated embryos (Figure 1l) exhibited gooseoid expression comparable to controls.

About 70% of stage-4 embryos attained stage 5 by the end of 6 h of incubation. In control embryo, gooseoid expression was detected at the tip of the Hensen’s node, in the head process, neural plate and primitive streak (Figure 1m). More than 70% (18/25) of the FGF-treated embryos showed reduced gooseoid expression in the neural plate, head process and primitive streak (Figure 1n). Suramin-treated embryos (21/21) showed gooseoid expression comparable to controls (Figure 1o).

Northern hybridization revealed about 20% reduction in the relative abundance of gooseoid transcripts in FGF-treated embryos and around 12% enhancement in suramin-treated embryos at the end of 2 h treatment (Figure 1p and q). The modulations in gooseoid expression levels were statistically significant in both FGF and suramin-treated embryos.

Effects of FGF and suramin on ERNI expression

At the end of 2 h incubation, ERNI transcripts were detected in the prospective neural plate in over 93% (27/29) of control embryos (Figure 2a). Ectodermal cells of the prospective neural plate (Figure 2b), and ectodermal as well as a few mesodermal cells of the Hensen’s node (Figure 2c) were clearly positive for ERNI transcripts. Cells in the posterior two-thirds of the primitive streak (Figure 2a and d) were faintly stained, if at all. FGF-treatment led to an increase in ERNI expression in the ectodermal cells of the prospective neural plate (Figure 2e–g) and in both ectodermal and mesodermal cells of the Hensen’s node (Figure 2g). Compared to the controls (Figure 2a and d), ERNI expression domain was extended in the posterior one-third region of the primitive streak after FGF treatment (Figure 2e and h). A few endodermal cells also exhibited de novo expression of ERNI in these embryos (Figure 2f and g). More than 80% (27/33) of FGF-treated embryos exhibited modulation of ERNI expression. In about 88% (24/27) of suramin-treated embryos (Figure 2i) ERNI expression was slightly upregulated in the prospective neural plate compared to controls (Figure 2a), without alteration in the pattern of tissue-specific distribution of transcripts (Figure 2j–l).

Control embryos showed expression of ERNI in the neural plate and tip of the Hensen’s node (Figure 2m) by
the end of 4 h of incubation. FGF-induced upregulation of ERNI in the neural plate continued in about 75% (18/24) embryos (Figure 2 n), with the expression domain (Figure 2 n) comparable to controls (Figure 2 m). In about 60% (17/28) of suramin-treated embryos (Figure 2 o), the staining intensity was more in the neural plate than controls (Figure 2 m), but still lower than FGF-treated embryos (Figure 2 n).

By 6 h of incubation, ERNI expression started getting cleared from the centre and became restricted to the border of the neural plate in control embryos (Figure 2 p). Upregulation of ERNI expression was seen in 75% (21/28) of FGF-treated embryos (Figure 2 q) in areas comparable to controls (Figure 2 p). In suramin-treated embryos (25/25), ERNI expression was comparable to the controls in terms of both intensity and spatial distribution (Figure 2 r).

The upregulation of ERNI expression observed within 2 h was about 37% and 12% in FGF- and suramin-treated embryo respectively, as seen from northern analysis (Figure 2 s and t).

Effects of FGF and suramin on brachyury expression

Earlier, we had observed significant down-regulation of brachyury expression in FGF-treated embryos after 2 h of treatment. About 60% of suramin-treated embryos showed slight upregulation of brachyury expression all along the primitive streak, while in about 30% embryos cells from the posterior two-thirds region of the primitive streak failed to express brachyury. These studies were extended up to 6 h.

Figure 1. Effects of fibroblast growth factor (FGF) and suramin on goosecoid expression. a−i. Two hours treatment: a−c, Expression in control embryo. a, Note staining on lateral sides of the Hensen’s node (Hn) and very faint staining in the primitive streak (ps). T.S. passing through the Hn exhibits transcripts in ectodermal (ec), mesodermal (me) and endodermal (en) cells (b) and barely detectable levels in the post-nodal primitive streak (c). d−f. Reduced expression in FGF-treated embryo in tissues comparable to control. g−i, Suramin-treated embryo. Note enhanced expression on lateral sides of the Hn (g) with tissue-specific distribution identical to control (h, i). j−l. Four hours treatment: j, Control embryos at stage 4+ show goosecoid expression at the tip of the Hn, in the head process (hp), neural plate (np) and ps. k, FGF-treated embryo with reduced staining in the hp, np and ps. l, Goosecoid expression in suramin-treated embryo is comparable to controls. m−o. Six hours treatment: m, Control embryos show goosecoid expression at the tip of the Hn, hp, np and ps. n, Reduced expression in comparable tissues of FGF-treated embryo. o, Suramin-treated embryo with staining comparable to controls. p, (Upper panel) Northern blot showing goosecoid transcript levels at the end of 2 h treatment in control [1], FGF-treated [2] and suramin-treated [3] embryos. (Lower panel) Northern blot signal intensities were normalized against the 28S and 18S bands of ethidium bromide-stained RNA from the respective group. q, Histogram depicting the relative abundance of transcripts from the three groups.
At the end of 4 h incubation, control embryos that attained stage 4” exhibited \textit{brachyury} expression in the cells all along the primitive streak with intense staining at the Hensen’s node (Figure 3a). The newly emerging head process also expressed \textit{brachyury} (not shown). Almost two-thirds (19/30) of FGF-treated embryos showed continued down-regulation of \textit{brachyury} expression in comparable embryonic regions (Figure 3b). In a large proportion (about 71%, 20/28) of suramin-treated embryos (Figure 3c), the expression of \textit{brachyury} was comparable to the controls. A small proportion of the suramin-treated embryos (around 28%, 8/28) that were still at stage 4 showed absence of \textit{brachyury} transcripts in the posterior two-thirds primitive streak (Figure 3d).

In control embryos \textit{brachyury} was expressed in the cells of the primitive streak, Hensen’s node and head process by the end of 6 h of incubation (Figure 3e). Down-regulation of \textit{brachyury} expression was persistent in the primitive streak cells and head process in about 65% (16/24) embryos treated with FGF (Figure 3f). In majority of suramin-treated (20/25) embryos, expression of \textit{brachyury} was comparable to controls (Figure 3g) while in the rest of the embryos, cells of the posterior one-third primitive streak were faintly stained (Figure 3h).

Northern analysis of embryos treated for 2 h with either saline, FGF or suramin revealed around 26% reduction after FGF treatment, whereas in suramin-treated embryos the alteration in abundance of \textit{brachyury} transcripts was not statistically significant (Figure 3i and j).

\textbf{Figure 2.} Effects of FGF and suramin on \textit{ERNI} expression. \textit{a}-\textit{j}. Two hours treatment: \textit{a-\textit{d}}, Expression in control embryo. \textit{a}. Note stained prospective neural plate (pnp) and unstained primitive streak (ps). In T.S., note transcripts mainly in the ectodermal cells (ec) in the pnp (b) and in both ec and a few mesodermal cells (me) in the Hensen’s node (Hn); ec are not stained (arrowhead, c). Posterior region of the ps is barely stained (arrows in \textit{a}, \textit{d}. \textit{e}-\textit{h}, Expression in FGF-treated embryo. \textit{e}-\textit{g}, FGF treatment causes upregulation of \textit{ERNI} expression in the ec cells of the pnp, and in the Hn both ec and me cells show enhanced expression of \textit{ERNI}. Note de novo expression in a few endodermal cells (arrowhead, \textit{g}) and extended expression domain in the posterior one-third of the ps (arrows in \textit{e}, \textit{h}). \textit{i}-\textit{t}, ERNI expression in suramin-treated embryo. Note upregulated expression in ec of the pnp (\textit{j}) and in both ec and me cells of the Hn (arrow, \textit{k}). Note the absence of transcripts in the ec (\textit{k}) and in the posterior region of the ps (\textit{l}, arrow in \textit{i}). \textit{m-\textit{o}}, Four hours treatment: \textit{m}, Staining is seen in the Hn and neural plate (np) in control embryo. \textit{n}, \textit{ERNI} expression is upregulated in FGF-treated embryo in a domain comparable to control (\textit{m}). \textit{o}, In suramin-treated embryo the expression levels are higher than controls (\textit{m}) but lower than FGF (\textit{n}). \textit{p-\textit{r}}, Six hours treatment: \textit{p}, Control embryo with intense staining at the border of the np and tip of the Hn. \textit{q}, FGF-induced up-regulation of \textit{ERNI} expression is still persistent in the np. \textit{r}, Suramin-treated embryo shows expression domain and staining intensity comparable to the control. \textit{s}, (Upper panel) Northern blot with \textit{ERNI} expression levels at the end of 2 h incubation in control [1], FGF-treated [2] and suramin-treated [3] embryos. (Lower panel) Ethidium bromide-stained RNA from the respective groups used for normalization of signal intensities. \textit{t}, Histogram displaying relative abundance of transcripts.
Effects of FGF and suramin on noggin expression

Noggin transcripts were detected at the tip of the Hen- 
sen’s node, while the rest of the primitive streak was 
faintly stained in control embryos at the end of 2 h incu-
bation. Besides the enhanced expression along the 
primitive streak and Hensen’s node, de novo expression 
of noggin was detected in the prospective neural plate in FGF-
treated embryos. In suramin-treated embryos, noggin ex-
pression was enhanced along the primitive streak and 
specifically on the lateral sides of the Hensen’s node.

At the end of 4 h incubation, control embryos showed 
noggin expression mainly in the neural plate. The primi-
tive streak, Hensen’s node and newly formed head 
process exhibited very low levels of noggin expression 
(Figure 4a). More than 80% (24/29) of FGF-treated em-
byos showed enhanced expression in the neural plate and 
the at the tip of the Hensen’s node (Figure 4b). In the sura-
min-treated group, the expression was slightly enhanced in 
the neural plate in half (14/27) the embryos (Figure 4c).

In comparison with 4 h incubated control embryos 
(Figure 4a), noggin expression was slightly enhanced at 
the tip of the Hensen’s node, in the neural plate and head 
process in 6 h incubated control embryos (Figure 4d). 
FGF treatment appeared to upregulate noggin expression 
in the comparable regions (Figure 4e) in about 75% 
(16/21) of the embryos. In suramin-treated embryos, 
expression was enhanced only in the neural plate, while 
the Hensen’s node was almost negative for noggin tran-
scripts in about 55% (11/20) embryos (Figure 4f).

In chick, two noggin transcripts of 5.3 and 2.9 kb have 
been reported. The 5.3 kb band was used for intensity 
analysis as it was consistently more prominent in all the 
blots. Enhancement in the number of noggin transcripts was 
around 40% in FGF-treated and 50% in suramin-treated 
embryos at the end of 2 h incubation (Figure 4g and h).

Discussion

We have treated in vitro cultured chick embryos with 
either exogeneous FGF or suramin to alter the endogenous 
levels of FGF signalling. Although suramin affects a variety 
of growth factors, it predominantly inhibits FGF action. 
Suramin has been used to determine the role of FGF sig-
alling in the development of mesodermal structures and 
neural retina cells. Brachury and noggin, genes 
crucial in mesoderm and neural development, are 
amongst the mediators of the molecular cascades initiated 
and sustained by FGF. In view of the array of abnormalities 
induced by FGF and suramin, we have further studied 
the effects of altered FGF signalling on goosecoid and 
ERNI, genes important in the affected processes, and have 
extended our studies on the effects of altered FGF signal-
ling on brachury and noggin. Thus we report modulation 
of expression of brachury, goosecoid, ERNI and noggin 
for up to 6 h post-treatment with either excess FGF or 
suramin, and the probable mechanism of FGF action.
Effects of FGF and suramin on goosecoid and brachyury expression

Our results show that altered FGF signalling modulates goosecoid and brachyury expression. FGF treatment caused down-regulation of both the genes within 2 h, which was maintained for at least 6 h post-treatment. Cells expressing goosecoid participate in gastrulation movements, while sufficient amounts of brachyury are essential for the convergent extension movements in embryos. The data suggest that altered expression of goosecoid and brachyury may have led to improper gastrulation movements, resulting in abnormal morphogenesis in embryos with altered FGF signalling. FGF has indeed been shown to be necessary for cell movements during streak formation and mesoderm differentiation in chick embryos. FGF-treated embryos exhibited improperly formed notochord and significant reduction in the body axis. Goosecoid expressing cells contribute to dorsal mesoderm, mainly the notochord. Brachyury too is necessary for specification of the notochord, and its expression below threshold levels leads to reduced axial mesoderm. Antero-posterior body axis is determined by the neural tube which is formed and patterned through the action of certain paracrine signals, emanating mainly from the underlying notochord. It is likely that the reduced amounts of goosecoid and brachyury transcripts together result in the formation of lesser amounts of notochord tissue which, in turn, may lead to shortening of body axis in FGF-treated embryos.

Embryos treated with suramin regain normal expression of goosecoid within 4 h and brachyury (80% embryos) by the end of 6 h incubation. Both, brachyury and goosecoid encode transcription factors, which regulate the expression of a number of downstream genes. Low levels of both these transcripts in the initial 2 h of development of stage-4 embryos seem to be enough to bring about abnormal development of mesodermal structures in suramin-treated embryos. Recovery of expression of these genes during subsequent development, thus, is not capable of alleviating the developmental abnormalities brought about by the initial reduction in their expression.

Figure 4. Effects of FGF and suramin on noggin expression. a-c. Four hours treatment: a. Staining seen mainly in the neural plate (np), while the head process (hp), Hensen’s node (Hn) and primitive streak (ps) exhibit low levels of transcripts in control embryos. b. Treatment with FGF leads to enhanced noggin expression in the np and tip of the Hn. c. Slightly upregulated expression seen in the np of suramin-treated embryos. d-f. Six hours treatment: In comparison with 4 h incubated control embryos (a), 6 h incubated control embryo shows elevated expression in the np, hp and Hn (d). e. Note upregulated noggin expression in comparable regions of FGF-treated embryos. f. Enhanced expression is seen only in the np of suramin-treated embryos, while Hn and hp show absence of transcripts. g. (Upper panel) Northern blot with noggin expression levels at the end of 2 h treatment in control [1], FGF-treated [2] and suramin-treated [3] embryos. Out of two bands obtained on the blot, the higher molecular weight band was used for intensity analysis as it was consistently more prominent in all blots. (Lower panel) Northern blot signal intensities are normalized against the 28S and 18S bands of ethidium bromide-stained RNA from the respective groups. h. Histogram showing relative abundance of transcripts.

Effects of FGF and suramin on ENR1 and noggin expression

FGF is known to induce transient expression of early pre-neural markers. FGF-soaked beads induce ENR1 as quickly and strongly as Hensen’s node within 2 h after implantation. We detected enhanced expression of ENR1 within 2 h after FGF treatment, which was maintained for 6 h. FGF led to de novo expression of ENR1 in the posterior one-third primitive streak cells and in a few endodermal cells by 2 h. However, at later time-points the expression domain became comparable to the respective controls. The present results support earlier studies wherein FGF was shown to be sufficient to induce early steps of activation during neural induction. However,
subsequent maintenance mechanisms are required to stabilize the neural fate.  

Significant upregulation of ERNI expression in the prospective neural plate region of suramin-treated embryos within 2 h was surprising. These observations do not agree with an earlier report which shows that the Hen sen's node graft cannot induce ERNI in the presence SU5402 (FGF-receptor inhibitor). ERNI encodes a coiled-coil protein that inhibits premature expression of Sox2, the earliest definitive neural plate marker. Low levels of FGF signaling are thought to sensitize the ectoderm to other neural-inducing cues in vivo. Absence of down-regulation of ERNI expression in suramin-treated embryos could be due to reduced but persisting FGF signaling. However, the cause of significant upregulation of ERNI expression observed in the present work is not yet known. With the increase in treatment time from 2 to 4 and 6 h, the proportion of suramin-treated embryos with normal pattern and levels of ERNI expression increased, suggesting that ERNI expression is only transiently affected in these embryos.

We have reported that noggin expression is elevated in both FGF- and suramin-treated embryos within 2 h. In the present study, we show that the elevated levels of noggin expression are maintained in FGF- and suramin-treated embryos for 6 h of treatment. Noggin, a secreted molecule, participates in the patterning of neural tube and somites. Altered levels of noggin expression thus could be partially responsible for abnormal development of the nervous system and somites in FGF- and suramin-treated embryos.

Summary and conclusions

Our work shows that the abnormal development of neural and mesodermal structures in FGF- and suramin-treated embryos is not only accompanied by, but appears to be an outcome of the rapid modulation of brachyury, goosecoid, ERNI and noggin. We thus demonstrate that FGF signaling plays an important role in the cascade of molecular events during the development of nervous system and mesodermal structures in chick embryo. We propose brachyury, goosecoid, ERNI and noggin to be amongst the crucial mediators of FGF action. The interplay between the various components of this complex network remains to be elucidated.


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