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FGF signalling in the early specification of mesoderm in *Xenopus*

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SUMMARY

We have examined the role of FGF signalling in the development of muscle and notochord and in the expression of early mesodermal markers in *Xenopus* embryos. Disruption of the FGF signalling pathway by expression of a dominant negative construct of the FGF receptor (XFD) generally results in gastrulation defects that are later evident in the formation of the trunk and tail, though head structures are formed nearly normally. These defects are reflected in the loss of notochord and muscle. Even in embryos that show mild defects and gastrulate properly, muscle formation is impaired, suggesting that morphogenesis and tissue differentiation each depend on FGF. The XFD protein inhibits the expression of the immediate early gene *brachyury* throughout the marginal zone, including the dorsal side; it does not, however, inhibit the dorsal lip marker *goosecoid*, which is expressed in the first involuting mesoderm at the dorsal side that will underlie the head. The XFD protein also inhibits *Xpo* expression, an immediate early marker of ventral and lateral mesoderm. These results suggest that FGF is involved in the earliest events of most mesoderm induction that occur before gastrulation and that the early dorsal mesoderm is already composed of two cell populations that differ in their requirements for FGF.

Key words: *Xenopus*, FGF receptor, mesoderm induction

INTRODUCTION

Following a series of rapid cell divisions, the amphibian embryo increases in complexity by a number of cell-cell interactions or inductions. The first known induction leads to the formation and patterning of the mesoderm in the equatorial region of the embryo at the blastula and gastrula stages (reviewed by Nieuwkoop, 1973). Although the behavior of signalling and responding cells was well-established over 60 years ago (reviewed by Hamburger, 1988), the molecular nature of the signals responsible for this induction has only recently been discovered. Several members of the fibroblast growth factor (FGF) family and the transforming growth factor-β (TGF-β) family are now implicated in this process (reviewed by Kimelman et al., 1992). Members of both families induce mesoderm in epithelial cells of animal cap blastomeres of *Xenopus* embryos (Kimelman and Kirschner, 1987; Rosa et al., 1988; Smith et al., 1990; Thomsen et al., 1990). The mRNAs that encode members of the FGF family (bFGF and XeFGF) and the TGF-β family (Vg1, Activin B, and BMP-4) are present at the time of mesoderm induction, further implicating these growth factors in the specification and patterning of the mesoderm (Kimelman and Kirschner, 1987; Isaacs et al., 1992; Weeks and Melton, 1987; Thomsen et al., 1990; Koster et al., 1991; Dale et al., 1992). In addition, other growth factor-like proteins, namely members of the Wnt family and Noggin, are also present in the early embryo and can influence the type of the mesoderm that is induced (Sokol et al., 1991; Smith and Harland, 1991, 1992; reviewed by Moon and Christian, 1992). Though a role for peptide growth factors in the induction of mesoderm in the amphibian embryo is now well-established, the specific process of tissue specification of the mesoderm remains unclear.

While studies of explanted animal hemisphere cells have been useful in demonstrating the capacity of early embryonic tissues to be respecified, they cannot be used to define the endogenous role of putative signals. For example, there is considerable overlap in the responses of animal caps to FGF and activins, so that it is difficult to determine their roles in vivo. Moreover, it is at present difficult to obtain information concerning the spatial and temporal localization of the growth factors and their posttranslational activation. For this reason we have focused on inhibiting the endogenous FGF signalling pathway in the intact embryo and on assaying the consequences of these inhibitions.

Recently, we succeeded in inhibiting the endogenous FGF receptor signalling pathway in developing embryos by expressing a dominant negative construct of the FGF receptor (XFD), comprising the extracellular and transmembrane domains (Amaya et al., 1991). This defective receptor, which lacks the catalytic cytoplasmic domain, presumably forms non-functional heterodimers with endogenous FGF receptors, thereby blocking their ability to phosphorylate and activate the vicinal catalytic domains of the native receptors and to phosphorylate other targets. Embryos expressing this XFD construct show specific defects in gas-

trulation as well as major deficiencies in trunk and posterior development. By stage 35, these embryos have grossly abnormal trunks but surprisingly normal heads. The defects are reflected in the apparently normal involution of the most dorsal mesoderm but the failure of lateral and ventral mesoderm to involute properly. The effect on lateral and ventral mesoderm suggests a very specific role for FGF, although in the absence of biochemical and immunocytochemical data, it was not possible to determine whether in these experiments all regions of the embryo were equally accessible to the dominant negative FGF receptor construct.

In this report, we have studied the effect of inhibiting FGF signalling on the expression patterns of genes expressed early as well as late in gastrulation. Using immunocytochemistry and in situ hybridization, we have determined the effect of the dominant negative FGF receptor construct on differentiation of muscle and notochord in older embryos and on the expression of several early mesodermal transcription factors at the gastrula stage. We have found that expression of this construct inhibited expression of early mesodermal transcription factors and that this inhibition occurred before any morphogenetic abnormality was evident in the embryos. Furthermore, expression patterns of some dorsal mesoderm markers were dependent on FGF while others were not. These differences on the dorsal side identified two distinct cell populations of the early involuting mesoderm involved in head and notochord specification. Inhibition of mesoderm formation continued into the tailbud stages, during which cells expressing the dominant negative construct failed to differentiate into muscle or notochord. These results show a broad pattern of FGF dependence in the earliest signalling events.

MATERIALS AND METHODS

Plasmids and in vitro transcriptions

The dominant negative construct XFD/Xss and the non-functional construct d50/Xss of the FGF receptor have been described previously (Amaya et al., 1991). The non-functional construct HAVØ has a three amino acid deletion in the extracellular domain (histidine 162, alanine 163 and valine 164) (Byers et al., 1992). The *Hin*dIII site at nucleotide site 482 of the wild-type FGF receptor corresponds to the codon for histidine 162. An oligonucleotide of the sequence 5′AAGAAGCTTCCAGCAGCAAAAACTG3′ was used to create an in frame *Hin*dIII site at nucleotide 491 by polymerase chain reaction mutagenesis (Vallette et al., 1989). The 523 bp *Hin*dIII-*Acc*I fragment within XFD/Xss was replaced by the 514 bp *Hin*dIII-*Acc*I PCR fragment thus deleting the three intervening amino acids. XFD/Xss, d50/Xss and HAVø were linearized with *Eco*RI and transcribed with SP6 RNA polymerase as previously described (Melton et al., 1984). The β-galactosidase construct (pSP64nucβgal) has been described previously (Smith and Harland, 1991; Vize et al., 1991). The plasmid was linearized with *Xho*I and transcribed with SP6 RNA polymerase.

Embryo injections

Xenopus laevis albino or pigmented females were injected, in the dorsal lymph sac, with 500-1000 IU of human chorionic gonadotropin (Sigma) to induce ovulation. Eggs were stripped from the ovulating females and fertilized as previously described (Newport and Kirschner, 1982). After 45 minutes the embryos were dejellied in 2% cysteine pH 7.8 (Sigma), transferred into 0.1× MMR (Newport and Kirschner, 1982) and maintained at 16°C. To maximize the available time for injections, fertilizations were performed 30-45 minutes apart until the embryos from the first fertilization divided. At the two-cell stage, the embryos were transferred into a solution containing 1× MMR, 6% Ficoll (Sigma), penicillin and streptomycin. One or both blastomeres of embryos at the two-cell stage were injected with 15-25 nl of 0.1-0.15 mg/ml RNA. The needle was always inserted into the marginal zone of the embryos (the border between the pigmented half and the non-pigmented half), or in some cases the region just below this zone. For injections into the dorsal marginal zone, pigmented embryos that showed a clear dorsal-ventral axis at the four-cell stage (i.e., embryos with two blastomeres that were more pigmented than the other two blastomeres) were selected. The two dorsal (less pigmented) blastomeres were injected with 10-15 nl of 0.1-0.15 mg/ml RNA. 1-2 hours after injection, embryos were transferred to 0.1× MMR, 6% Ficoll, penicillin and streptomycin, and then incubated at 18°C until needed. The embryos were staged according to Nieuwkoop and Faber (1967).

Antibody preparation

The *Xenopus* FGF receptor antibody (R#1) was generated using a glutathione transferase-partial FGF receptor fusion protein as previously described (Amaya et al., 1991). The R#1 antiserum was produced in a different rabbit than the FGF receptor antiserum, which was used in the earlier report. We have found the R#1 antibody to be of superior quality for immunoblotting and immunocytochemistry. The R#1 antibody was affinity purified on either western strips (Smith and Fisher, 1984) or columns containing bound *Xenopus* FGF receptor (Harlow and Lane, 1988).

Immunoblotting

Ten embryos from each stage were transferred into an Eppendorf tube (1.5 ml) containing 0.5 ml of ice-cold lysis buffer (20 mM Tris pH 8, 100 mM NaCl, 1 mM EDTA, 1% Triton X-100, 10% glycerol, 0.1 mM PMSF, and 10 μg/ml chymostatin, leupeptin, and aprotinin). The embryos were incubated on ice for 15 minutes and lysed by disrupting them through a yellow pipette tip. The lysed embryos were centrifuged at 16,000 r.p.m. for 15 minutes at 4°C, the clear supernatants were transferred, and 50 μl of wheat germ agglutinin beads (Pharmacia) was added to each supernatant. After an overnight incubation at 4°C, the beads were washed three to four times with fresh lysis buffer, eluted into sample buffer with 0.1 M DTT and boiled for 5 minutes. The eluted proteins were separated on a 5-15% polyacrylamide gradient gel (Laemmli, 1970) and transferred to Immobilon (Millipore). After blocking with 5% nonfat milk (in 1× PBS and 0.1% Tween 20), the membrane was incubated with affinity purified R#1 antiserum (1:2000) followed by anti-rabbit secondary IgG conjugated with horseradish peroxidase. The blot was exposed for 30 seconds to 5 minutes to visualize the proteins using enhanced chemiluminescence (Amersham).

Collagen type II gene isolation

The collagen type II gene was cloned by screening a *Xenopus laevis* stage 17 cDNA library (λgt10; Kintner and Melton, 1987) with the collagen type I gene from the chick (plasmid: pCg45; Lehrach et al., 1978). Several clones that were isolated contained inserts greater than 5 kb. Sequence analysis of the 3′ end of one of these clones showed highest sequence similarity with collagen type II genes from other species. The same *Xenopus* collagen type II gene has been more thoroughly characterized (Su et al., 1991; Bieker and Yazdani-Buicky, 1992).

In situ hybridizations

The protocols for probe preparation and for the whole-mount in situ hybridization procedure have been described previously (Har-

land, 1991). The proteinase K step in the procedure could be deleted for the *brachyury*, collagen type II and cardiac actin in situ hybridizations with little effect on the strength of the hybridization signal. The cardiac actin probe was generated by transcribing *Pvu*II-digested AC100 with Sp6 (Hemmati-Brivanlou et al., 1990); the collagen type II probe was generated by transcribing a 450 bp fragment in the 3′ end of the gene (plasmid: pXK500); full length *brachyury*, *Xpo* and *goosecoid* probes were transcribed with T7 RNA polymerase (plasmids: pXT1, XpoHK and gs, respectively).

Pigmented embryos at the gastrula stages were not cleared with the benzyl alcohol:benzyl benzoate (1:2) mixture after the in situ procedure. The pigment in these embryos did not interfere with the in situ label, since the markers that were studied did not label the animal pole. When the embryos were cleared, transmitted light was used; when the embryos were not cleared, reflected light from fiber optic light sources was used. Embryos were photographed with Kodak Ektachrome 160T film using a Zeiss Axiophot microscope.

Immunocytochemistry

Whole-mount immunocytochemistry was performed essentially as previously described (Hemmati-Brivanlou and Harland, 1989). The 12/101 hybridoma cell line (Kintner and Brockes, 1984) was obtained from the Developmental Studies Hybridoma Bank maintained by the Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine, Baltimore MA, USA. The affinity purified R#1 antibody was used at a 1:500 dilution and the 12/101 monoclonal supernatant was used at a 1:5 dilution. Whenever immunocytochemistry and in situ hybridizations were done on the same embryos, the in situ procedure was performed first and the proteinase K step in the in situ protocol was eliminated.

β-galactosidase assay

The vitelline membrane was manually removed from the embryos using watchmakers forceps and the embryos were fixed at room temperature for 15 minutes in 1% gluteraldehyde in 50 mM sodium cadodylate buffer at pH 7.3. The fixed embryos were washed for 5 minutes in 0.5 ml of Fe/NaP solution (7.2 mM Na_2HPO_4, 2.8 mM NaH_2PO_4, 150 mM NaCl, 1 mM $MgCl_2$, 3 mM $K_3(Fe(CN)_6)$, 3 mM $K_4(CN)_6$), pH 7.2). Staining was performed in 0.5 ml of fresh Fe/NaP solution containing 0.1% Triton X-100 and 0.027% X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) at 37°C for 1-2 hours. When the intensity of staining was adequate the embryos were refixed for 1 hour in MEMFA buffer (0.1 M MOPS, 2 mM EGTA, 1 mM $MgSO_4$, and 3.7% formaldyhyde, pH 7.4) and stored overnight in 100% methanol. The β-galactosidase stained embryos were then used for either in situ hybridizations or immunocytochemistry.

RESULTS

Expression of FGF receptor constructs in embryos

In earlier experiments we inhibited the FGF signalling pathway in embryos by expressing a truncated form of the FGF receptor (XFD) that interfered with the activity of endogenous FGF receptors (Amaya et al., 1991). In these first experiments the effect of XFD expression was assayed biochemically and morphologically but the exact distribution and persistence of the XFD protein was not determined.

We have now examined the level of XFD expression at different stages of embryo development. Embryos expressing the dominant negative construct (XFD embryos) were generated by injecting embryos at the two- to four-cell stage with XFD RNA. As a control, RNA encoding a non-functional form of XFD that contains a three amino acid deletion in the extracellular domain (HAV∅, ''HAV not'') was injected (Byers et al., 1992). At various developmental stages the glycosylated proteins (bound to wheat germ agglutinin beads) were analyzed by western blot using anti-FGF receptor antibody. XFD and HAV∅ RNAs were efficiently translated and glycosylated throughout early development to stage 26 (mid tail bud). At the 16-cell stage (st. 4), the proteins were already abundant but not fully glycosylated (Fig. 1A). At the gastrula stage (st. 11), the proteins were fully glycosylated and had reached their highest level. By the mid tailbud stage (st. 26) the XFD protein was still abundant, whereas the HAV∅ construct had declined. The endogenous FGF receptor could be detected when this blot was overexposed (data not shown), indicating that the translated levels of the truncated receptors were at least 10- to 20-fold higher than the endogenous FGF receptor.

We also studied the distribution of the translated constructs by whole-mount immunocytochemistry using the same *Xenopus* FGF receptor antibody. The endogenous receptor is present throughout the animal hemisphere as shown by Ding et al. (1992) who stained embryos with a human anti-FGFR1 antibody. We have repeated these experiments with the antibody to the *Xenopus* FGF receptor with the same results (P. S. and T. J. M., unpublished observation). In particular, at the early gastrula stage, the receptor was present throughout the animal cap and the entire marginal zone, including the prospective prechordal and chordamesoderm. In embryos injected with XFD RNA, the protein could be detected easily from the 16-cell stage to the late tailbud stage (data not shown). The staining at each stage appeared to be at the cell surface (Fig. 1B,C). Even when we injected all of the blastomeres at the two- or four-cell stage with XFD RNA, we were unable to generate embryos that expressed the constructs uniformly throughout the embryo. We believe that this result is due to the slow diffusion of the RNA from the injection site and the rapid partitioning of the cytoplasm during cleavage. As a consequence XFD embryos were somewhat mosaic in their expression and presumably in their inhibition of FGF receptor function. This non-uniformity in distribution is most likely responsible for some of the variability in the observed defects (Amaya et al., 1991). To analyze such experiments we had to develop other methods of analysis that allowed us to ascertain the pattern of expression of the injected RNA.

Effects of inhibiting FGF receptor function on muscle and notochord differentiation

At neurula and tailbud stages the most abundant recognizable mesodermal derivatives are muscle and notochord. We have examined the effects of expressing the dominant negative construct of the FGF receptor on the differentiation of these tissues. Although we had previously shown that muscle actin expression was diminished in XFD embryos, these earlier studies did not take into account the mosaic pattern of expression of the injected RNAs. To assess fairly the inhibition of actin it was necessary to examine

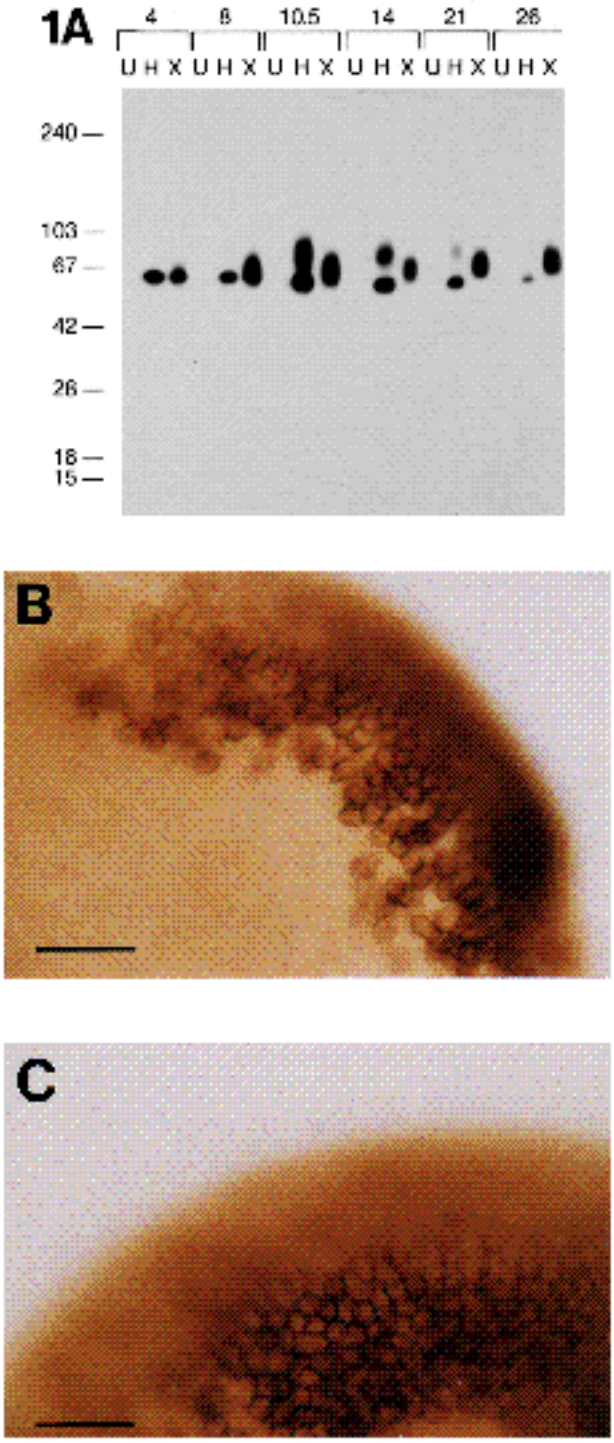


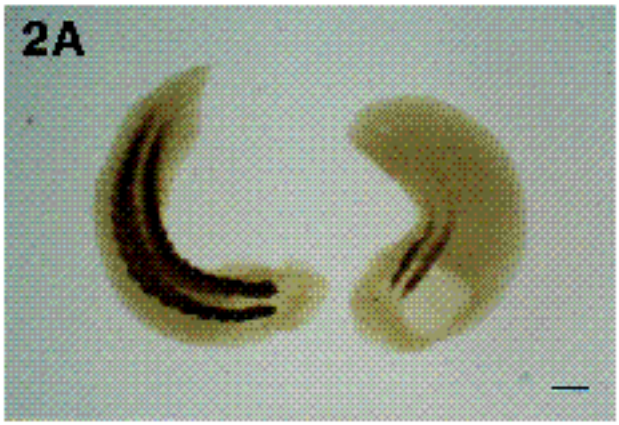
Fig. 1. Translation of the FGF receptor constructs during early development. (A) One blastomere of two-cell stage embryos were injected with HAV∅(H) or XFD (X) RNA. At stages 4, 8, 10.5, 14, 21 and 26, the glycosylated proteins from the injected and uninjected (U) embryos were separated on an acrylamide gel, transferred to a nylon membrane and incubated with an affinity purified FGF receptor antibody. Molecular mass standards ($\times 10^{-3}$) are indicated on the left. (B, C) Two-cell stage embryos were injected with HAV∅RNA (B) and XFD RNA (C). At the mid gastrula stage (st. 11) the embryos were fixed and the expressed truncated FGF receptors were localized by immunohistochemistry using an FGF receptor antibody. Scale bars, 60 μm.

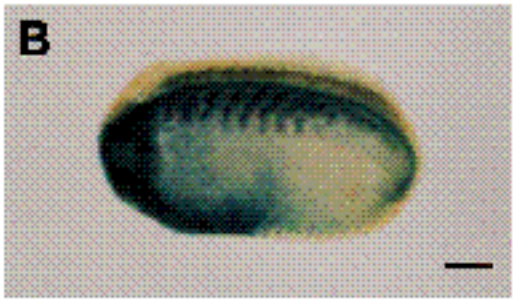
expression in the local regions that expressed the XFD constructs, using whole-mount immunocytochemistry and in situ techniques rather than bulk protein and RNA analysis.

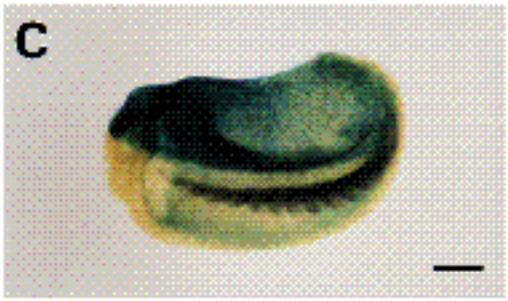
We stained XFD and control embryos with either a muscle-specific monoclonal antibody (12/101; Kintner and Brockes, 1984) or an in situ digoxigenin probe for the muscle actin transcript (Hemmati-Brivanlou et al., 1990). Nearly all of the embryos injected with the XFD construct showed deficiencies in muscle differentiation when assayed with these probes. In contrast only a few embryos injected with control RNAs had improper somite differentiation. XFD embryos commonly exhibit an open blastopore as a consequence of an impaired gastrulation process (Amaya et al., 1991). Over 50% of the embryos injected with XFD RNA had gastrulation defects. The most common muscle defect in these embryos was a deficiency in or total absence of a row of somites on one side of the open blastopore. In the few control embryos that failed to gastrulate normally, somites always developed on both sides of the open blastopore. Even embryos that did not have defects in gastrulation showed major deficiencies in muscle differentiation (see Fig. 2A,C). These embryos were most illuminating since they clearly showed that the defects in muscle formation were not a result of the gastrulation defects, and suggested that gastrulation movements and tissue-specific differentiation were not necessarily linked.

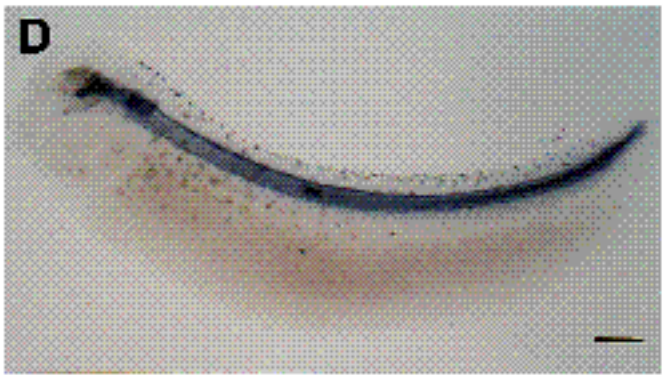
After analyzing over a hundred XFD embryos, it became evident that different regions of the somites were missing in different embryos. This was most likely due to the injection the XFD RNA randomly with respect to the embryonic axes and its poor diffusion in the embryo. To evaluate the phenotypes of the XFD embryos, we needed to determine which regions had inherited the XFD RNA. To determine the location of the XFD RNA, we co-injected the XFD and control constructs with a reporter construct that encoded β-galactosidase and which possessed a nuclear localization signal for more sensitive localization (Smith and Harland, 1991; Vize et al., 1991). The location of the XFD could then be inferred by staining for β-galactosidase activity. Since the β-galactosidase is localized to the nucleus we could easily co-stain the same cells with other specific, non-nuclear probes.

To determine whether the location of β-galactosidase activity reflected the distribution of the XFD protein, we stained gastrula stage embryos that had been injected with XFD and β-galactosidase RNA with the β-galactosidase substrate and with the FGF receptor antibody. There was a close but not perfect correlation between the β-galactosidase nuclear staining and the XFD plasma membrane staining. All XFD-positive cells were stained for β-galactosidase, but about 10% of the cells around the margin expressing β-galactosidase did not stain with the antibody for XFD. This discrepancy may simply reflect the different sensitivity for detection of β-galactosidase and the XFD product, since the β-galactosidase activity is concentrated in the nucleus whereas the XFD protein is dispersed throughout the plasma membrane. Also, the sequential staining procedures may have caused a loss of sensitivity in detecting the XFD protein with the FGF receptor antibody. Therefore, while co-injection seemed to be a reliable assay for localizing XFD expression, it may not be precise









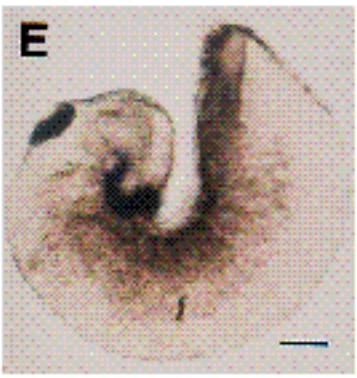


Fig. 2. Effects of overexpressing the control (d50, HAVØ) and dominant negative FGF receptor (XFD) constructs on muscle and notochord formation. (A) Embryos were injected at the two-cell stage with either d50 (left) or XFD RNA (right). At the tailbud stage (st. 28), the embryos were fixed and subjected to immunocytochemistry with the muscle-specific monoclonal antibody, 12/101 (brown stain). (B, C) One blastomere at the two-cell stage was co-injected with HAVØand β-galactosidase RNA (B) or with XFD and β-galactosidase RNA (C). At the early tailbud stage (st. 22) the embryos were fixed, stained for β-galacosidase activity (blue stain) and then subjected to whole-mount in situ hybridization with a muscle actin probe (black stain). (D, E) The two dorsal blastomeres for four-cell stage embryos were injected with HAVØ (D) or XFD RNA (E). At the late tailbud stage (st. 30) the embryos were fixed and subjected to whole-mount in situ hybridization with a collagen type II probe which labels the notochord (blue stain). Scale bars, 250 μm.

enough for some experiments requiring a cell by cell evaluation.

About fifty XFD and control HAVØ embryos were co-injected with β-galactosidase RNA and analyzed for β-galactosidase and either muscle actin staining or 12/101 staining. While somites stained for β-galactosidase were found in the control embryos, no stained somites were detected in XFD embryos. Fig. 2B and C show a HAVø embryo and a XFD embryo respectively, at stage 20, where half of each embryo stained for β-galactosidase. Note that the half of the HAVØ embryo that stained with β-galactosidase was also labelled by the muscle actin probe, whereas the half of the XFD embryo that stained for β-galactosidase contained virtually no muscle actin labelling. Furthermore, in this particular case both embryos gastrulated completely, yet the XFD embryo had a severe deficiency in somite formation in the half expressing β-galactosidase. The inhibition in those embryos that gastrulated normally indicates that the loss of muscle differentiation may be more sensitive to the inhibition of FGF signalling than the gastrulation defects that are commonly seen in XFD embryos.

Since the notochord is the most dorsal derivative of the mesoderm and is generally not induced by FGF in animal cap assays, we were interested in determining whether FGF signalling is required for normal notochord differentiation. For this purpose we have generated a useful probe for the notochord. The notochord in the chick embryo and sturgeon produce collagens specific to adult cartilage (Linsenmayer et al., 1973; Miller and Mathews, 1974). We, therefore, attempted to use collagen type II, which is specific for cartilage, as a marker for notochord formation. The *Xenopus* collagen type II gene was isolated by probing a stage 17 *Xenopus* cDNA library with a chicken collagen probe. In situ staining for collagen type II mRNA labelled the notochord quite efficiently and specifically during the tailbud stages (Fig. 2D). Recently Su et al. (1991) and Bieker and Yazdani-Buicky (1992) have also demonstrated the utility of collagen type II as a notochord marker. Since collagen type II mRNA is a cell autonomous marker for the

notochord, it is a more accurate probe for notochord expression than the antibody staining of components of the extracellular matrix of the notochord, which might be concentrated in the notochord but produced by cells outside the notochord.

To assay the effects of blocking FGF signalling on notochord differentiation we injected XFD RNA into the most dorsal quadrant of blastomeres from four-cell stage embryos, which gives rise to the notochord. When the embryos were co-injected with β-galactosidase RNA, most embryos were labelled within 30 degrees of the middle dorsal lip at the early gastrula stage, which corresponds to the cells that will differentiate into the notochord. (Unfortunately we were unable to detect β-galactosidase staining unambiguously in the differentiated notochord of any embryo, including control embryos, which may be related to vacuolation of notochord cells.) Most of the XFD embryos injected in this region had serious deficiencies in notochord formation. The most common defect in XFD embryos was that the notochord was absent in the one side of the open blastopore that stained for β-galactosidase. In a minority of cases the notochord of XFD embryos failed to differentiate appreciably at either side of the blastopore (Fig. 2E). These results are significant because in the few cases where HAVØ control embryos failed to gastrulate normally, their notochord was generally split resulting in two notochord structures running down both sides of the open blastopore. In the vast majority of cases, HAVØ embryos gastrulated normally and formed normal notochord. The percentage of XFD embryos with gastrulation defects was higher when the RNA was injected into the dorsal quadrant of the embryos than when the RNA was injected more randomly along the marginal zone.

Effects of inhibiting FGF receptor function on expression of mesoderm markers at gastrula stages

Many of the tissue-specific markers for mesoderm appear well after gastrulation and for this reason cannot distinguish a role of FGF in early cell specification, morphogenesis, or terminal differentiation. Specifically, since the level of the dominant negative FGF receptor construct remained high through gastrulation and neurulation into the tailbud stages, its ability to inhibit muscle and notochord formation may have been due to a requirement of the FGF signalling pathway for terminal differentiation of these tissues, rather than for early specification at the blastula and gastrula stages. To assay putative early events in gastrulation, we have examined the effects of XFD and of control constructs on the expression of mesodermal markers at the early gastrula stage before there are obvious morphological differences between XFD and control embryos.

The *Xenopus brachyury* (*Xbra*) gene is expressed throughout the marginal zone and serves as a general mesodermal marker (Fig. 3A; Smith et al., 1991). It is induced by either activin A or bFGF in animal caps in a cycloheximide independent manner, suggesting that it is a general early response gene for mesodermal induction (Smith et al., 1991). To test the effects of XFD, approximately 100 embryos were injected with XFD or HAVØ RNA and examined for the expression of *Xbra* at the mid gastrula stage (st. 11). Most of the embryos were injected into one blastomere at the two-cell stage. Control embryos (Fig. 3A) displayed a complete circle of *Xbra* expression throughout the marginal zone. XFD embryos lacked *Xbra* expression in a quarter to a half of the marginal zone (Fig. 3A, right). Embryos co-injected with the β-galactosidase and XFD RNA showed complementary distributions of reporter and marker staining; the region of the marginal zone that stained for β-galactosidase in the XFD embryos lacked *Xbra* expression (Fig. 3E, and data not shown). Some of the *Xbra* in situ hybridizations were further stained with the anti-FGF receptor antibody. In the XFD embryos the region lacking *Xbra* expression stained with antibody to the FGF receptor (Fig. 3C). In contrast the control HAVø embryos shared extensive co-staining with the *Xbra* probe and the FGF receptor antibody (Fig. 3B).

The development of the dorsal mesoderm was of particular interest, since it alone seemed refractile to XFD inhibition. At the gastrula stage the *Xbra* gene is expressed in the dorsal mesoderm, as well as lateral and ventral mesoderm. We injected XFD RNA into the dorsal two blastomeres at the four-cell stage, which led to its localized expression in the dorsal marginal zone. Co-injection of the β-galactosidase construct confirmed that XFD RNAs were found in the dorsal marginal zone when injected in the dorsal blastomeres. In XFD embryos *Xbra* expression was inhibited in the dorsal marginal zone (Fig. 3E), while HAVØ embryos exhibited dorsal expression of *Xbra* (Fig. 3D). Therefore it seems that the FGF signalling pathway is required for the induction of *Xbra* expression anywhere in the marginal zone.

Xbra is only one of several early genes that are expressed in the early mesoderm. We extended our study to include two genes with polarized distributions, *Xpo* and *goosecoid.* At the early neurula stages *Xpo* is expressed in the posterior mesoderm and anterior neural plate. Furthermore *Xpo* gene expression is induced in animal caps by either activin A or bFGF (Sato and Sargent, 1991). We found that the *Xpo* gene was expressed strongly at the mid gastrula stage in the lateral and ventral marginal zones and not in the dorsal marginal zone as described by Sato and Sargent (1991; Fig. 4A). While we saw heavy staining in posterior structures at stage 13, the notochord was clearly unlabeled (data not shown). At stage 11, XFD embryos were partially inhibited in *Xpo* expression (Fig. 4B), whereas HAVØ control embryos had the full pattern of *Xpo* expression throughout the lateral and ventral marginal zones (Fig. 4A). Since in situ hybridizations performed for *Xpo* stain the nuclei strongly at the mid gastrula stage, we were unable to co-stain these embryos for β-galactosidase.

The *goosecoid* gene is expressed at the early gastrula stage in the dorsal lip in a region that ultimately generates prechordal mesoderm and perhaps some chordamesoderm (Cho et al., 1991). It is therefore expressed in a complementary pattern to *Xpo. Goosecoid* is induced by activin in animal caps but not by bFGF (Cho et al., 1991) whereas *Xpo* is more strongly induced by FGF than by activin A (Sato and Sargent, 1991). We injected four-cell stage embryos in the dorsal blastomeres as described above to ensure expression in the dorsal marginal zone. At the early gastrula stage (st. 10), XFD embryos showed normal pat-

terns of *goosecoid* expression (Fig. 4D), indistinguishable from uninjected (Fig. 4C) or HAV∅ embryos (data not shown). Sibling embryos injected with β-galactosidase RNA stained in the region of the dorsal lip, where *goosecoid* is expressed (data not shown). Again we were unable to co-stain embryos for *goosecoid* and β-galactosidase expression due to the high level of *goosecoid* staining in the nucleus at this stage.

DISCUSSION

We showed previously that the expression of a truncated version of the FGF receptor can act as a dominant negative mutant: (1) by blocking FGF signalling in *Xenopus* oocytes expressing the full length receptor, (2) by blocking mesoderm induction in response to FGF in explanted animal caps, and (3) by grossly inhibiting trunk formation in intact embryos (Amaya et al., 1991). These defects could be corrected by overexpression of the wild-type receptor, strongly implicating FGF in mesoderm formation. These experiments, however, did not define the role of FGF. First, the animal cap assay only measured tissue differentiation and not tissue morphogenesis, and these under artificial conditions. Second, in the intact embryo, characteristic defects appeared only after extensive morphogenesis and it was difficult to determine (a) whether the defects were due to inappropriate inductive events, (b) whether the failure of tissue differentiation was a result of morphogenetic defects, or (c) whether morphogenetic defects were inherently linked to tissue differentiation. Third, paradoxically, though there were gross defects in trunk and tail formation, there were only mild defects in head formation, both of which involve the same types of tissues, such as the neurons, cartilage and muscle. The limitations of the previous experiments suggested two directions for future work: more studies in complete embryos where the natural context for FGF function is preserved, and studies of earlier markers, where early inductive effects might be distinguishable from later morphogenesis.

An important technical limitation to these intact embryo experiments was the incomplete diffusion of the injected RNA. This introduced a mosaic expression of the dominant negative FGF receptor construct (XFD) and variability in the defects. For this reason bulk measurements of expression were of limited utility. A satisfactory determination of the role of FGF signalling during mesoderm formation required that we know which cells in the embryo expressed the injected RNAs. To identify those cells we co-injected RNA that encodes β-galactosidase together with the experimental RNAs. When embryos were co-stained with an antibody to the FGF receptor, we could show that the β-galactosidase RNA was localized in the same region as the expressed receptor.

Using this procedure, we have found that cells that expressed the XFD construct failed to differentiate into muscle or notochord, while no effects were seen with controls. The failure to express markers of dorsal mesoderm differentiation was consistent with the gross loss of tail and trunk in the most extreme phenotypes. Since bFGF has been shown to induce muscle in animal caps (Slack et al., 1987; Kimelman and Kirschner, 1987), the FGF requirement for muscle formation was not surprising. But the requirement for an intact FGF signalling pathway for the differentiation of the notochord was unexpected, since bFGF on its own is not capable of inducing notochord in animal cap explants (Slack et al., 1987; Green et al., 1990; Christian et al., 1992; Green et al., 1992). However, the animal cap assays are limited because they test only for the sufficiency of FGF for signalling in tissues that will not normally form mesoderm. Recently Christian et al. (1992) have shown that FGF can induce notochord in animal caps if Xwnt-8 is co-expressed, and Kimelman and Maas (1992) have shown that injection of RNA for bFGF can cause notochord formation in isolated animal caps. These results leave open the question of whether FGF is sufficient for notochord formation in the marginal zone where the mesoderm normally forms.

It is as yet unproven whether FGF or activin provide initial inductive signals or whether they or other molecules sustain an induction that has been initiated in some other way. In support of the view that FGF and activin are the primary mesoderm inducers, both are found in the early embryo and both induce mesoderm in animal caps (Kimelman et al., 1988; Thomsen et al., 1990). However, the persistence of the XFD protein to tailbud stages raised the possibility that FGF plays its role late in the tissue differentiation process and not at the earliest stages. It is also possible that the major effects of FGF are not on differentiation but on the complex and extensive cell movements of gastrulation and neurulation. We have therefore asked whether the XFD construct affects early mesoderm markers, like *brachyury*, *Xpo* and *goosecoid*, which are expressed before tissue morphogenesis. *Xpo* and *Xbra* are both inducible by bFGF in animal caps (Sato and Sargent, 1991; Smith et al., 1991). A detailed reinvestigation of the pattern of *Xpo* expression by whole-mount methods showed that in gastrula stage embryos, *Xpo* gene is expressed on the lateral and ventral marginal zone only. It is excluded from a narrow zone on the dorsal side. We interpret the original report describing *Xpo* expression in dorsal mesoderm to be due to slightly skewed mid-sagittal sections (Sato and Sargent, 1991). *Xbra* by contrast is expressed throughout the entire marginal zone and is therefore a more general mesodermal marker. *Goosecoid* is inducible with activin at high concentrations and is a marker for the first involuting mesoderm on the dorsal side. It is not induced by bFGF (Cho et al., 1991). These markers are early response genes (inducible in the absence of protein synthesis) (Almendral et al., 1988) for mesoderm induction, and are expressed at the early to mid gastrula stages, before gastrulation defects occur. They are all thought to be transcription factors.

The expressions of *Xbra* and *Xpo* were completely inhibited by the expression of the XFD construct. Analysis of the expression of *Xbra* in embryos injected with XFD RNA indicated that FGF signalling was required for the expression of *Xbra* throughout the marginal zone, including the dorsal quadrant. Thus FGF signalling was not only required for the induction of ventral and lateral mesodermal markers, but also for some dorsal mesodermal markers. Furthermore, translation of the XFD construct also

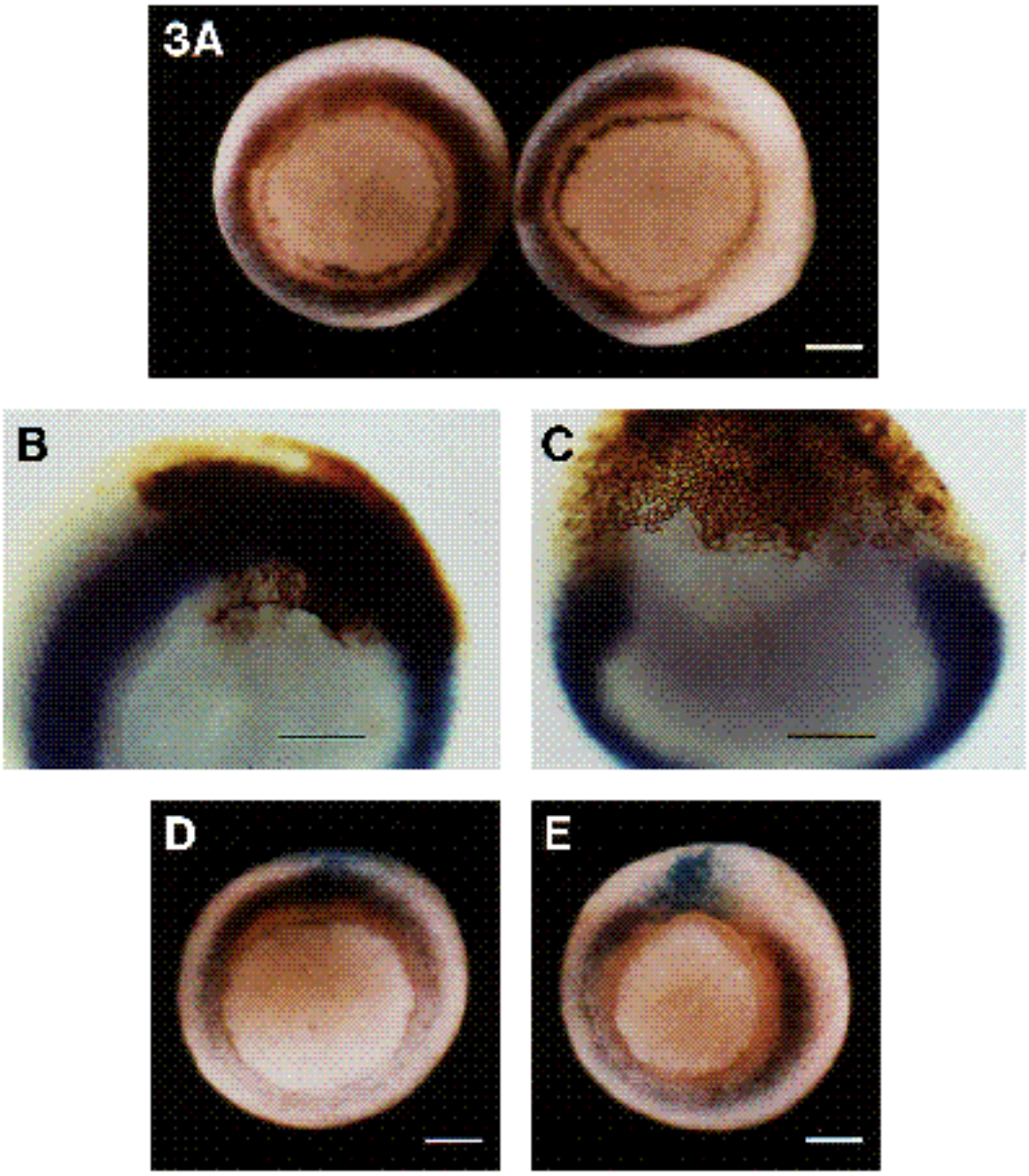


Fig. 3. Effects of overexpressing the control (HAVØ) and dominant negative FGF receptor (XFD) constructs on *brachyury* expression. (A) One blastomere at the two-cell stage was injected with either HAVØ (left) or XFD (right) RNA. At the mid gastrula stage (st. 11), the embryos were fixed and subjected to whole-mount in situ hybridization with a *Xenopus brachyury* (*Xbra*) probe (brown stain). After staining for *Xbra* some embryos were then stained with the FGF receptor antibody. The embryo in B was injected with HAVØ RNA and the embryo in C was injected with XFD RNA. In these panels the stain for *brachyury* is blue while the FGF receptor protein stain is red-brown. (D, E) The two dorsal blastomeres of four-cell stage embryos were co-injected with HAVø and β-galactosidase RNA (D) or XFD and β-galactosidase RNA (E). At the mid gastrula stage (st. 11) the embryos were fixed and stained for β-galactosidase activity (blue stain) and then subjected to in situ hybridization with the *brachyury* (*Xbra*) probe (brown stain). The embryos in D and E are oriented with the dorsal side toward the top of the page. To emphasize the dorsal side, the embryos were not cleared and tilted ventrally, resulting in the bleached appearance of the ventral side from reflected light. Scale bars, 250 μm.

inhibits the expression of *Xnot*, a gene that is expressed in the dorsal marginal zone (von Dassow et al., 1993).

There has always been a wide range of severity of gastrulation defects when XFD RNA was injected randomly into the embryo. Even in the absence of these gastrulation defects muscle actin expression may still be inhibited (Fig. 2C). Whatever the site of injection, there was a more or less normal development of the head and loss of trunk and tail structures. When we targeted the RNA to the dorsal quadrant, we found more uniform and severe defects in gastrulation but mostly in the lateral and ventral marginal zone. Since injection of XFD RNA on the dorsal side led to gastrulation defects in the lateral and ventral marginal zone, we may conclude that inhibition of FGF signalling on the dorsal marginal zone must have long range effects. Although the XFD protein should be cell autonomous, in

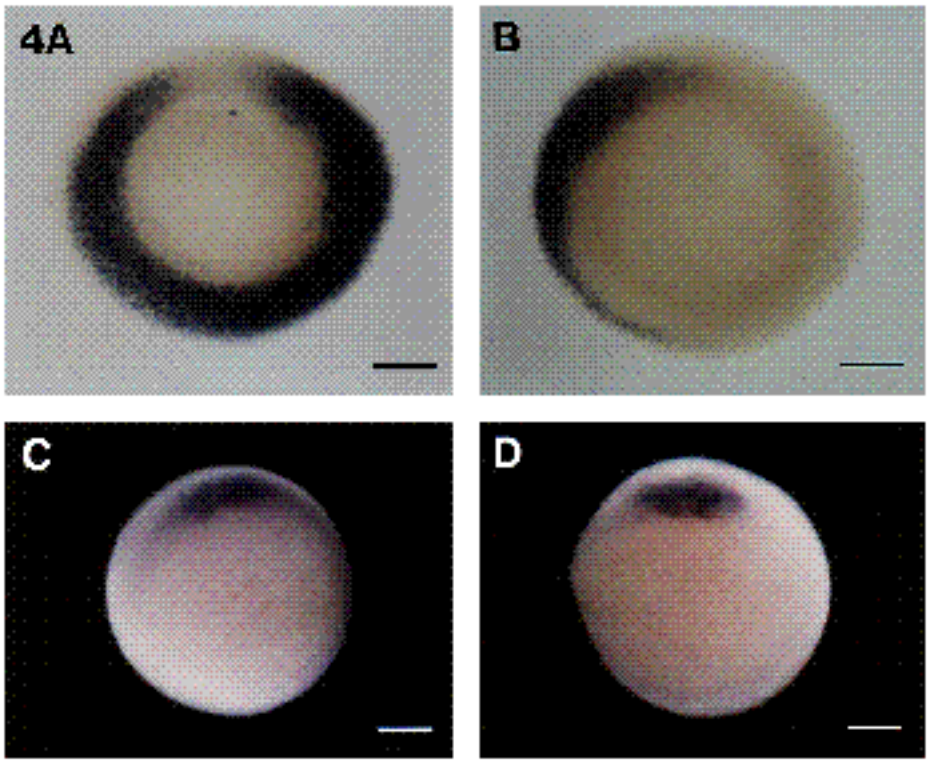


Fig. 4. Effects of overexpressing the control (HAV∅) and dominant negative FGF receptor (XFD) constructs on *Xpo* and *goosecoid* expression. One blastomere of the two-cell stage embryo was injected with either HAV∅ (A) or XFD (B) RNA. At the mid gastrula stage (st. 11), the embryos were fixed and subjected to whole-mount in situ hybridization with an *Xpo* probe (black stain). In A the dorsal side is oriented toward the top of the page. (C, D) Embryos were not injected (C) or the two dorsal blastomeres of four-cell stage embryos were injected with XFD RNA (D). At the early gastrula stage (st. 10) the embryos were fixed and then subjected to in situ hybridization with the *goosecoid* probe (dark purple stain). The embryos are oriented with the dorsal side toward the top of the page. Scale bars, 250 μm.

the whole embryo the effects did not seem to be cell autonomous, suggesting that FGF signalling may be required for the production of other signals that further pattern the embryo.

The expression of XFD did not inhibit *goosecoid* expression. *Goosecoid* is expressed in the first involuting mesoderm (Cho et al., 1991), that will form the prechordal (head) mesoderm and which may be involved in the specification of the brain. Chordamesoderm follows the prechordal mesoderm, generates the notochord, underlies and may be involved in inducing the trunk neural tube. It is this region on the dorsal side that expresses *brachyury* (Smith et al., 1991). The formation of a nearly normal head with no trunk or notochord in the XFD embryos is explainable by the differential action of the XFD construct on *goose - coid* and *brachyury*. Globally, FGF signalling is required for *brachyury* expression but seems dispensable for the *goosecoid* pathway. These results suggest that the prechordal mesoderm and the adjacent chordamesoderm differ in their requirements for FGF and are presumably distinguishable at these very early stages, even though the FGF receptor itself appears to be present in both these tissues at the blastula and gastrula stages (Ding et al., 1992). These tissues, though, differ in their migratory behavior. The prechordal mesoderm *diverges* after involution and actively migrates along the blastoceol roof where it underlies the future brain (Winklbauer et al., 1991). The chordamesoderm as well as other more lateral and posterior mesoderm shows an opposite cell behavior, called convergent extension, where the cells *converge* by intercalation on the dorsal midline (Keller et al., 1992). If FGF is required for convergence and intercalation on the dorsal side, it would explain the severe gastrulation defects seen in XFD embryos. The first prechordal mesoderm might involute normally in the absence of FGF but the chordamesoderm cells require FGF. Expression of the XFD protein in the chordamesoderm at the dorsal midline would disrupt the convergence of the more lateral mesoderm cells, resulting in the failure of these embryos to complete gastrulation.

In a previous study, we showed that the dominant negative FGF receptor construct does not inhibit the ability of activin to induce mesoderm in animal caps (Amaya et al., 1991). Since activin can induce head structures and *goose - coid* in animal caps, we might conclude that the in vivo role for activin may be limited to the induction of anterior structures. However, studies with a dominant negative activin receptor construct indicate that activin signalling is also required for the induction of mesoderm in the embryo (Hemmati-Brivanlou and Melton, 1992). Therefore, while either growth factor alone can induce mesoderm in animal caps, in the embryo both factors appear to be required for the formation of most mesoderm. It is possible that the marginal zone has subthreshold levels of FGF and activin such that neither growth factor alone can fully induce mesoderm; instead the two factors may act synergistically to induce most of the mesoderm, as was seen in experiments with FGF and TGF-β1 or Activin A (Kimelman and Kirschner, 1987; Green et al., 1992). Alternatively, the animal cap may be lacking inhibitors found in the marginal zone of the intact embryo, where activin and FGF may be mutually dependent.

In summary our results indicate that the FGF signalling pathway is important for the induction of most but not all mesoderm. Early FGF signals precede gastrulation movements and are involved in early specification. There are different requirements of mesodermal tissues for FGF sig-

nalling that define two distinct cell populations of the early involuting mesoderm. These cells are further differentiated by their behavior during gastrulation movements: the prechordal (FGF insensitive) mesoderm diverges during active migration while the chordamesoderm (FGF requiring) participates in convergent extension. We cannot distinguish whether the loss of muscle and notochord is due entirely to the initial specification events or due to a subsequent requirement in tissue differentiation. For most of the mesoderm, FGF and activin do not seem to be clearly distinguishable by the types of tissue they induce, nor by the region of the embryo in which they function. They may be distinguishable by the cell behaviors they induce and thus may have important consequences for the types of tissue formed. It seems most likely that both activins and FGF will cooperate in generating the mesoderm pattern, particularly on the dorsal side and that FGF may participate in setting up long range inductions as well as short range signalling.

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REFERENCES

Almendral, J. M., Sommer, D., Macdonald-Bravo, H., Burckhardt, J., Perera, J. and Bravo, R. (1988). Complexity of early genetic response to growth factors in mouse fibroblasts. *Mol. Cell Biol.* **8**, 2140-2148.

Amaya, E., Musci, T. J. and Kirschner, M. W. (1991). Expression of a dominant negative mutant of the FGF receptor disrupts mesoderm formation in *Xenopus* embryos. *Cell* **66**, 257-270.

Bieker, J. J. and Yazdani-Buicky, M. (1992). Distribution of type II collagen mRNA in *Xenopus* embryos visualized by whole-mount in situ hybridization. *J. Histochem. Cytochem.* **40**, 1117-1120.

Byers, S., Amaya, E., Munro, S. and Bluschuk, O. (1992). Fibroblast growth factor receptors contain a conserved HAV region common to cadherins and influenza strain A hemagglutinins. *Dev. Biol.* **152**, 411-414.

Cho, K. W. Y., Blumberg, B., Steinbeisser, H. and De Robertis, E. M. (1991). Molecular nature of Spemann's organizer: The role of the *Xenopus* homeobox gene *goosecoid*. *Cell* **67**, 1111-1120.

Christian, J. L., Olson, D. J. and Moon, R. T. (1992). Xwnt-8 modifies the character of mesoderm induced by bFGF in isolated *Xenopus* endoderm. *EMBO J.* **11**, 33-41.

Dale, L., Howes, G., Price, B. M. J. and Smith, J. C. (1992). Bone morphogenetic protein 4: a ventralizing factor in early *Xenopus* development. *Development* **115**, 573-585.

Ding, X., McKeehan, W. L., Xu, J. and Grunz, H. (1992). Spatial and temporal localization of FGF receptors in Xenopus laevis. *Roux's Arch. Dev. Biol.* **201**, 334-339.

Green, J. B. A., Howes, G., Symes, K., Cooke, J. and Smith, J. C. (1990). The biological effects of XTC-MIF: quantitative comparison with *Xenopus* bFGF. *Development* **108**, 173-183.

Green, J. B. A., New, H. V. and Smith, J. C. (1992). Responses of embryonic *Xenopus* cells to activin and FGF are separated by multiple dose thresholds and correspond to distinct axes of the mesoderm. *Cell* **71**, 731-739.

Hamburger, V. (1988). *The Heritage of Experimental Embryology: Hans Spemann and the Organizer* Oxford: Oxford University Press.

Harland, R. M. (1991). In situ hybridization: an improved whole-mount method for *Xenopus* embryos. *Meth. Cell Biol.* **36**, 685-695.

Harlow, E. and Lane, D. (1988). *Antibodies: A Laboratory Manual* Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

Hemmati-Brivanlou, A., Frank, D., Bolce, M. B., Brown, B. D., Sive, H. L. and Harland, R. M. (1990). Localization of specific mRNAs in *Xenopus* embryos by whole-mount *in situ* hybridization. *Development* **110**, 325-330.

Hemmati-Brivanlou, A. and Harland, R. M. (1989). Expression of an *engrailed*-related protein is induced in the anterior neural ectoderm of early *Xenopus* embryos. *Development* **106**, 611-617.

Hemmati-Brivanlou, A. and Melton, D. A. (1992). A truncated activin receptor inhibits mesoderm induction and formation of axial structures in *Xenopus* embryos. *Nature* **359**, 609-614.

Isaacs, H. V., Tannahill, D. and Slack, J. M. W. (1992). Expression of a novel FGF in the *Xenopus* embryo. A new candidate inducing factor for mesoderm formation and anteroposterior specification. *Development* **114**, 711-720.

Keller, R. E., Shih, J. and Domingo, C. (1992). The patterning and functioning of protrusive activity during convergence and extension of the *Xenopus* organizer. *Development* **Supplement**, 81-91.

Kimelman, D., Abraham, J. A., Haaparanta, T., Palisi, T. M. and Kirschner, M. W. (1988). The presence of fibroblast growth factor in the frog egg: Its role as a natural mesoderm inducer. *Science* **242**, 1053-1056.

Kimelman, D., Christian, J. L. and Moon, R. T. (1992). Synergistic principles of development: overlapping patterning systems in *Xenopus* mesoderm induction. *Development* **116**, 1-9.

Kimelman, D. and Kirschner, M. (1987). Synergistic induction of mesoderm by FGF and TGF-β and the identification of an mRNA coding for FGF in the early *Xenopus* embryo. *Cell* **51**, 869-877.

Kimelman, D. and Maas, A. (1992). Induction of dorsal and ventral mesoderm by ectopically expressed *Xenopus* basic fibroblast growth factor. *Development* **114**, 261-269.

Kintner, C. R. and Brockes, J. P. (1984). Monoclonal antibodies identify blastemal cells derived from dedifferentiating muscle in newt limb regeneration. *Nature* **308**, 67-69.

Kintner, C. R. and Melton, D. A. (1987). Expression of *Xenopus* N-CAM RNA in ectoderm is an early response to neural induction. *Development* **99**, 311-325.

Koster, M., Plessow, S., Clement, J. H., Lorenz, A., Tiedemann, H. and Knochel, W. (1991). Bone morphogenetic protein 4 (BMP-4), a member of the TGF-β family, in early embryos of *Xenopus laevis*: analysis of the mesoderm inducing activity. *Mech. Dev.* **33**, 191-200.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**, 680-685.

Lehrach, H., Frischauf, A. M., Hanahan, D., Wozney, J., Fuller, F., Crkvenjakov, R., Boedtker, H. and Doty, P. (1978). Construction and characterization of a 2.5-kilobase procollagen clone. *Proc. Natl. Acad. Sci. USA* **75**, 5417-5421.

Linsenmayer, T. F., Trestad, R. L. and Gross, J. (1973). The collagen of chick embryonic notochord. *Biochem. Biophys. Res. Comm.* **53**, 39-45.

Melton, D. A., Krieg, P. A., Rebagliati, M. R., Maniatis, T., Zinn, K. and Green, M. R. (1984). Efficient in vitro synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. *Nucl. Acids Res.* **12**, 7035-7056.

Miller, E. J. and Mathews, M. B. (1974). Characterization of notochord collagen as cartilage-type collagen. *Biochem. Biophys. Res. Comm.* **60**, 424-430.

Moon, R. T. and Christian, J. L. (1992). Competence modifiers synergize with growth factors during mesoderm induction and patterning in *Xenopus*. *Cell* **71**, 709-712.

Newport, J. and Kirschner, M. (1982). A major developmental transition in early *Xenopus* embryos: I. Characterization and timing of cellular changes at the midblastula stage. *Cell* **30**, 675-686.

Nieuwkoop, P. D. (1973). The 'organization center' of the amphibian embryo: Its origin, spatial organization and morphogenetic action. *Adv. Morph.* **10**, 1-39.

Nieuwkoop, P. D. and Faber, J. (1967). *Normal Table of Xenopus laevis (Daudin)* Amsterdam: North Holland

Rosa, F., Roberts, A. B., Danielpour, D., Dart, L. L., Sporn, M. B. and Dawid, I. B. (1988). Mesoderm induction in amphibians: The role of TGF-β2-like factors. *Science* **239**, 783-785.

Sato, S. M. and Sargent, T. D. (1991). Localized and inducible expression of *Xenopus-posterior* (*Xpo*), a novel gene active in early frog embryos, encoding a protein with a 'CCHC' finger domain. *Development* **112**, 747-753.

Slack, J. M. W., Darlington, B. G., Heath, J. K. and Godsave, S. F. (1987). Mesoderm induction in early *Xenopus* embryos by heparin-binding growth factors. *Nature (London)* **326**, 197-200.

Smith, D. E. and Fisher, P. A. (1984). Identification, developmental regulation and response to heat shock of two antigenically related forms of a major nuclear envelope protein in *Drosophila* embryos: Application of an improved method for affinity purification of antibodies using polypeptides immobilized on nitrocellulose blots. *J. Cell Biol.* **99**, 20-28.

Smith, J. C., Price, B. M. J., Green, J. B. A., Weigel, D. and Herrmann, B. G. (1991). Expression of a *Xenopus* homolog of *Brachyury* (T) is an immediate-early response to mesoderm induction. *Cell* **67**, 79-87.

Smith, J. C., Price, B. M. J., van Nimmen, K. and Huylebroeck, D. (1990). Identification of a potent *Xenopus* mesoderm-inducing factor as a homolog of activin A. *Nature (London)* **345**, 729-731.

Smith, W. C. and Harland, R. M. (1991). Injected Xwnt-8 RNA acts early in *Xenopus* embryos to promote formation of a vegetal organizing center. *Cell* **67**, 753-765.

Smith, W. C. and Harland, R. M. (1992). Expression cloning of *noggin*, a new dorsalizing factor localized to the Spemann organizer in *Xenopus* embryos. *Cell* **70**, 829-840.

Sokol, S., Christian, J. L., Moon, R. T. and Melton, D. A. (1991). Injected *Wnt* RNA induces a complete body axis in *Xenopus* embryos. *Cell* **67**, 741-752.

Su, M.-W., Suzuki, H. R., Bieker, J. J., Solursh, M. and Ramirez, F. (1991). Expression of two nonallelic type II procollagen genes during *Xenopus laevis* embryogenesis is characterized by stage-specific production of alternatively spliced transcripts. *J. Cell Biol.* **115**, 565-575.

Thomsen, G., Woolf, T., Whitman, M., Sokol, S., Vaughan, J., Vale, W. and Melton, D. A. (1990). Activins are expressed early in *Xenopus* embryogenesis and can induce axial mesoderm and anterior structures. *Cell* **63**, 485-493.

Vallette, F., Mege, E., Reiss, A. and Adesnik, M. (1989). Construction of mutant and chimeric genes using the polymerase chain reaction. *Nucl. Acids Res.* **17**, 723-733.

Vize, P. D., Melton, D. A., Hemmati-Brivanlou, A. and Harland, R. M. (1991). Assays for gene function in developing *Xenopus* embryos. *Meth. Cell Bio.* **36**, 367-387.

von Dassow, G., Schmidt, J. and Kimelman, D. (1993). Induction of the *Xenopus* organizer: Expression and regulation of *Xnot*, a novel FGF and activin-inducible homeobox gene. *Genes Dev.* (in press).

Weeks, D. L. and Melton, D. A. (1987). A maternal mRNA localized to the vegetal hemisphere in *Xenopus* eggs codes for a growth factor related to TGF-β. *Cell* **51**, 861-867.

Winklbauer, R., Selchow, A., Nagel, M., Stoltz, C. and Angres, B. (1991). Mesoderm cell migration in the *Xenopus* gastrula. In *Gastrulation: Movements, Patterns and Molecules,* (ed. R. Keller, W. H. Clark Jr. and F. Griffin) New York: Plenum Press.

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