Analysis of FGF function in normal and no tail zebrafish embryos reveals separate mechanisms for formation of the trunk and the tail

Kevin Griffin, Roger Patient and Nigel Holder*
Developmental Biology Research Centre, Division of Biomedical Science, The Randall Institute, King’s College, 26-29 Drury Lane, London WC2B 5RL, UK
*Author for correspondence

SUMMARY
To analyse the roles of FGF activity and brachyury during gastrulation we have directly compared the consequences of inhibition of FGF-receptor signalling with the phenotype of the zebrafish brachyury mutant, no tail (ntl). We show that expression of ntl is regulated by FGF and that inhibition of FGF receptor-signalling leads to complete loss of the trunk and tail. Since the ntl mutant lacks the tail and notochord but has an otherwise normal trunk, this demonstrates that trunk development is dependent upon an unidentified gene, or set of genes, referred to as no trunk (ntk) which is regulated by FGF. We propose a model to explain the FGF-dependent regulation of ntl and ntk that accounts for the above phenotypes. Consistent with this model, over-expression of eFGF led to suppression of anterior fates and development of trunk and tail derivatives only. In addition, widespread activation of convergence and extension movements resulted in the formation of multiple axis-like structures. Expression of eve1 and cad1 was also regulated by FGF activity, suggesting that during gastrulation FGF activity is normally restricted to the germ ring where these genes, and ntl, are expressed. Taken together these data suggest that the germ ring acts as a posteriorising centre during AP patterning, mediated by FGF activity in this tissue.

Key words: eFGF, zebrafish, gastrulation, anteroposterior, mesoderm, brachyury, dominant negative FGFR

INTRODUCTION
Developmental mechanisms in vertebrate embryos are proving to be remarkably conserved (Beddington and Smith, 1993). The ease with which Xenopus embryos are surgically manipulated has been invaluable in analysing early inductive interactions, but a genetic approach is not feasible with this organism. The zebrafish, on the other hand, offers a rapidly growing number of mutations affecting early development (Mullins et al., 1994; Driever et al., 1994) and, like Xenopus, can be used for transient assays of injected mRNAs. We have, therefore, used the zebrafish to extend our understanding of the part played by fibroblast growth factors (FGFs) in mesoderm induction (Kimelman and Kirschner, 1987; Slack et al., 1987) and anteroposterior (AP) patterning of the embryo (Isaacs et al., 1994; Schulte-Merker and Smith, 1995) into a developmental context.

The mesoderm and AP axis of the vertebrate embryo are first identifiable during gastrulation. Mesoderm is formed by an inductive interaction occurring in the blastula (Slack, 1994), whereas formation of the AP axis is intricately linked to the gastrulation movements themselves (Gerhart et al., 1989). Members of the FGF family are thought to be important in both of these processes since many family members can induce mesoderm from explanted tissue, which would normally not adopt this fate in vivo (Kimelman and Kirschner, 1987; Slack et al., 1987), and expression of a dominant negative FGF receptor (FGFR) during Xenopus development leads to loss of the posterior body axis (Amaya et al., 1991). But it has proved difficult to determine when FGF activity is important in these processes in vivo and what the underlying mechanisms are. It is likely that the relationship between FGF and brachyury expression is important. Smith and co-workers have shown that expression of Xenopus brachyury (Xbra) is an immediate-early response to mesoderm induction (Smith et al., 1991) and may act as a genetic switch, conferring mesodermal identity (Cunliffe and Smith, 1992). Both Xbra and FGF induce ventroposterior mesoderm from animal cap tissue (Cunliffe and Smith, 1992; Ruiz i Altaba and Melton, 1989a), and the phenotypes of homozygous brachyury mutant mice (Chesley, 1935) and zebrafish (Halpern et al., 1993) are similar to the phenotype of Xenopus embryos after expression of a dominant negative FGFR. Consistent with these similarities, it has recently been shown that embryonic FGF (eFGF) and Xbra, both of which are expressed in the blastopore lip (Isaacs et al., 1992; Smith et al., 1991), can induce expression of each other, and their continued expression during gastrulation is co-dependent and forms a positive-feedback regulatory loop (Isaacs et al., 1994; Schulte-Merker and Smith, 1995). However, the precise role of brachyury in mesoderm formation remains to be determined and whilst it is frequently referred to
as a pan-mesodermal marker it is, at least in zebrafish, also expressed by some populations of cells which do not form mesoderm or involute at gastrulation (Halpern et al., 1993).

Despite this, expression of Xbra cannot be the only response to FGF treatment since Xenopus animal caps injected with Xwnt8 mRNA and treated with FGF are capable of notochord differentiation (Christian et al., 1992), whereas those injected with Xbra and Xwnt8 mRNAs are not (Cunliffe and Smith, 1994). In order to determine the relative importance in vivo of the relationship between FGF and brachyury versus other genes which respond to FGF, the effects of loss of brachyury function need to be compared directly with those due to loss of function of FGF activity. This is possible using zebrafish embryos because a brachyury mutant, no tail (Halpern et al., 1993), is available.

We have used injection of mRNAs into zebrafish embryos to express either dominant negative FGFR or eFGF in order to examine the roles of FGF activity during early development. Expression of the dominant negative FGFR led to loss of ntI expression and complete absence of the trunk and tail. Since no tail mutants only lack the tail and notochord and have an otherwise normal trunk, the difference between these two phenotypes indicates that different mechanisms control trunk and tail development. We suggest that an additional gene or set of genes, which we call no trunk (ntk), is necessary for trunk development and propose a model, based on positive feedback regulatory loops between FGF and these two genes, to explain the role of FGF activity in axis formation. In addition we show that (a) FGF activity is a key regulator of gene expression in the germ ring, the site of mesoderm formation, and (b) overexpression of eFGF results in a loss of head structures and widespread convergence and extension movements in the zebrafish.

MATERIALS AND METHODS

Maintenance of fish
Breeding fish were maintained at 28.5°C on a 14-hour light/10-hour dark cycle. Embryos were collected by natural spawning and raised in 10% Hank’s buffered saline at 28.5°C and staged according to Westerfield (1993).

Preparation of synthetic RNA and RNA injections
All plasmids were manipulated using standard techniques and conditions, according to Sambrook et al. (1989), or manufacturers recommendations. pSP64-eFGF and pSP64-ΔeFGF plasmids were prepared as follows. Full length eFGF coding sequence (Isaacs et al., 1992) was isolated as a HinI fragment. pSP64-eFGF(–) was produced by cutting the eFGF cDNA at an internal HindIII site and with BamHI, this removed the secretory signal peptide and left an in-frame methionine residue. Both fragments were blunt-end cloned into the BglII site of pSP64T (Krieg and Melton, 1984). pSP64-eFGF(–) contained full-length eFGF in the antisense orientation. The dominant negative FGFR receptor, XFD, and the control receptor, Δ50, were those of Amaya et al. (1991), pSP64-β-galactosidase, containing a nuclear localisation signal, was a gift from D. Wilkinson. Capped RNAs were prepared according to the method of Cornell and Kimmelman (1994), and tested by in vitro translation (Boehringer Mannheim). Translation reactions were separated by SDS-tricine PAGE (Schägger and Von Jagow, 1987) and visualised by autoradiography. Chorionated embryos were injected at the 1-8 cell stage with 0.7-1.5 ng of FGFR RNAs with or without 100 pg of lac-Z RNA, or with 125 pg of eFGF RNAs unless indicated.

Whole-mount in situ hybridisation
Whole-mount in situ hybridisations were performed according to the method of Thirse et al. (1994) with the following modifications. Hybridisations were carried out at 65°C, and anti-digoxigenin Fab fragment was used at 1:2,500 dilution. Colour development was stopped by two washes in PBS/0.1% Tween-20 (PBT) and one 30 minute wash in 100% methanol to remove background colour. Stained embryos were stored in PBT at 4°C and photographed in 1% methyl cellulose.

Whole-mount antibody staining and assay for β-gal activity
All steps were at room temperature unless indicated. Embryos were fixed in 4% paraformaldehyde, PBS (pH 7.4), overnight at 4°C, washed several times in PBT (PBS, 0.1% Tween-20, Sigma), manually dechorionated, washed five times with PBT and stored in methanol at –20°C until required. If appropriate, β-gal activity was assayed prior to storing in methanol as follows. Embryos were rinsed in buffer A (0.1 mM MgCl2, 15 mM K-FeCN6, 12 mM KFe(CN)6) for five minutes, then at 37°C in buffer A including X-Gal (Strata-gene) at 800 µg/ml until colour had developed, washed several times and stored in methanol, as normal. Embryos were removed from methanol by washing with PBT (5 washes, 5 minutes each) blocked in 10% normal goat serum, PBT (PBT-NGS) for 2 hours. Primary antibodies were incubated overnight at 4°C in PBT-NGS. Nil antibody was used according to the method of Schulte-Merker et al. (1992) and A41025 (Pavlath et al., 1989) was a mouse monoclonal supernatant (IgG; to detect myosin) and used at 1:15. Antibodies were detected using Vectastain ABC elite (Vector), secondary antibodies were used at 1:400 and the ABC complex at 1:1000. Embryos were washed in PBT six times (15 minutes each) between incubations. Prior to develop-oment, embryos were washed twice in PBS (5 minutes each) to remove detergent. Staining was detected using peroxidase substrate kit (Vector) and colour development monitored visually. The reaction was stopped with three washes in PBT. Embryos were stored in 70% glycerol and photographed on a dissecting microscope using Kodak Ektar (25 ASA) film.

RESULTS

Expression of dominant negative FGFR results in loss of the trunk and tail
Injection of RNA encoding a dominant negative FGFR into 1-8 cell embryos, but not of a non-functional FGFR, profoundly affected the formation of the body axis (Table 1). By 24 hours post fertilisation (hpf), three-quarters of embryos injected with the mutant receptor showed graded deficiencies in trunk and tail development with severely affected embryos lacking the body axis from the level of the first somite; head development appeared normal and the otic vesicle was visible (Fig. 1). This large defect is in marked contrast to those seen in the zebrafish brachyury mutant, no tail, which only lacks the notochord and the posterior body axis posterior to somite 17-19 (Halpern et al., 1993; Fig. IC).

Inactivation of the FGF signalling affects mesoderm phenotype but not its formation
Given the potential role for FGF in mesoderm induction we looked for effects on the expression of two putative mesoder-
eral markers, ntl and snail-1 (sna1). These genes are expressed throughout the presumptive mesoderm in the blastula, though ntl is also expressed by some non-mesodermal cells such as in the margin of the enveloping layer (Schulte-Merker et al., 1992; Hammerschmidt and Nusslein-Volhard, 1994; Thiese et al., 1994). At the onset of gastrulation ntl transcripts are detected throughout the germ ring of normal embryos (Fig. 2A), but gaps were detected in this ring in injected embryos (Fig. 2B). The extent of these gaps was variable but some expression was always found. To determine the distribution of the injected RNA with respect to the changes in expression of ntl, embryos were co-injected with lac-Z RNA (Amaya et al., 1993). When β-gal activity and Ntl protein distribution were detected in the same embryo, the gaps in Ntl staining coincided with enzyme activity (blue nuclei), indicating that the defects were due to the dominant negative FGFR (Fig. 2C).

In a separate set of experiments, we looked for changes in snail expression relative to the predictable effects on ntl expression. Batches of injected embryos were divided in two: half were probed for ntl expression, and the other half for snail. In contrast to ntl expression, which we consistently found to be affected in a large proportion of embryos, snail expression was never found to be affected in the epiblast margin at 50% epiboly (pre-involution) and normal snail-expressing hypoblast was found at the shield stage (data not shown).

Since these results indicate that the primary effect of the dominant negative FGFR was on mesodermal phenotype (ntl, snail1+) and that hypoblast formation was normal, we looked for effects on notochord and somitic muscle formation using antibodies to Ntl and myosin heavy chain (Pavlath et al., 1989), respectively (Fig. 3A). Large deficiencies in notochord formation were found at 16 hpf (12-15 somites). One third of embryos lacked notochord, except at its rostral limit in the region of the hind-brain, in one third it was short and nodular and did not extend into the tail-bud, in the remainder it was normal (Fig. 3B). Similar defects were found in muscle formation. In normal embryos at 16 hpf, myosin is detected in the adaxial cells of the somites (Fig. 3A). In injected embryos unilateral or bilateral defects in this pattern were seen, which could be as extensive as total absence of staining up to and inclusive of the first somite (Fig. 3B).

It is significant that β-gal activity did not co-localise with the defects, as seen with ntl expression during gastrulation, indicating that they were not simply due to a block in cell differentiation, but more likely due to prior effects on cell specification or movement. In fact, the distribution of β-gal-stained cells in injected embryos at this stage was intriguing. Blue-cells were evenly distributed throughout the anteroposterior axis of embryos injected with lac-Z and the control FGFR (Fig. 3C), but were found predominantly in the head of two thirds of embryos injected with the dominant negative FGFR (Fig. 3D), the significance of which is unknown.

**Over-expression of eFGF affects gastrulation movements and morphogenesis**

To ascertain what processes were dependent upon FGF signalling and, therefore, likely targets of the dominant negative FGFR, we ectopically expressed eFGF, a secreted member of the family. Embryos injected with as little as 4 pg of full-length eFGF RNA showed dramatic changes in morphogenesis, whereas those injected with eFGF lacking its secretory signal sequence were normal (Table 1). Abnormalities were first apparent during early epiboly, which was retarded and the cell sheet was often of uneven thickness. Gastrulation began at the same time postfertilisation as normal embryos, but before 50% epiboly. The germ ring of injected embryos was much more prominent than normal (Fig. 4B,D) whereas the shield, the zebrafish equivalent to the dorsal lip of amphibia, appeared unaffected. The cells of the yolk syncytial layer had frequently migrated further vegetally than the epiblast margin, suggesting that epiboly had been delayed. In some embryos this delay was significant, and the vegetal yolk cell remained uncovered throughout the remainder of development. In all embryos the thickened hypoblast remained obvious, even during the late stages of gastrulation, as a collar surrounding the whole circumference of the embryo.

Although the shield formed normally its migration appeared to be affected. No hypoblast was present at the animal pole of injected embryos at 80% epiboly, when the shield cells should have reached this location, and a prominent bulge was present.

**Fig. 1.** Effect of inhibition of the FGF-receptor on normal development. Live embryos at 48 hpf. (A) Normal embryo, arrows indicate otic vesicle (1), somite 1 (2), and somite 17 (3). (B) Extreme phenotype resulting from expression of the dominant negative FGF-receptor; all structures posterior to the otic vesicle are missing. (C) Homozygous no tail mutant, which lacks only the body axis posterior to somite 17-19, and the notochord.
on the dorsal side near the pole (Fig. 4E,F). Another bulge in the hypoblast was often present on the posterior-ventral side. Embryos at the tail bud stage were lozenge-shaped and the yolk plug was invariably open (Fig. 4H). After gastrulation, the hypoblast formed into a number of separate accumulations of cells, apparently at random. These remained continuous with each other toward the animal pole and later formed axis-like extensions over the yolk cell. By 18 hpf the embryos were grossly abnormal (Fig. 4I). Most embryos consisted of a cap of tissue atop the yolk cell, while others had a belt of tissue around the yolk. Variable numbers of finger-like extensions, often three, spread toward the opposite pole from this main clump. Morphological differentiation consisted of, at most, broad blocks of segmented tissue and a single, convoluted notochord. The embryos became necrotic after 24 hpf. Thus over-expression of eFGF caused profound changes in morphogenesis from gastrulation onwards, and the embryos showed only scant morphological differentiation.

**Effect of over-expression of eFGF on mesodermal gene expression and behaviour**

Since FGF is a potent mesoderm inducing factor in *Xenopus* animal cap assays, the dramatic development of the germ ring described above might indicate that more mesoderm was forming in these embryos. To test this possibility we compared the effect of eFGF on expression of *ntl* and *snail*. After

---

Table 1. Numbers of embryos affected after RNA injection

<table>
<thead>
<tr>
<th>RNA</th>
<th>Amount (pg)</th>
<th>n</th>
<th>Normal (%)</th>
<th>Abnormal (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>XFD</td>
<td>700</td>
<td>84</td>
<td>25</td>
<td>75</td>
</tr>
<tr>
<td>d50</td>
<td>700</td>
<td>86</td>
<td>92</td>
<td>8</td>
</tr>
<tr>
<td>ΔeFGF</td>
<td>126</td>
<td>37</td>
<td>95</td>
<td>5</td>
</tr>
<tr>
<td>eFGF(−)</td>
<td>126</td>
<td>52</td>
<td>92</td>
<td>8</td>
</tr>
<tr>
<td>eFGF</td>
<td>126</td>
<td>146</td>
<td>7</td>
<td>93</td>
</tr>
<tr>
<td>eFGF</td>
<td>42</td>
<td>33</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>eFGF(−)</td>
<td>12.6</td>
<td>60</td>
<td>5</td>
<td>95</td>
</tr>
<tr>
<td>eFGF</td>
<td>4.2</td>
<td>48</td>
<td>2</td>
<td>98</td>
</tr>
<tr>
<td>eFGF</td>
<td>1.4</td>
<td>32</td>
<td>47</td>
<td>53</td>
</tr>
<tr>
<td>eFGF(−)</td>
<td>0.4</td>
<td>38</td>
<td>92</td>
<td>8</td>
</tr>
</tbody>
</table>

XFD is the *Xenopus* dominant negative receptor, d50 is the non-functional receptor. ΔeFGF is a non-secreted mutant of eFGF, and eFGF(−) is in the antisense orientation.
injection of eFGF the spatial regulation of ntl expression (Fig. 5A) was lost, and expression was induced throughout the epiblast at 50% epiboly (Fig. 5B). This widespread expression was at a lower level than in the germ ring, and persisted throughout gastrulation. In contrast, the distribution of sna1 expression in the blastula and early gastrula was unaffected by ectopic eFGF activity (Fig. 5C), though it is possible that quantitative changes may have occurred. Therefore, eFGF did not have a general effect on genes expressed in the presumptive mesoderm. The lack of an effect on sna1 expression might indicate that the size of the mesodermal compartment was unaffected. This contention is supported by the observation, described later, that many neural markers were expressed at later stages, despite the virtually ubiquitous expression of ntl during earlier development.

At later stages, however, the hypoblast showed definite abnormalities in gene expression and behaviour. At 9 hpf, when sna1 expression should be restricted to the hypoblast underlying the germ ring and the adaxial cells of the somitic mesoderm (Fig. 5D), sna1 was detected throughout the entire hypoblast except for the presumptive dorsal mesoderm (Fig. 5E,F). And at 18 hpf Ntl was detected in small groups of mesenchymal cells located consistently at the ends of the axis-like extensions, giving them the appearance of tail buds (Fig. 5G). Notochord was only ever detected in one of the axis-like structures. At this stage myosin staining was detected in several separated regions (Fig. 5H,I), usually in association with the axis-like extensions (compare 5G,H). In summary, we detected one axis containing muscle and a notochord, plus one or more additional axis-like extensions which lacked a notochord but contained disorganised muscle and a structure resembling a tail-bud. Using notochord tissue and goosecoid (gsc) expression accumulation of cells is forming (asterisk). Note the increased thickness of the ventral hypoblast (arrowheads). (G) Normal embryo at 10 hpf (arrowhead = tail bud). (H) An injected embryo at the same stage showing an abnormal accumulation of cells at the top of the embryo (asterisk) and more than one thickening in the region of the tail-bud (arrowheads). (I) Embryos at 18 hpf showing a range of abnormal morphologies. The central embryo has a large accumulation of tissue on top of the yolk cell, whereas the left and right embryos have belts of tissue constricting the yolk cell. A variable number of axis-like extensions (arrowheads) have formed.
as dorsal markers, the embryos were not either dorsalised or ventralised.

**Effects of eFGF over-expression are not dependent upon ntl**

To determine whether the effects we observed were simply due to widespread activation of ntl, we injected eFGF RNA into no tail mutant embryos. *no tail* is a recessive mutation (Halpern et al., 1993) and 25% of injected embryos were expected to be homozygous mutant. All injected embryos developed the thickened germ ring and hypoblast and showed abnormal morphogenesis after gastrulation. Since homozygous *ntl* mutant embryos accumulate little or no Ntl protein (Schulte-Merker et al., 1994), we stained with the Ntl antibody to verify that a proportion of the embryos were *ntl* homozygous mutants: 9/47 embryos surviving to 18 hpf did not label using this antibody and were presumed to be homozygous mutants. A similar proportion (6/31) of embryos left un.injected developed the mutant phenotype. At the very least, therefore, the effects of eFGF over-expression on gastrulation movements and morphogenesis do not require functional Ntl protein. This may indicate that either another gene with similar properties to *ntl* is being activated by eFGF, or that eFGF is responsible for the downstream effects of *ntl*. We have yet to determine which aspects of the phenotype of embryos over-expressing eFGF are dependent upon *ntl* function.

**Markers of anterior and posterior fates are differentially regulated by FGF activity**

The striking correlation observed between FGF activity and the development of the trunk and tail tissue is consistent with FGF being involved in posteriorising the zebrafish embryo, as it appears to be in *Xenopus* (Isaacs et al., 1994). To understand the mechanism underlying this, we...
examined the effects of FGFR inhibition or eFGF over-expression on the expression patterns of genes potentially involved in this process. *cad1* (Joly et al., 1992) and *eve1* (Joly et al., 1993) are zebrafish homologues of the *Drosophila* *caudal* (Mlodzik et al., 1985) and *even-skipped* (Harding et al., 1986) genes. Members of these gene families are implicated in the specification of posterior fates and suppression of anterior ones (Ruiz i Altaba and Melton, 1989b; Mlodzik et al., 1990; Joly et al., 1993; Barro et al., 1994). We found that, as with *ntl*, expression of *cad1* and *eve1* was dependent upon FGF activity. *eve1* and *cad1* are usually expressed in the epiblast margin and later in the germ ring (Fig. 6A,D). After inhibition of the FGFR, gaps were found in the normal expression domains of both genes (Fig. 6B,E), and after over-expression of eFGF their transcripts were detected throughout the entire epiblast of the early and late gastrula (Fig. 6C,F). At 18 hpf, when *eve1* expression is normally restricted to the tail-bud mesenchyme and *cad1* expression to the tail, these genes were still expressed throughout most of the embryo (Fig. 6G,H). Thus FGF activity regulates the expression of two genes with posteriorising activity.

A possible target for posteriorising activity is *gsc* expression, which is a marker of dorsoanterior fate (Beddington and Smith, 1993). *gsc*-expressing cells are the first to involute and their subsequent migration toward the animal pole, the future anterior end, is the first morphological indication of the AP axis (Stachel et al., 1993). *gsc* expression was unaffected by FGFR inhibition, consistent with normal head development in these embryos (data not shown). After over-expression of eFGF the initial expression of *gsc* and involution of the shield cells was unaffected, indicating that specification of the dorsal axis was normal (Fig. 7A, B). But during gastrulation, when *gsc* is strongly expressed in the migrating anterior mesodermal cells (Fig. 7C), expression in injected embryos was significantly weaker, expressing cells were no longer tightly grouped and the extent of migration was highly variable (Fig. 7D). By 11 hpf, when *gsc* is usually detected in the pillow and prechordal plate, expression was no longer detected or was very weak and in abnormal locations (data not shown). eFGF over-expression, therefore, results in ectopic expression of posteriorising genes and abnormalities in phenotype and behaviour of anterior cells expressing *gsc*.

### Over-expression of eFGF results in loss of anterior neural markers

To analyse further the effects of the changes in *eve1*, *cad1* and *gsc* expression on AP patterning, we looked at a number of genes which show restricted expression along the AP axis. During early somitogenesis *pax2* is initially expressed at the prospective midbrain-hindbrain border, and *krox-20*/*krx-20* is expressed exclusively in rhombomeres 3 and 5 (Krauss et al., 1991; Oxtoby and Jowett, 1993; Fig. 8A). After over-expression of eFGF these domains were located much nearer the presumptive anterior end of the embryo and *pax2* could be found at the anterior limit in some embryos (Fig. 8B). Furthermore over 95% of injected embryos at the 10 somite stage expressed *krox-20*, usually but not always in a pair of stripes, but only two-thirds expressed *pax2*. Thus expression of *pax2* is more sensitive to eFGF treatment than *krox-20*, suggesting that the effects are primarily on anterior tissue.

To rule out the possibility that *pax2* was in a normal position injected with eFGF RNA and showing ubiquitous expression of *eve1*. (D-F) Lateral views of *cad1* expression during early gastrulation. (D) Normal embryo at 65% epiboly showing strong *cad1* expression throughout the germ ring. (E) Embryo injected with dominant negative FGFR RNA, with a clear gap (arrowhead) in *cad1* expression. (F) Embryo injected with eFGF RNA; *cad1* is expressed ubiquitously. (G,H) Lateral views of *cad1* expression at 18 hpf. (G) Normal embryo, anterior to the right, with yolk cell dissected away. *cad1* is only expressed in the tail region, especially in the tail bud (arrowhead). (H) Injected embryo showing that ubiquitous expression of *cad1* persists to this stage.
relative to the anterior end and that the AP axis was not bent back on itself by abnormal morphogenesis, we looked at expression of sonic hedgehog (shh), which is initially expressed by the entire axial mesoderm (Krauss et al., 1993). Injected embryos at the 10 somite stage expressed shh in a single band, which was considerably wider than that seen in normal embryos, and expression was always restricted to one side of the embryo (data not shown). Thus expression of pax2 and krx-20 appeared to be shifted anteriorly relative to the anterior limit of shh expression in the axial midline.

To confirm this apparent loss of anterior tissue we looked for expression of islet-1 (isl1), which is initially only expressed in the pillow (Inoue et al., 1994), which is the most anterior structure and probably derives from the shield. Though we predicted that isl1 expression would be lost, the majority of eFGF-injected embryos continued to express isl1 in a relatively normal pattern, at the base of the cap of cells. The persistence of isl1 expression probably indicates that this gene and gsc are expressed in different cells at the anterior end. But, whatever the reason, isl1-expressing cells remain a useful marker for the anterior end. In normal embryos isl1 and krx-20 labelling are widely separated by the forebrain, midbrain and anterior hindbrain (Fig. 8C); this distance is greatly reduced in eFGF-injected embryos, suggesting that the anterior neural plate was lost (Fig. 8D). To confirm this we looked at expression of receptor tyrosine kinase-1 (rtk1), which is expressed in the forebrain, hindbrain rhombomeres 3 and 5, and notochord (Xu et al., 1994; Fig. 8E). Injected embryos at the equivalent of 14 somites all showed expression in the notochord, most showed segmental hindbrain expression, but none showed staining anterior to this (Fig. 8F). Taken together these data show that over-expression of eFGF causes loss of anterior neural plate from the forebrain through to the hind-brain, although the most anterior mesoderm, expressing isl1, was unaffected.

DISCUSSION

Separate mechanisms control trunk and tail development

The dramatic difference between the phenotype of zebrafish embryos expressing the dominant negative FGFR and that of the no tail mutant demonstrates that a functional FGFR signalling pathway is essential for the development of a larger domain of the embryo than brachyury. While brachyury is clearly necessary for the formation of the tail, our results show that another gene or set of genes, also dependent upon FGFR, are required for the development of the trunk. Prior to this study the only clear evidence showing that FGF must have other targets besides brachyury came from animal-cap experiments in which injection of Xwnt8 mRNA and treatment with FGF induced notochord differentiation (Christian et al., 1992), whereas injection of a combination of Xbra and Xwnt8 mRNAs did not (Cunliffe and Smith, 1994). Our data demonstrate, in the intact embryo, that additional targets for eFGF must exist.

In order to investigate these targets further, we over-expressed eFGF in ntl mutant embryos to analyse the response to eFGF in the absence of ntl function. The result, that ntl embryos showed the same morphological changes (thickened germ ring and abnormal morphogenesis) as wild-type embryos, unambiguously demonstrates that ntl function is not required for all the effects of eFGF over-expression on morphogenesis (we have yet to analyse other aspects of the response). At this level of analysis, then, the other targets of eFGF appear to act in parallel and function in an analogous manner to ntl; or else ntl is not involved in the morphogenetic processes affected by eFGF. Further detailed analysis of the response to eFGF over-expression in ntl embryos will allow us to determine which components of the phenotype require ntl and to shed light on the function of ntl in fish development.

Are convergence and extension movements the main target of alterations in FGF signalling?

The complementary phenotypes caused by the dominant negative FGFR and eFGF over-expression (loss of trunk and tail versus anterior suppression) correlates with a fundamental
Role of FGF activity in axis formation

2991

difference in morphogenetic behaviour between these two regions of the body. The mesoderm progenitors of the head actively migrate, whereas more posterior regions undergo convergence and extension (CE) movements (Keller and Danilchik, 1988). FGF activity may be crucial in controlling CE and when this is defective, trunk and tail development is blocked. The phenotype after eFGF over-expression is consistent with this. The hypoblast no longer converges onto the dorsal side, but instead CE is activated to such a degree that multiple foci of CE develop throughout the embryo, giving rise to the axis-like extensions and a redistribution of mesoderm. It is noteworthy that the axis-like structures are incomplete since they lack notochords, in contrast to Li+-induced multiple axes, which appear to be fully competent in this respect. Similarly, eFGF did not ventralise the embryo since gsc expression and dorsal mesoderm (notochord) formed normally.

A role for FGF in controlling cell movements is consistent with the results of Isaacs et al. (1994), who found that Xenopus dorsal mesoderm expressing the dominant negative FGFR failed to converge and extend properly, and with the phenotypes of FGFR mutants in Drosophila where cell migration is affected (Shishido et al., 1993; Reichman-Fried et al., 1994). Our data do not indicate how direct this control is but there may be a link with snail, since over-expression of eFGF caused abnormally widespread expression of snail throughout the hypoblast of the late gastrula. The normal expression pattern of snai in zebrafish, and the snail mutant phenotype in Drosophila (Alberga et al., 1991), suggest that this gene is involved in morphogenetic movements.

A model for the role of FGF activity in trunk and tail development

We propose a model for the role of FGF activity in trunk and tail development which not only accounts for the severity of the effect of the dominant negative FGFR, but also provides a satisfactory explanation for two observations regarding the regulation and function of brachyury. Firstly, brachyury is expressed throughout the presumptive mesoderm yet the only tissues directly affected by loss-of-function mutations are the notochord and posterior body axis (Halpern et al., 1993; Chesley, 1935). The sparing of the trunk may indicate that brachyury is not required in this tissue (Beddington et al., 1992; Stott et al., 1993). Alternatively, ntl may function throughout the mesoderm but its function is redundant during trunk development due to the expression of another gene, or set of genes, with similar properties. Secondly, in Xenopus mesoderm, expression of eFGF and Xbra is linked by an auto-regulatory loop (Isaacs et al., 1994; Schulte-Merker and Smith, 1995), implying that functional brachyury protein indirectly maintains its own expression. Yet in no tail mutants, where this protein is non-functional, the mutant RNA is still expressed throughout gastrulation (Schulte-Merker et al., 1994). Since FGF activity regulates ntl, the expression of ntl RNA in the mutant may indicate that another factor is activating eFGF, which then maintains ntl expression in the absence of a functioning auto-regulatory loop.

Fig. 9A shows a cartoon of a zebrafish embryo divided into domains based on the phenotypes of the dominant negative FGFR and the ntl mutant. We propose that an unidentified

Fig. 8. Expression patterns of anterior markers after eFGF over-expression. (A) A normal 3-somite embryo viewed from the anterior end showing pas2 RNA at the midbrain-hindbrain border (large arrow) and krx-20 RNA in hindbrain rhombomeres 3 and 5 (small arrows). (B) Lateral view, anterior to the left, of eFGF-injected embryo showing pas2 and krx-20 close to the presumptive anterior limit of the embryo. (C) Normal expression pattern of isl1 in the pillow (large arrow) and krx-20 in the hindbrain (small arrows) in a 3-somite embryo (lateral view, anterior to the left). (D) Injected embryo at a similar stage (orientated as in C), showing the marked decrease in the distance between these two markers. (E) Dorsal view of flat-mounted 3 somite embryo, anterior to the left, showing the normal expression domains of rkt1. From left to right these are the forebrain (large arrow), rhombomeres 3 and 5 (small arrows), and notochord (large arrow). (F) Dorsal view of injected embryo, anterior to left, showing strong expression in the hindbrain rhombomeres and the notochord, which is broader than normal, but with no discernible forebrain expression.
gene, or set of genes, which is regulated by FGF, is essential for the development of the domain between the anterior limits of these phenotypes. This gene, referred to as no trunk (ntk), functionally substitutes for ntl in the trunk, allowing this to develop in the no tail embryo. In wild-type embryos expression of ntl and ntk is activated by a transient mesoderm inducing signal, and both genes activate eFGF expression (Fig. 8B). eFGF maintains expression of both genes through auto-regulatory loops.

In no tail embryos (Fig. 9C), mesoderm induction and ntl and ntk activation are unaffected. However, since Ntl protein is non-functional, eFGF-activation and trunk development are entirely dependent upon ntk but eFGF maintains expression of both genes. ntk is not active in the tail-bud, leading to loss of the tail. (D) Expression of the dominant negative FGFR interrupts the regulatory loops maintaining ntl and ntk expression. This blocks the function of eFGF, and expression of ntl and ntk cannot be maintained. Trunk and tail development are inhibited.

How are FGF activity and ntl expression related to mesoderm induction?

The widespread ectopic expression of ntl and the thickening of the hypoblast after eFGF over-expression might indicate enhanced mesoderm formation; FGF treatment or expression of brachyury alone is sufficient to confer mesodermal identity in Xenopus animal cap assays. But the normal expression of snail in the blastula after eFGF over-expression suggests that the size of the mesodermal compartment was unaffected, and the thickened hypoblast could be accounted for by alterations in epiboly and morphogenesis, which we know are substan-
tially affected. If mesoderm formation is not affected then eFGF activity cannot be sufficient to induce mesoderm in vivo. It also raises the issue of what exactly can be inferred from expression of *ntl* alone: expression may reflect more accurately the size of the mesodermal compartment. Perhaps in the intact animal, expression of *ntl* alone cannot confer mesodermal identity, but must be in the correct context, for example, in combination with *twist* and *snail*.

**Role of FGF activity and the germ ring in AP patterning**

Ectopic expression of eFGF led to suppression of anterior development, evident at late stages as loss of anterior neural plate, *isl1* expression in the anterior mesoderm was not affected. A simple explanation for the direct cause of the deficiency is that the prechordal plate, the anterior inducing tissue, has been transformed by expression of posterior-type genes, and its specific inducing properties have been lost or overridden. The loss of *gsc* expression during shield migration and the similarity in the axial expression domains of *RTK-1* (normally in the notochord) and *shh* (normally in both the prechordal plate and the notochord) supports this. *eve1* and *cad1* are obvious candidates for mediating this posteriorisation, since these genes were widely expressed after eFGF over-expression, and ectopic expression of other members of these families is sufficient to suppress anterior development in *Drosophila, Xenopus* and zebrashift (Ruiz i Altaba and Melton, 1989b; Mlodzik et al., 1990; Barro et al., 1994).

Whether this is related to the mechanism of AP patterning is unclear. The FGF-dependent regulation and ectopic expression of *eve1, cad1* and *ntl* demonstrated here shows that active FGF ligands must be restricted to the germ ring during normal development since all cells in the embryo can respond to FGF stimulation. This restriction may be significant since studies of amphibian development suggest that the micro-enviornment at the blastopore lip, the equivalent to the germ ring, acts as a posteriorising centre (Gerhart et al., 1989; Isaacs et al., 1994). According to this view, proximity to or increasing exposure to this environment positively instructs posterior positional values. Our results are broadly consistent with this, since ectopic expression of eFGF led to the induction throughout the embryo of an environment which was similar in many respects to that found at the germ ring, i.e. eFGF, *ntl*, *eve1*, *cad1*. Consequently the migration of anterior cells away from the germ ring did not remove them from its influence and their anterior character was lost. However, the persistence of *isl1* expression conflicts with this interpretation as this shows that at least some anterior tissue was unaffected. If this effect reflects a role for FGF activity in AP patterning, then FGF must regulate only certain elements of AP patterning, for example the inducing properties of the axial mesoderm.

We are especially grateful to Jonathan Slack for eFGF cDNA and for communicating results prior to publication, Enrique Amaya for the FGFR constructs, and Stefan Schulte-Merkier, David Wilkinson, Hitoshi Okamoto, Simon Hughes, Trevor Jowett, Qiling Xu, Jean-Stephane Joly, Bernard Thissen, Phil Ingham for other reagents used in this study. We thank Stefan Schulte-Merkier, Jeff Williams, Steve Wilson, Alex Gann and Carolyn Viviano for critical comments on the manuscript, and the anonymous reviewers, whose comments helped to clarify this work. N. H. is a BBSRC Senior Research Fellow. This work was supported by a project grant from the MRC to R.P. and N.H.

**REFERENCES**


of zebrafish pax genes suggest a role in early brain regionalisation. Nature 333, 267-270.


Shishido, E., Higashijima, S., Enomoto, Y. and Saijo, K. (1993). TGF- 
orpehomologues of Drosophila one is expressed in mesodermal primordium in early embryos. Development 117, 751-761.


