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Original Article

Evidence for non-axial A/P patterning in the nonneural ectoderm of *Xenopus* and zebrafish pregastrula embryos

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ABSTRACT Recent studies in early *Xenopus* and zebrafish embryos have demonstrated that posteriorizing, non-axial signals arising from outside the organizer (or shield) contribute to A/P patterning of the neural axis, in contradiction to the classical Spemann model in which such signals were proposed to be solely organizer derived. Our studies on the early expression of the transcription factors GATA-2 and 3 in both *Xenopus* and zebrafish nonneural ectoderm lend support to the existence of such non-axial signaling in the A/P axis. Thus we find that the earliest expression of GATA-2 and 3 is located in nonneural ectoderm and is strongly patterned in a graded manner along the A/P axis, being high anteriorly and absent from the most posterior regions. This results by early neurula stages in three broad zones: an anterior region which is positive for both GATA-2 and 3, a middle region which is positive for GATA-2 alone and a posterior region in which neither gene is expressed. These regions correspond to head, trunk and tail ectoderm and may represent the beginnings of functional segmentation of nonneural ectoderm, as suggested in the concept of the 'ectomere'. We find that A/P patterning of GATA expression in nonneural ectoderm may occur as early as late blastula/early gastrula stages. We investigate which posteriorizing signals might contribute to such distinct non axial ectodermal patterning in the A/P axis and provide evidence that both FGF and a Wnt family member contribute towards the final A/P pattern of GATA expression in nonneural ectoderm.

KEY WORDS: *A/P patterning, nonneural ectoderm, GATA factors, Xenopus, zebrafish, FGF, Wnt*

Introduction

The induction and patterning of the embryonic nervous system has been the focus of much experimental effort over a number of years [for reviews see (Doniach, 1992; Lumsden and Krumlauf, 1996)]. In contrast, very little is known about the formation and patterning of nonneural ectoderm. Recent experiments in early *Xenopus* and zebrafish embryos reveal the activity of posteriorizing, non-axial signals arising from outside the organizer (or shield) in contradiction to the classical Spemann model in which such signals were proposed to be solely organizer derived (Bang *et al.*, 1997; Woo and Fraser, 1997). Clearly such radial A/P signals should operate within nonneural as well as neural ectoderm, but, other than isolated reports of the expression in nonneural ectoderm of single hox genes (Condie and Harland, 1987; Kolm and Sive, 1995; von Bubnoff *et al.*, 1995), patterned gene expression within nonneural ectoderm has not been reported.

The importance of the nonneural ectoderm in early development is indicated by its role in a number of inductive epithelial-mesenchymal interactions which are required for early embryonic patterning. Thus ectodermal signaling to mesenchyme is important for initial patterning events in tooth development in urodeles (Lumsden, 1988), for the directional guidance of neural crest and migrating pronephric duct in axolotl (Lofberg *et al.*, 1985; Drawbridge *et al.*, 1995), and for the stimulation of hematopoietic differentiation within the blood islands in *Xenopus* (Maeno *et al.*, 1996). In early cranio-facial development, matrix mediated interactions between epithelia and mesenchyme, operating locally, are

Abbreviations used in this paper: A/P, antero/posterior; D/V, dorsal/ventral; nne, nonneural extoderm; bFGF, basic fibroblast growth factor; eFGF, embryonic fibroblast growth factor; RA, retinoic acid; BMP, bone morphogenic protein; MBT, mid blastula transition; TGF, transforming growth factor.

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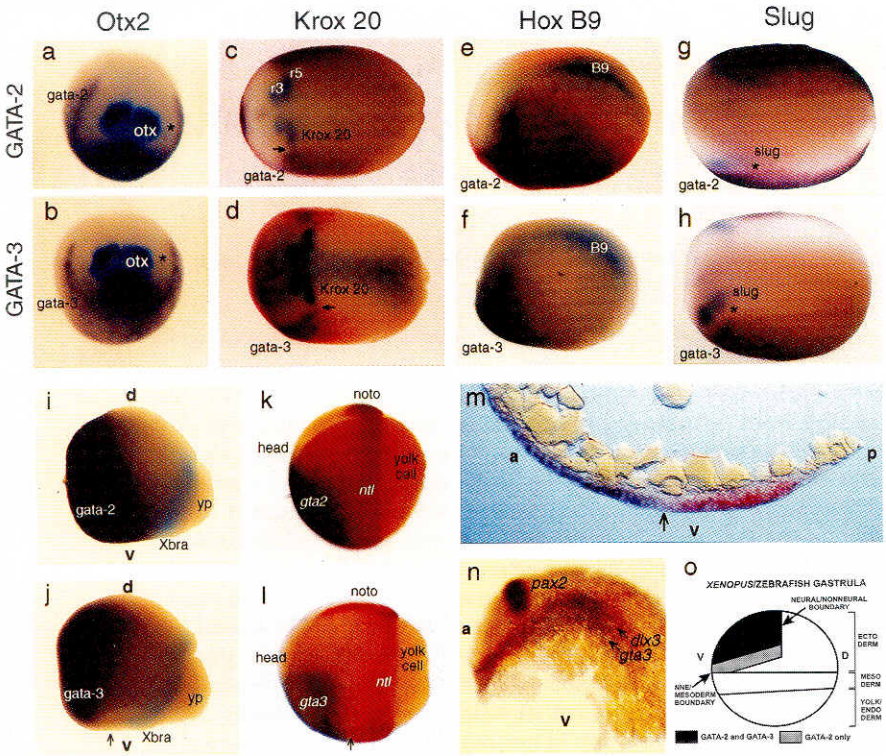

Fig. 1. Conserved boundaries of expression of GATA factors in the nonneural ectoderm of frog and fish gastrulae. *Double whole-mount in situ hybridizations of Xenopus early neurula embryos stained for GATA-2 (a,c,e,g) or GATA-3 (b,d,f,h) with the anterior neural marker, Otx2 (a and b), the hindbrain marker Krox20 (c and d), the spinal cord marker HoxB9 (e and f) and neural crest marker, slug (g and h). Anterior is to the left and posterior to the right except for a and b which are anterior views.* **(a)** *Embryo stained for Otx2 (turquoise) and GATA-2 (purple). At the most anterior end GATA-2 is expressed in the cement gland and abuts Otx2 whereas more posteriorly a gap can be seen between GATA-2 and Otx2 (asterisk).* **(b)** *Double staining for GATA-3 (purple) and Otx2 (turquoise). At the most anterior end GATA-3 expressed in the cement gland overlaps the expression of Otx2. More posteriorly, a gap exists between GATA-3 and Otx2 expression (asterisk).* **(c)** *Dorsal view of an embryo stained for GATA-2 (purple) and Krox 20 (turquoise) marking rhombomeres 3 and 5 of the hindbrain (r3 and r5). A gap can be seen between GATA-2 and Krox20 expression in r5 (arrow).* **(d)** *Dorsal view of an embryo stained for GATA-3 (purple) and Krox20 (turquoise). A gap is seen between GATA-3 and Krox20 staining in r5 (arrow).* **(e** *and* **f)** *Lateral view of embryos stained for GATA-2 and 3 (purple) and HoxB9 (B9, turquoise). Note that GATA-2 is expressed more posteriorly than GATA-3.* **(g** *and* **h)** *Dorsal views of embryos stained for GATA-2 (purple, g) or GATA-3 (purple, h) and the neural crest marker slug (turquoise, g and h). Note the gap between GATA and slug expression (asterisk, g and h).* **(i** *and* **j)** *Double whole-mount in situ hybridization of Xbra (turquoise, i and j) with GATA-2 (dark blue, i) and GATA-3 (dark blue, j) in Xenopus mid-gastrula embryos (the notocordal Xbra signal is not yet visible, stage 11). Note that GATA-2 abuts Xbra expression, (i), but a gap is seen between GATA-3 and Xbra expression, (arrow, j). yp, yolk plug; d, dorsal; v, ventral; anterior is to the left.* **(k)** *A 70% epiboly (gastrula) zebrafish embryo, stained for gta2 (blue) and ntl (red). gta2 expression abuts ntl at all gastrula stages.* **(l)** *An intact 70-80% (gastrula) zebrafish embryo stained for gta3 (blue) and ntl (red). Note the gap between gta3 and ntl (arrow). This gap is also seen at earlier gastrula stages (data not shown). At late gastrula, ntl is expressed in the notochord (noto).* **(m)** *17 mm saggital section through the region marked by the arrow in l showing the gap (arrow) between gta3 (blue) and ntl (red) expression. a, anterior; p, posterior; v, ventral.* **(n)** *A flat mount of a zebrafish embryo stained for gta3 (blue) and dlx3 (expressed strongly in placodal tissue at this stage) and pax2 (a marker of the midbrain/hindbrain border) both stained in red. Note the gap between dlx3 and pax2 staining representing neural crest. a, anterior; p, posterior; v, ventral.* **(o)** *Diagrammatic representation of a Xenopus/zebrafish gastrula embryo showing the boundaries of expression of GATA-2 and 3 between neural and nonneural ectoderm and between nonneural ectoderm and mesoderm which are conserved between the two species.*

thought to play a role in directing head mesenchyme differentiation into cartilage and bone, thus specifying skeletal pattern (Thorogood, 1988). In addition, instructive interactions from nonneural to neural ectoderm have been demonstrated in the induction of dorsal neural tube cell types including neural crest (Dickinson *et al.*, 1995; Liem *et al.*, 1995). Such diversity of function implies that the nonneural ectoderm cannot exist as a blank sheet but must be patterned in order that a variety of regional inductive, instructive or permissive interactions can occur. Indeed support for the concept of patterning in the nonneural ectoderm came from studies involving transplantation of anterior neural crest and associated superficial nonneural ectoderm between quail and chick neurula embryos. These experiments led to the suggestion that not only is the neural tube segmentally divided into units (the neuromeres) along the A/P axis but so too is neural crest and nonneural ectoderm, thus defining an early developmental unit –the 'ectomere' (Couly and Le Douarin, 1990).

The identity of nonneural ectoderm is dependent on BMP-4. In the dorsoventral (D/V) axis, BMP-4 promotes epidermal development and inhibits neural tissue formation (Graff *et al.*, 1994; Sasai *et al.*, 1995; Wilson and Hemmati-Brivanlou, 1995; Neave *et al.*, 1997). Inhibition of BMP receptor activity by injection of a dominant negative BMP2/4 receptor into ventral regions of the *Xenopus* embryo results in formation of a second neural axis (Graff *et al.*, 1994; Suzuki *et al.*, 1994; Schmidt *et al.*, 1995). The same effect is produced by ventral injections of RNA coding for cleavage mutant BMP2, 4 and 7 proteins (Hawley *et al.*, 1995). In addition, neuralization of dissociated animal cap ectodermal cells is now thought to be due to dilution of epidermal promoting BMP-4 in the animal cap (Wilson and Hemmati-Brivanlou, 1995). Inhibition of BMP-4 receptor activity in animal caps leads to formation of neural tissue and BMP-4 expressed during gastrulation blocks neural induction by noggin and follistatin (Sasai *et al.*, 1995). More recently, both noggin and chordin have been shown to antagonize BMP-4 by direct interaction between the proteins, preventing binding of BMP-4 to its receptor (Piccolo *et al.*, 1996; Zimmerman *et al.*, 1996). Thus BMP-4 activity is an essential requirement for nonneural ectoderm identity and inhibition of BMP-4 activity is necessary for neural tissue formation.

In the animal/vegetal axis of late blastula/early gastrula stages (which for ectoderm is roughly equivalent to the A/P axis), prospective nonneural ectoderm is in apposition to the ring of mesendoderm induced around the equator of

the embryo. So the signaling molecules likely to define the limits of nonneural ectoderm in this axis are the mesoderm inducing growth factors belonging to the FGF and TGFβ families (Smith, 1989). In addition, recent studies in *Xenopus* from a number of labs have demonstrated that, during gastrula stages, bFGF is capable of inducing neural tissue directly, independent of mesoderm induction (Kengaku and Okamoto, 1993; Kengaku and Okamoto, 1995; Lamb and Harland, 1995; Launay *et al.*, 1996), and of posteriorizing anterior neural tissue induced by noggin (Lamb and Harland, 1995), follistatin or a dominant negative activin receptor, Δ1XAR1 (Cox and Hemmati-Brivanlou, 1995). Furthermore, a dose-dependent effect of bFGF on neural posteriorization has been demonstrated (Kengaku and Okamoto, 1995). In *Xenopus*, FGF is enriched in posterior/vegetal regions of the gastrula embryo in and around the blastopore (Isaacs *et al.*, 1994) and could therefore posteriorize nonneural as well as neural ectoderm.

Retinoic acid (RA) has also been demonstrated to act as a posteriorizing molecule during gastrula stages. Addition of exogenous RA during blastula and gastrula stages results in concentration-dependent disruptions of the A/P axis, affecting both mesodermal and ectodermal tissues (Dursten *et al.*, 1989; Sive *et al.*, 1990; Ruiz i Altaba and Jessell, 1991). Molecular analysis shows that RA treatment can lead to a concentration-dependent decrease in some anteriorly expressed genes and increase of some posterior genes (Sive *et al.*, 1990; Papalopulu and Kintner, 1996). Therefore, although reports on the location of retinoid activity in the embryo are variable, RA is another candidate for posteriorization during the blastula and gastrula stages (Dursten *et al.*, 1989; Chen *et al.*, 1994; Creech Kraft *et al.*, 1994).

Members of the *Wnt* family have also been shown to have posteriorizing activities within *Xenopus* neural ectoderm. Although incapable of inducing neural tissue per se, both *XWnt3A* RNA and *XWnt8* in a DNA construct which delays expression until after MBT are capable of posteriorizing anterior neural tissue (McGrew *et al.*, 1995; Fredieu *et al.*, 1997). Very recently *XWnt3A* was reported to be expressed in posterior mesoderm and ectoderm during gastrula and neurula stages (McGrew *et al.*, 1997), and at least one other member of the *Wnt* family –*XWnt8* is also expressed in posterior non-axial mesoderm during gastrulation (Christian and Moon, 1993). Therefore, like FGF, these *Wnts* could posteriorize nonneural as well as neural ectoderm during the period of early neural induction. The *Wnts*, therefore, represent a third possible posteriorizing activity which might pattern the nonneural ectoderm during gastrulation.

Expression of the genes involved in early ectomesenchymal signaling activities will be driven by transcription factors located in the ectoderm at the appropriate time in development. Although the transcription factors GATA-2 and 3 are more commonly associated with blood, we have shown that the earliest expression of these factors in *Xenopus* and zebrafish embryos is primarily in ventral ectoderm rather than mesoderm. This GATA factor expression in the ectoderm is transitory starting before the onset of gastrulation and declining during neurula stages (Walmsley *et al.*, 1994; Neave *et al.*, 1995; Bertwistle *et al.*, 1996). The importance of GATA factors for early ectodermal identity is clearly indicated by mutations in the *Drosophila* and *C. elegans* GATA homologs, *pannier* and *ELT-1* (Ramain *et al.*, 1993; Page *et al.*, 1997). In this study, using positionally defined neural, placodal and mesodermal markers, we demonstrate that GATA-2 and 3 predominate in the nonneural ectoderm of both frogs and fish during late blastula, gastrula and early neurula stages, and that the boundaries of expression are conserved between the two species and may be defined by the same signaling molecules. We show that GATA expression is patterned in a graded manner in the A/P axis being high anteriorly and absent from posterior regions resulting in three broad zones corresponding to head, trunk and tail ectoderm. This distinctive A/P patterning of the GATA factors does not appear to result from RA activity. We present evidence that, as reported for neural ectoderm, FGF and *XWnt3A* can contribute to this graded pattern of expression in nonneural ectoderm, suggesting that these molecules play a role in patterning all ectoderm and represent at least part of the non-axial signaling which patterns pregastrula embryos in the A/P axis.

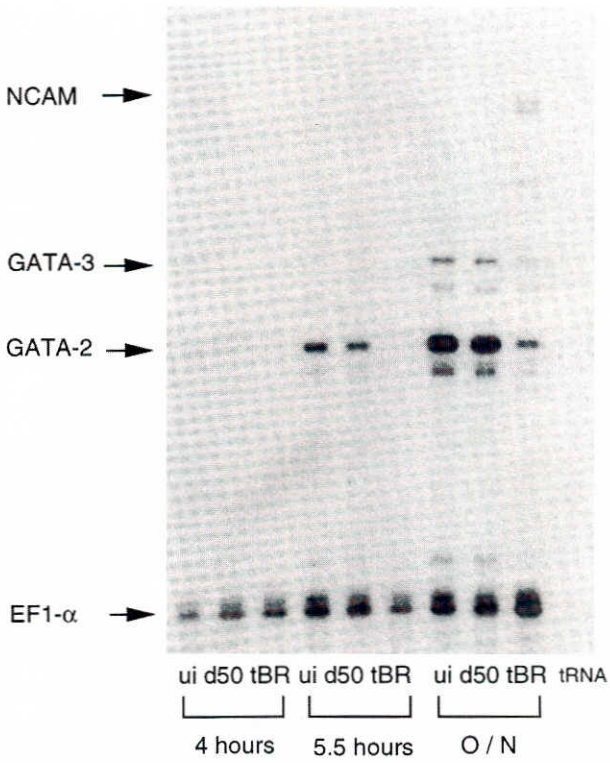

Fig. 2. Expression of GATA-2 and 3 in nonneural ectoderm is dependent on BMP-4 signaling. *1ng of a dominant negative truncated BMP receptor RNA (lanes tBR) or of a control RNA (d50), was injected in to the animal pole of all four blastomeres of 4-cell stage embryos. Animal caps were removed at stage 8 along with uninjected caps (ui lanes), and cultured for 4 h, 5.5 h or overnight to stage 14 (O/N lanes). RNA prepared from caps was probed by ribonuclease protection for the expression of GATA-2 and 3, NCAM and EF1-α as a loading control.*

Results

GATA-2 and 3 expression in Xenopus ectoderm is patterned along the D/V and A/P axes

In the D/V axis at neurula stages, both GATA-2 and 3 are expressed in the sensorial layer of the ectoderm from the ventral mid line to a point very close to the edge of the neural plate (Fig. 1e and f). This indicates that, at this stage, both factors may be expressed only in the nonneural ectoderm. The separation of the GATA-2 and 3 domains from the neural plate is demonstrated by a small gap that can be seen between the limits of both GATA

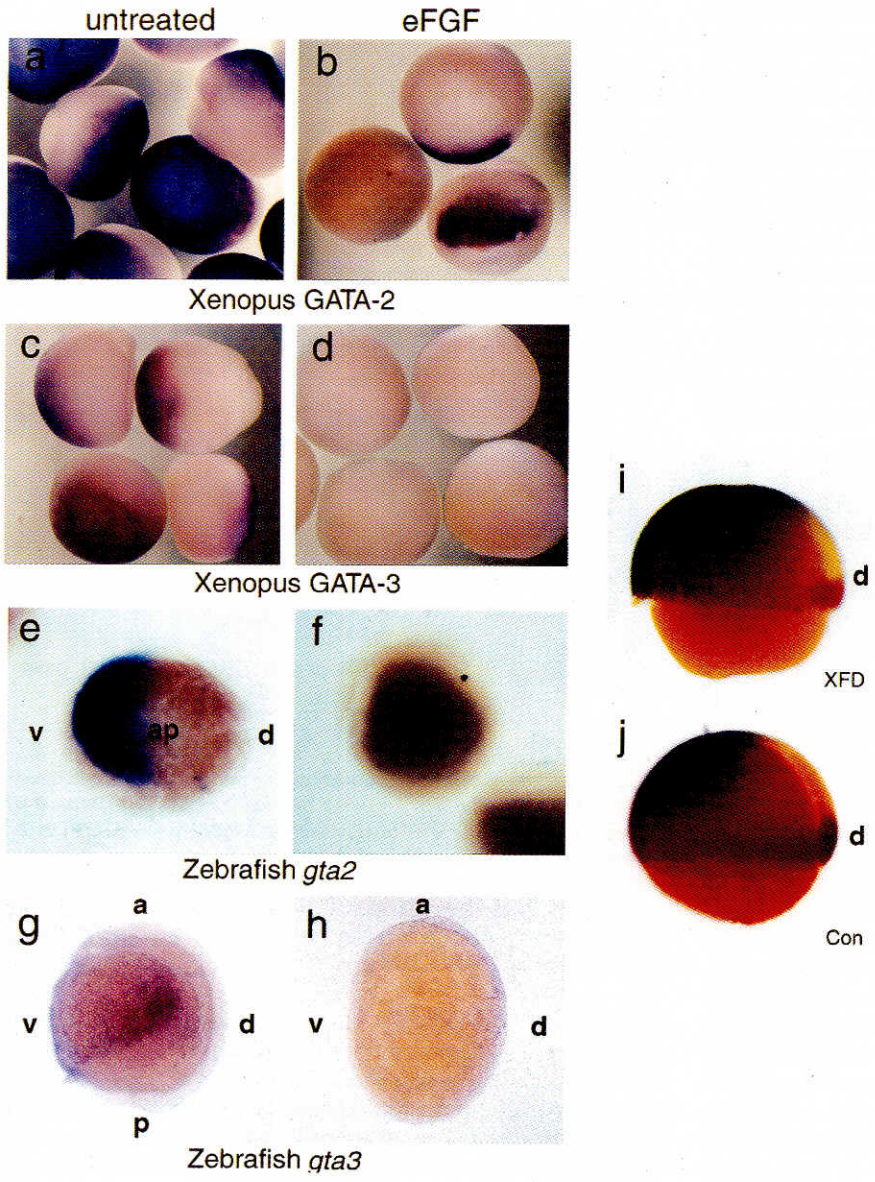

Fig. 3. FGF represses the expression of GATA-2 and 3 (*gta2* and 3) in the nonneural ectoderm of frog and fish embryos and restricts expression to regions outside the germ ring. *Xenopus* **(a-d)** *and zebrafish* **(e-j)** *embryos were either untreated (a,c,e,g and j) or injected with 2pg of eFGF RNA at the single cell stage for Xenopus (b and d) or with 220 pg eFGF RNA (note that this RNA was not made with the Megascript kit and was capped less successfully, thus more was needed to produce an effect) at the 1-4 cell stage for zebrafish (f and h) or with 40pg XFD RNA at the 1-4 cell stage (i). Embryos were examined by whole-mount in situ hybridization staining for GATA-2 (a and b), GATA-3 (c and d), gta2 (e and f) and gta3 (g and h). Note that there is no gta2 staining in f): the background is caused by yolk which has been lost from the embryo in e). (i) Zebrafish gastrula embryo injected with XFD RNA and stained for gta2 (blue) and ntl (red). Note that gta2 expression reaches down to the involuting edge on the ventral side where ntl has been eliminated.*

factors and *Krox20* expression (Bradley *et al.*, 1992) in rhombomere 5 (Fig. 1c and d, arrow). To define more closely the gap between neural and nonneural ectoderm in anterior regions, embryos were stained for *slug*, a marker of neural crest (Mayor *et al.*, 1995), along with GATA-2 and 3. In this case, we could still detect a gap (asterisk, Fig. 1g and h) between GATA positive ectoderm and slug positive neural crest. Therefore the gap seen between GATA-2 and 3 and *Krox20* (Fig. 1c and d) represents both early neural crest and placodal precursors. This was also demonstrated in zebrafish. Figure 1n shows a flat mount of a zebrafish embryo at 95-100% epiboly probing for *gta3* in purple, marking ventral ectoderm, *pax2* in red, marking neural ectoderm (Krauss *et al.*, 1991), and *dlx3* in red, which at this stage is strongly expressed in presumptive placodal tissue (Akimenko *et al.*, 1994). As in *Xenopus*, *gta3* expression in the D/V axis does not abut the neural plate. It does, however, abut the *dlx3* expressing placodal precursors. The gap between *dlx3* and the neural plate marker *pax2* represents neural crest precursors. Thus GATA expression is confined to the nonneural ectoderm.

In the A/P axis, expression of GATA-2 and 3 are both patterned in a rostro-caudal gradient with GATA-2 being expressed more posteriorly than GATA-3 (Fig. 1c-h). In order to define the boundaries of expression of GATA-2 and 3 in nonneural ectoderm within this axis, early neurula *Xenopus* embryos were examined by double whole-mount *in situ* hybridization, probing for the GATA factors and the neural markers *Otx2* (defining anterior neurectoderm –Pannese *et al.*, 1995), *Krox20* (expressed in hindbrain rhombomeres 3 and 5 –Bradley *et al.*, 1992) and HoxB9 which is expressed in the spinal cord (Wright *et al.*, 1990; Godsave *et al.*, 1994). Both GATA-2 and 3 are strongly expressed at the most anterior edge of the nonneural ectoderm as defined by the position of *Otx2* expression (Fig. 1a and b) and their expression decreases gradually in a posterior direction (Fig. 1c-f). Both GATA factors are also expressed in the cement gland overlying the ventral forebrain with GATA-3 being expressed more dorsally then GATA-2 (Fig. 1b). Lateral views demonstrate that, for both GATA factors, expression falls away from the dorsal midline resulting in their expression extending further posteriorly on the ventral side of the embryo (Fig. 1e and f). The ventral posterior limit of GATA-3 expression lies somewhere between rhombomere 5 in the hindbrain, as defined by *Krox20* (Fig. 1d), and the anterior limit of the spinal cord defined by *HoxB9* staining (Fig. 1f), whereas GATA-2 expression extends more posteriorly to a position well down the spinal cord (Fig. 1e). Dorsally, near the boundary of the neural plate, GATA-3 expression falls off just posterior to rhombomere 5 (Fig. 1d and f), whereas GATA-2 expression reaches as far as the anterior limit of *HoxB9* staining, the junction between hindbrain and spinal cord (Fig. 1c and e).

In summary, GATA-2 and 3 are expressed in an antero-posterior gradient in nonneural ectoderm, high in the anterior and low or absent in the more posterior regions of the embryo. Both dorsally

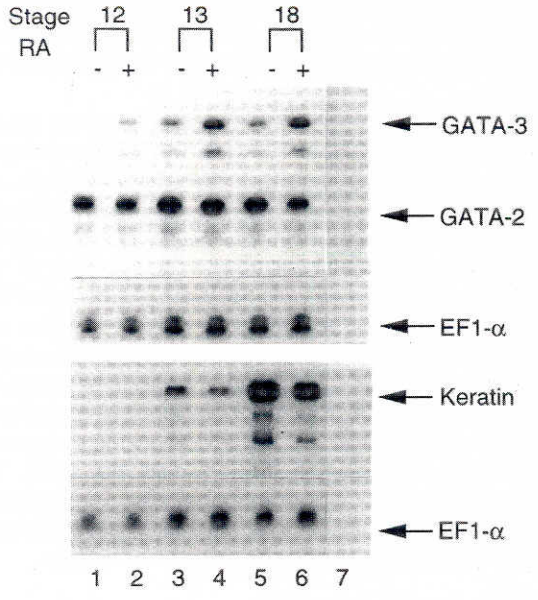

Fig. 4. RA is not responsible for patterning GATA-2 and 3 in nonneural ectoderm. *Animal caps were isolated at stage 8-9, and treated with 10^{-7}M RA until stage 11. Caps were then transferred and allowed to develop without RA. RNase protection was carried out on treated caps (+) and on untreated controls (-) taken at stages 12, 13 and 18. In this experiment there is little effect on GATA-2 but, GATA-3 is increased in RA treated caps at all stages. In the same experiment epidermal keratin is decreased. EF1-α was used as loading control and tRNA was probed as a negative control (lane 7). The downregulation of epidermal keratin probably represents an effect of the RA on differentiation of the ectoderm.*

and ventrally, GATA-2 expression extends more posteriorly than GATA-3. Both factors are expressed strongly ventrally and extend laterally to outline the anterior neural plate, with a small gap between the boundaries of neural and nonneural ectoderm which may represent neural crest and placodal tissue. The distance from the dorsal midline of GATA-2 and 3 expression increases considerably towards the posterior end of the embryo, where the neural plate border is less well defined, and expression extends more posteriorly on the ventral side.

To understand how these patterns of expression in neurula embryos might have been established we looked at mid to late gastrula stage *Xenopus* embryos probed by double whole-mount *in situ* for GATA-2 or 3 and the mesodermal marker *Xbra* (Smith *et al.*, 1991). At these early stages, within the ectodermal layer, the animal/vegetal axis is roughly equivalent to the future A/P axis. A lateral view of such embryos shows that, even during early gastrulation, GATA-2 is expressed more posteriorly than GATA-3 (Fig. 1i and j). Whereas ventrally, the limit of GATA-2 extends posteriorly to touch the boundary of expression of *Xbra*, GATA-3 expression falls short of it (arrow, Fig. 1j). In addition, the expression of both GATA-2 and 3 has already been restricted dorsally. Thus, in *Xenopus*, the localization of GATA expression to the nonneural ectoderm is established during gastrulation. Completion of the gastrulation movements will lead to the pattern seen in neurula embryos. In the zebrafish, similar boundaries of expression can be seen during gastrulation (Fig. 1k and l). A gastrula stage embryo probed for *gta2* and *ntl* (the zebrafish homolog of Xbra, (Schulte-Merker *et al.*, 1994) demonstrates that *gta2* abuts *ntl* ventrally (Fig. 1k), whereas there is a clear gap between the ventral boundaries of *ntl* and *gta3* (Fig. 1l, arrow). In the D/V axis, *gta2* and *3* have been restricted dorsally and expression is seen only in ventral nonneural ectoderm (Fig. 1k and l). A saggital section of the embryo shown in Figure 1l clearly demonstrates the gap between *gta3* and *ntl* expression (arrow, Fig. 1m).

If we combine the data from frogs and fish, we may define the expression domains of GATA-2 and 3 (*gta2* and *3*) as nonneural

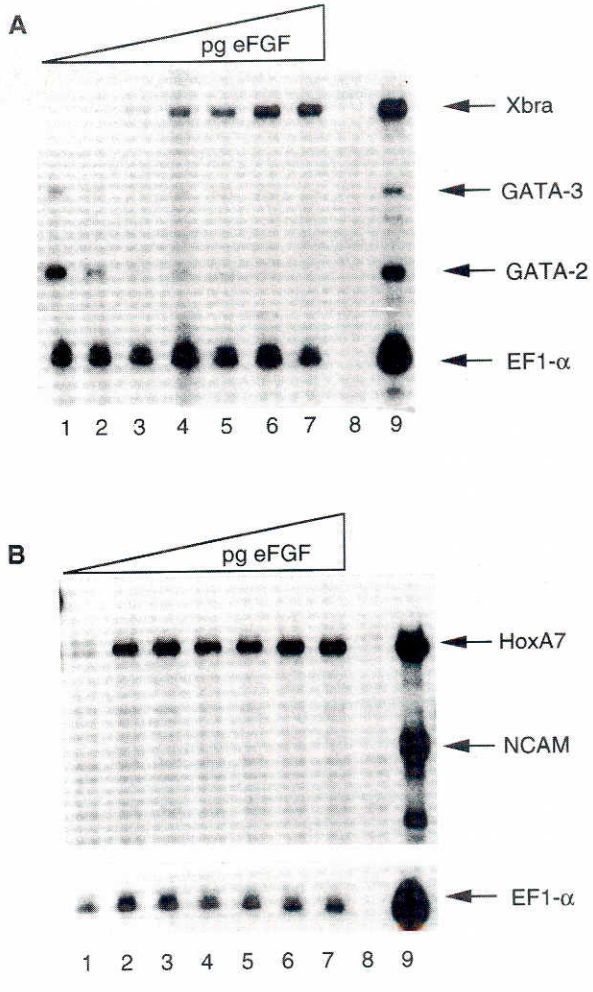

Fig. 5. FGF posteriorizes blastula nonneural ectoderm and down regulates GATA-2 and 3 in the absence of mesoderm and neural induction. (A) *GATA-2 and 3 are down regulated in the absence of and at low levels of mesoderm induction. eFGF RNA was injected in to the animal pole of single cell stage Xenopus embryos over a range of 60 fg to 2.5 ng (lanes 1 to 7 represent 0, 60 fg, 125 fg, 0.25 pg, 0.5 pg, 1 ng and 2.5 ng of injected eFGF RNA respectively). Animal caps were removed at stage 8, cultured to stage 10.5 and processed for RNase protection. Probing was carried out for GATA-2, GATA-3, Xbra and EF1-α as a loading control.* **(B)** *FGF does not induce NCAM but does posteriorize as shown by HoxA7 expression. Caps from the same experiment as (a) but aged to stage 21 probed for HoxA7, NCAM and EF1-α. This experiment was repeated three times to verify that the rate of GATA down regulation significantly exceeded the rate of induction of Xbra ($p < 0.05$).*

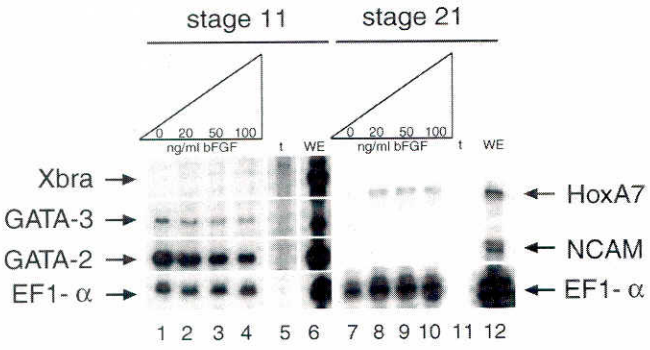

Fig. 6. FGF posteriorizes gastrula nonneural ectoderm and down regulates GATA-2 and 3 in the absence of mesoderm and neural induction. *RNA from ventral gastrula caps aged to stage 10.5 then treated with bFGF in the range 0-100 ng/ml and incubated to stage 11 (lanes 1-4) or stage 21 (lanes 7-10) was probed by ribonuclease protection for expression of GATA-2, GATA-3 and Xbra (lanes 1-6), NCAM (doublet) and HoxA7 (lanes 7-12) and EF1-α as a loading control. WE, whole embryo RNA at stage 11 (lane 5) and stage 21 (lane 11). Negative controls were 20 μg tRNA (t, lanes 6 and 12). HoxA7 up-regulation was confirmed by scanning densitometry taking in to account the loading controls.*

ectoderm, being excluded from neural ectoderm, neural crest and placodal precursors. This expression is strongest anteriorly, reducing to zero posteriorly. Thus both the presence and patterning of GATA-2 and 3 expression in nonneural ectoderm has been evolutionarily conserved between frogs and fish. Figure 1o depicts diagrammatically the conserved boundaries of GATA-2 and 3 expression in nonneural ectoderm of frogs and fish at gastrula stages.

Creating the D/V boundary: inhibition of BMP-4 signaling abolishes the expression of GATA-2 and 3 in Xenopus animal cap ectoderm

BMP-4 has recently been shown to play a vital role in defining ectodermal fate. To ask whether BMP-4 might be involved in defining the neural/nonneural boundary of GATA-2 and 3 expression, we injected RNA encoding a dominant negative truncated BMP-4 receptor (tBR) into the animal pole ectoderm of all four blastomeres at the four cell stage, isolated animal caps at stage 8 and looked for effects on GATA-2 and 3 expression over a time course of 4 h to approximately 14 h (O/N, Fig. 2). Both GATA-2 and 3 were down regulated by the inhibition of BMP-4 signaling resulting from injection of the truncated receptor (lanes tBR). The down regulation of GATA-2 can already be detected in caps incubated for 5.5 h (longer exposures show that this is true also for GATA-3, which is expressed at a lower level than GATA-2, data not shown). In caps incubated overnight to early neurula when NCAM expression starts to be detectable, the down regulation of GATA-2 and 3 expression is accompanied by the induction of NCAM transcription (Fig. 2, doublet in lane tBR of O/N sample) implying that a conversion of nonneural to neural ectoderm has taken place. These effects were not seen in uninjected embryos nor when a control mutant FGF receptor, d50 (Amaya *et al.*, 1991), was injected (Fig. 2, lanes ui and d50). In addition, injection of BMP-4 RNA in to the animal pole results in stimulation of GATA-2 expression in *Xenopus* embryos (data not shown). Similar effects of BMP-4 and a dominant negative BMP receptor RNA on expression of *gta*3 in the zebrafish have been reported by us (Neave *et al.*, 1997). Thus BMP-4 is implicated in defining the D/V boundary of GATA expression.

Several lines of evidence have indicated that the boundary between neural and nonneural ectoderm results from the antagonistic action of neural inducers and BMP-4 (Sasai *et al.*, 1995; Wilson and Hemmati-Brivanlou, 1995). We have previously shown that the neural inducer noggin plays a role in defining regions of GATA-2 expression in *Xenopus* embryos (Walmsley *et al.*, 1994). Thus noggin was shown to down regulate the expression of GATA-2 in animal cap ectoderm by converting GATA-2 positive animal cap ectoderm to GATA-2 negative, NCAM positive neural tissue. Using the same experimental approach, we have now shown that noggin has a similar effect on GATA-3 expression (data not shown) and we have recently reported that *Xenopus* noggin suppresses ectodermal *gta*3 expression in zebrafish (Neave *et al.*, 1997). Thus, in both frogs and fish, the neural/nonneural boundary of GATA expression may reflect the antagonism between the activities of BMP-4 and a dorsalizing activity such as noggin.

Mesoderm induction by FGF and activin down regulates GATA-2 and 3 expression in Xenopus and zebrafish embryos

In frog gastrulae, a ring of *Xbra* expression in the mesoderm around the blastopore is induced and maintained by FGF signaling (Isaacs *et al.*, 1994; Schulte-Merker and Smith, 1995). Similarly, in fish, *ntl* expression in the germ ring is dependent on FGF (Griffin *et al.*, 1995). We have shown that, in both frogs and fish, GATA-2 and 3 expression is excluded from the blastoporal mesoderm and the germ ring (Fig. 1). Thus FGF is present at the right time and place to play a role in setting up the GATA expression boundary between nonneural ectoderm and mesoderm.

To test for effects of FGF on the expression of GATA-2 and 3 we injected eFGF RNA in to *Xenopus* and zebrafish embryos and monitored GATA expression by whole-mount *in situ* hybridization. In both species GATA-2 and 3 were strongly down regulated by eFGF (Fig. 3a-h). When zebrafish embryos were injected with XFD, a dominant negative receptor for FGF (Amaya *et al.*, 1991), and doubly stained for *gta2* (purple) and *ntl* (red), ectopic *gta2* expression extended posteriorly into the ventral blastoderm margin where *ntl* expression was eliminated (Fig. 3i). However, *gta2* expression was not seen where XFD abolished *ntl* expression more dorsally. The normal expression of *ntl* and *gta2* in uninjected embryos is shown in Figure 3j. Similar results were found for XFD injected embryos probed for *gta3* and *ntl* (data not shown). Taken together these data indicate that FGF signaling is required to exclude GATA expression from the germ ring and that the dorsoventral patterning of GATA factors is maintained even during ectopic expression in the germ ring.

Confirmation of the loss of GATA expression in animal pole ectoderm resulting from eFGF injection of whole embryos was obtained in isolated *Xenopus* animal caps where both GATA-2 and 3 were downregulated by injection of eFGF RNA or exposure to bFGF protein (see Fig. 5A and data not shown). The downregulation of GATA-2 and 3 in caps was accompanied by induction of mesoderm as measured by *Xbra* expression. Similar results were obtained when animal caps were treated with activin (Walmsley *et al.*, 1994 and data not shown). Furthermore, in XFD injected caps, GATA expression levels were undisturbed and treatment of XFD injected caps with activin, where mesoderm was not induced, also

had no effect on GATA levels (data not shown). Taken together these experiments show that when FGF is absent or low in ectoderm, GATA expression is permitted, but raising the levels of FGF or of activin results in down regulation of GATA expression associated with the conversion of ectoderm to mesoderm. Therefore both FGF and FGF-dependent activin-like activities could be playing roles in setting up the boundary of GATA expression between nonneural ectoderm and mesoderm.

Patterning GATA-2 and 3 expression in the A/P axis

We next asked which signaling molecules might be responsible for refining the pattern of GATA-2 and 3 in ventral nonneural ectoderm along the A/P axis. In both *Xenopus* and zebrafish embryos, by early gastrula, expression of GATA-2 extends more posteriorly than that of GATA-3 and the most posterior ectoderm is GATA free (Fig. 1i-l). This suggested the existence of non-axial posteriorizing signals which might be patterning the nonneural ectoderm in a similar manner to that described for neural patterning in *Xenopus* and zebrafish embryos (Bang *et al.*, 1997; Woo and Fraser, 1997).

RA activity is not consistent with a role in patterning GATA factor expression in the nonneural ectoderm

RA has been demonstrated to act as a posteriorizing molecule during gastrula stages. Measurement of the levels of activity of endogenous retinoids in *Xenopus* embryos has revealed that their distribution is complex but may be present in a gradient in the anteroposterior axis, being high in the posterior and low in the anterior regions of the embryo (Chen *et al.*, 1994). We therefore asked if RA might play a role in patterning GATA-2 and 3 expression in the nonneural ectoderm along this axis. Animal cap ectoderm from stage 8-9 *Xenopus* embryos was treated with 10^{-7}M RA until stage 11, the period during which ectodermal patterning is taking place. The caps were then harvested at stages spanning optimal GATA-2 and 3 expression, and monitored by RNase protection. RA had little effect on the expression of GATA-2 but up-regulated the expression of GATA-3 at all stages tested (Fig. 4). The differential responses of the two GATA factors to RA treatment suggests that RA cannot be responsible for the graded anteroposterior patterning of GATA-2 and 3 in nonneural ectoderm. In the same experiment the expression of epidermal *keratin* was clearly down regulated (Fig. 4), indicating a possible change in cell fate. However, no such activity has been attributed to RA and, in this case, the down regulation of epidermal *keratin*, a marker of terminally differentiated epidermis, is more likely to represent an inhibition of epidermal differentiation by RA (Fuchs and Green, 1981). A role for GATA factors in maintaining the undifferentiated state has already been suggested (Briegel *et al.*, 1993; Gove *et al.*, 1997) and in this instance it seems likely that the responses of GATA-2 and 3 to RA are reflecting a block to differentiation rather than A/P patterning.

FGF posteriorizes blastula nonneural ectoderm and contributes to the patterning of GATA factors within this layer

FGF can posteriorize anterior neural tissue (Cox and Hemmati-Brivanlou, 1995; Lamb and Harland, 1995). During gastrulation, FGF expression is located in the posterior regions of *Xenopus* embryos (Isaacs *et al.*, 1992,1994; Isaacs, 1997) and is therefore present at the right time and place to posteriorize nonneural ectoderm during the period when posteriorization and patterning of the neural plate is occurring. To investigate the effects of FGF more

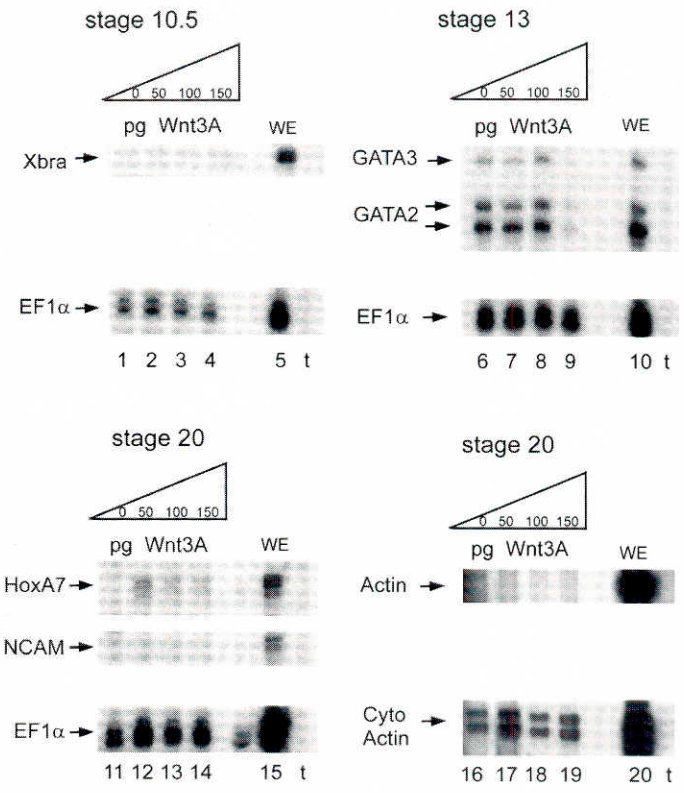

Fig. 7. XWnt3A posteriorizes blastula nonneural ectoderm and down regulates GATA factor expression in the absence of mesoderm and neural induction. *50, 100 and 150 pg XWnt3A RNA was injected in to the middle of the animal pole of single cell stage Xenopus embryos. Animal caps from uninjected and injected embryos were removed at stage 8 and were cultured in 1xMBS to stage 10.5, 13 or stage 20. RNA prepared from stage 10.5 caps was probed by ribonuclease protection for Xbra and EF1-α. RNA from stage 13 caps was probed for GATA-2 and 3 and EF1α and RNA from stage 20 caps was probed for HoxA7, NCAM and muscle actin. EF1-α and cytoskeletal actin were used as loading controls. Whole embryo RNA (lanes 5, 10 15 and 20) and tRNA (lanes marked t) were used as positive and negative controls respectively. HoxA7 up-regulation was verified by scanning densitometry taking into account the loading controls. Bands in the lane adjacent to lane 15 were due to leakage from lane 15.*

closely, we injected eFGF RNA over a wide concentration range and monitored expression in animal caps of GATA-2, GATA-3 and *Xbra* at stage 10.5 when *Xbra* expression, and therefore mesoderm, is first induced. It was important to monitor GATA expression at the same time as *Xbra* to ensure that any effects on GATA expression were not downstream of mesoderm induction. In addition we monitored expression of *NCAM* and *HoxA7* at stage 21, when both are easily detectable, as a measure of neural induction and posteriorization respectively. We did not attempt to measure GATA expression at this late stage since ectodermal GATA expression has declined to low levels by late neurula (Walmsley *et al.*, 1994).

Induction of *Xbra* at low levels of FGF was extremely weak yet strong down regulation of GATA-2 and 3 was observed (Fig. 5A). The weakness of the *Xbra* signals at low FGF levels indicates that very few ectodermal cells within the cap had been converted to mesoderm, so the strong reduction in GATA expression suggests

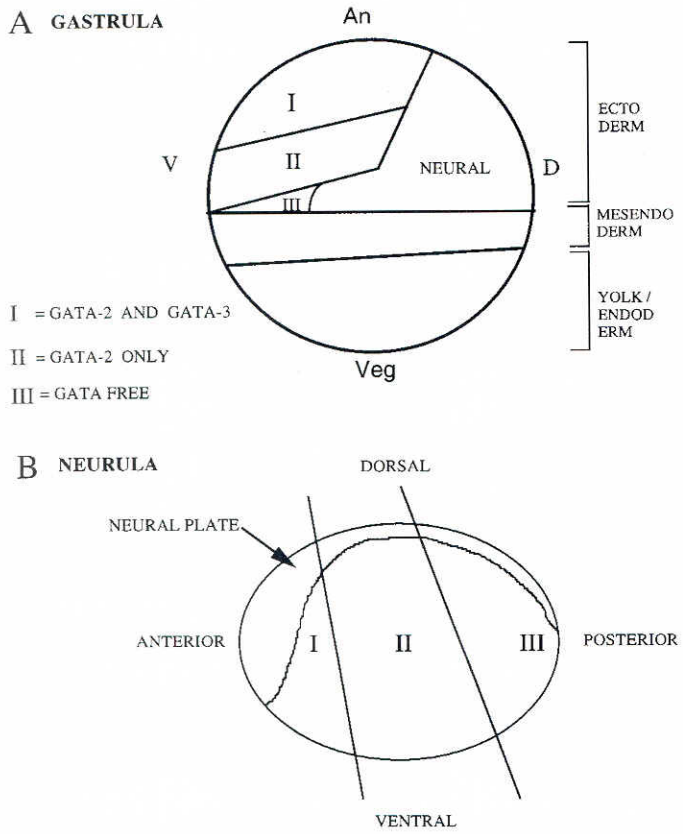

Fig. 8. A model for the patterning of GATA-2 and GATA-3 in the nonneural ectoderm. (A and B). *Diagrammatic representation of GATA expression in early gastrula and neurula embryos. Zones I-III represent the distinct areas of GATA expression within the A/P axis (which at blastula/gastrula stages is roughly equivalent to the animal/vegetal axis) of the nonneural ectoderm of frogs and fish as determined by whole-mount in situ hybridization. Patterning of GATA expression in to zones I-III by posteriorizing agents such as FGF and/or Wnts is set up during late blastula/early gastrula (A). Subsequent convergent extension movements lead to the pattern seen in early neurula embryos as demonstrated for Xenopus (B).*

that FGF has had an effect on ectoderm independent of mesoderm induction. Evidence for posteriorizing activity of FGF in these experiments came from expression of the posterior gene *HoxA7* which was strongly up regulated (Fig. 5B). As expected for blastula caps (Isaacs *et al.*, 1994), this posteriorization occurred in the absence of neural induction measured by *NCAM* expression (Fig. 5B). So the down regulation of GATA-2 and 3 does not represent conversion of nonneural to neural ectoderm. These results suggest that, at very low levels, eFGF can posteriorize nonneural ectoderm in the absence of mesoderm and neural induction, and that down regulation of GATA factor expression is a consequence of this posteriorization.

FGF posteriorizes gastrula nonneural ectoderm and influences GATA factor pattern within this layer

As an alternative way of studying the effects of FGF on GATA expression in the absence of mesoderm induction, presumptive epidermal cells were exposed to FGF after stage 10.5 when they are no longer competent to make mesoderm in response to FGF (Jones and Woodland, 1987). Caps were explanted at stage 10.5, biasing the explant towards the ventral side, and treated with bFGF over a concentration range of 0-100 ng/ml.

We found that GATA-2 and 3 levels in gastrula ectoderm were down regulated by bFGF in a concentration-dependent manner in the absence of mesoderm induction, as revealed by the lack of *Xbra* expression (Fig. 6, lanes 1-4). The down regulation, however, was relatively modest (approximately two-fold). Evidence for posteriorization in these experiments came from expression of the posterior gene *HoxA7* which was up regulated (allowing for loading differences and verified by scanning densitometry –Fig. 6, lanes 7-10). Posteriorization occurred in the absence of neural induction as measured by *NCAM* expression (Fig. 6, lanes 7-10). Therefore the down regulation of GATA-2 and 3 does not result from conversion of nonneural to neural ectoderm. These results demonstrate that FGF can posteriorize nonneural ectoderm from gastrulae in the absence of mesoderm and neural induction. However, this posteriorization is accompanied by a relatively modest down regulation of GATA factor expression raising the possibility that FGF functions predominantly during blastula stages and/or that another signal is normally involved.

XWnt3A posteriorizes blastula nonneural ectoderm and contributes to the patterning of GATA factors within this layer

Since the down-regulation of GATA expression by FGF in gastrula nonneural ectoderm was incomplete we looked for further posteriorizing activities which might contribute to patterning of the GATA factors within the nonneural ectoderm. Both *XWnt3A* and *XWnt8* have been reported to posteriorize anterior neural tissue (McGrew *et al.*, 1995; Fredieu *et al.*, 1997). We injected XWnt3A RNA over a concentration range, into the middle of the animal cap of single cell embryos, dissected out caps at stage 8 and cultured them to appropriate stages for molecular analysis by RNase protection. Posteriorization of ectoderm by *XWnt3A* was indicated by the induction of *HoxA7* in *Wnt* injected caps analyzed at stage 20 (taking into account loading differences and confirmed by scanning densitometry –Fig. 7, lanes 11-15), confirming a previous report (McGrew, *et al.*, 1995). Unlike FGF, *Wnt3A* does not induce mesoderm in animal cap ectoderm (confirmed below), we therefore analyzed GATA-2 and 3 around the peak of their ectodermal expression (stage 13). At this stage, both GATA 2 and 3 were down regulated but only at the highest concentration of *Wnt* used (Fig. 7, lanes 6-10), indicating that *HoxA7* is more sensitive to the *Wnt* signal than GATA-2 or 3. As reported previously (McGrew *et al.*, 1995), *XWnt3A* expression in caps did not induce neural tissue (indicated by *NCAM* expression, Fig. 7, lanes 11-15) nor did it induce dorsal mesoderm indicated by monitoring actin expression (Fig. 7, lanes 16-20). In addition, XWnt3A failed to induce Xbra expression in stage 10.5 caps (Fig. 7, lanes 1-5), indicating the absence of any mesoderm induction. Thus we have demonstrated that a Wnt family member can posteriorize nonneural ectoderm and down regulate the expression of GATA factors in this tissue in the absence of mesoderm and neural induction.

Discussion

We have described the early boundaries of expression of the transcription factors GATA-2 and 3 (*gta2* and *3*) in the nonneural ectoderm of frogs and fish and found them to be highly conserved between the two species. Two clear boundaries were observed, one between neural and nonneural ectoderm, the other between

nonneural ectoderm and mesoderm. In addition, along the A/P axis, expression of the two GATA factors is graded and distinct limits of expression are seen, GATA-2 being expressed more posteriorly than GATA-3 such that, by early neurula, three zones of distinct GATA patterning can be seen representing head, trunk and tail ectoderm (Fig. 8B). The possible functional significance of this distinctive patterning is suggested as follows. Zone I represents the ectoderm overlying the presumptive branchial arches. A role for ectoderm in specifying skeletal pattern in the head has been suggested (Thorogood, 1988) and the importance of GATA-3 in this region is suggested by the phenotype of the GATA-3 homozygous mutant mouse which commonly exhibits disruption of facial structures (Pandolfi *et al.*, 1995). In zone II, dorso-laterally, GATA-2 positive ectoderm covers prospective somite and dorsolateral plate (the region which includes future definitive blood, endothelial and nephric systems). Recent evidence in the axolotl embryo has demonstrated a role for ectoderm from this region in signaling to underlying mesoderm for the directional guidance of migrating pronephric duct and in somite fissure formation (Drawbridge *et al.*, 1995). Ventrally, GATA-2 positive ectoderm represents the superficial ectoderm of the future blood islands. Ventral ectoderm has been shown to stimulate hematopoietic differentiation (Maeno *et al.*, 1992) and GATA-2 has been strongly implicated in this ectoderm/mesoderm interaction (Maeno *et al.*, 1996). Finally, the GATA free region of the nonneural ectoderm (zone III) represents the ectodermal component of the future tail as defined in fate mapping studies in *Xenopus* (Tucker and Slack, 1995), suggesting that these GATA factors play no role in the activity of tail forming genes. These broad regions of transcription factor expression may represent the beginnings of functional segmentation of nonneural ectoderm along the A/P axis as suggested in the concept of the 'ectomere' (Couly and Le Douarin, 1990).

The dorsoventral axis

Our results suggest that the neural/nonneural boundary of expression of GATA factors in ectoderm is set up by the antagonistic action of dorsalizing activities such as noggin with BMP-4 (Walmsley *et al.*, 1994; Neave *et al.*, 1997; this study). Since BMP2 and 7 also interact with noggin and with chordin in a similar fashion to BMP-4 (Piccolo *et al.*, 1996; Zimmerman *et al.*, 1996) it is likely that some redundancy exists between the BMP family and organizer-associated dorsalizing molecules and that all of these molecules could play roles in restricting the expression of GATA factors to the nonneural ectoderm. In addition to representing the limits of the neural plate, this boundary has an important functional role. It is clear that the interaction of nonneural ectoderm with neural ectoderm is responsible for the formation of the neural folds and the neural plate border which contains both neural crest and placodal tissues (Moury and Jacobson, 1989; Dickinson *et al.*, 1995). GATA-2 and 3 may have important functions in defining this border.

A homolog of GATA-2 and 3 is the *pannier* gene in *Drosophila.* The *pannier* gene codes for a zinc finger protein with strong homology in the finger region to the zinc fingers of GATA factors (Ramain *et al.*, 1993). *Pannier* acts as a repressor of *achaete* and *scute,* transcription factors expressed at sites where neural precursors develop in the imaginal disc. *Pannier* mutants with lesions in the zinc finger domain display an overexpression of *achaete* and *scute* and the development of extra neural precursors. This *Drosophila* GATA homolog, therefore, plays an important role in neural/nonneural decisions within the imaginal disc. In the embryo proper, *pannier* is expressed in the dorsal ectoderm of *Drosophila* (Winick *et al.*, 1993) which, given the inversion of the dorsoventral axis between vertebrates and invertebrates (Holley *et al.*, 1995), is the equivalent location to GATA-2 and 3 in frogs and fish. Very recently, a *C. elegans* GATA homolog, *Elt-1*, has been shown to be absolutely required for epidermal cell fate (Page *et al.*, 1997). It therefore seems that GATA function as well as expression pattern in embryonic ectoderm has been highly conserved during evolution.

The anteroposterior axis

Creation of the boundary between nonneural ectoderm and mesoderm at late blastula stages seems to involve FGF and/or activin-like molecules. Both these molecules down regulate the GATA factors in ectoderm when present at concentrations sufficiently high to induce mesoderm. Therefore these molecules are likely to be involved in the suppression of GATA-2 and 3 in the region of the nonneural ectoderm around the blastopore. This expression pattern is refined at some point between late blastula and early neurula, such that the two factors become graded in the A/P axis, high anteriorly and low or absent in more posterior regions. An expression pattern such as this might well result from the activity of signals produced in posterior regions of the gastrula embryo acting negatively on GATA expression equivalent to the non-axial signals demonstrated in *Xenopus* and zebrafish to posteriorize neural ectoderm (Bang *et al.*, 1997; Woo and Fraser, 1997).

RA has been shown to be a potent posteriorizer of the neural plate during these stages but does not appear to be the agent that down regulates the GATA factors in nonneural ectoderm, since it has little effect on GATA-2 and strongly up regulates GATA-3. In this assay the expression of epidermal keratin (a marker of terminal epidermal differentiation) was shown to be repressed, indicating that differentiation of the ectoderm had been prevented. There is no evidence that RA treatment of animal cap ectoderm produces a change in cell fate, therefore the response of the two GATA factors is more likely to reflect a change in their expression levels at different stages of differentiation of the epidermal precursors.

Although a role for FGF in the induction and posteriorization of neural ectoderm has been claimed (Lamb *et al.*, 1993; Cox and Hemmati-Brivanlou, 1995; Kengaku and Okamoto, 1995), more recently Kroll and Amaya have shown that signaling through the FGF receptor is not required for neural induction or antero-posterior patterning in transgenic embryos (Kroll and Amaya, 1996). These contradictory results may be explained by a multiplicity of FGF receptors or alternative neural induction and posteriorization pathways employed in XFD transgenic embryos. In addition, Woo and Frazer when examining candidates for the non-axial signaling which patterns neural tissue of zebrafish embryos in the A/P axis concluded that FGF alone cannot fulfil this role (Woo and Fraser, 1997). In the *Xenopus* study too, Bang et. al. concluded that neither RA nor FGF acting alone could represent the non-axial posteriorizing activity. Here, we have provided evidence that FGF can posteriorize *Xenopus* nonneural ectoderm, as measured by the up-regulation of the posterior gene *HoxA7*, in the absence of mesoderm and neural induction. This activity of FGF appears to contribute to the down regulation of GATA expression in posterior regions of the developing embryo. From our data, the effect is stronger when the cells are exposed at late blastula stages (stage 8-10.5) and

decreases during gastrulation to about two-fold between stages 10.5 and 11. Thus in *Xenopus* embryos, much of the patterning of GATA factors within the ectoderm by FGF may have occurred before gastrulation. This would account for the relatively modest effect of FGF on GATA expression in gastrula caps. An additional possibility is that another factor is involved.

A candidate for such an additional factor is a member of the Wnt family. We demonstrate here that *XWnt3A* can posteriorize nonneural ectoderm and, at high concentration, can down regulate GATA factor expression in this tissue. Thus *XWnt3A* acts to posteriorize all ectoderm. Both *XWnt3A* and *XWnt8* are expressed in posterior ventro-lateral mesoderm during gastrulation in non-axial regions around the blastopore. So a number of *Wnt* family members may contribute to posteriorizing and patterning ectoderm at these early developmental stages. Our experiments do not address whether *Wnts* act directly on ectoderm to posteriorize it or whether *Wnts* trigger production of a dominant posteriorizing morphogen which then patterns ectoderm. Precedence for *Wnts* activating a secondary morphogen which then acts over a distance to alter cell fate has been reported for other vertebrate and invertebrate systems (see Fredieu *et al.*, 1997 and references therein).

The positive and negative effects of BMP-4 and posteriorizing agents respectively on GATA-2 and 3 suggest the following model for establishing the observed expression patterns (Fig. 8). At or just before the beginning of gastrulation, ectodermal GATA expression is activated by BMP-4 in regions beyond the influence of mesoderm induction and dorsalizing signals from the organizer (zones I and II, Fig. 8A). FGF and Wnts, produced initially in the mesoderm and maintained in posterior regions as gastrulation proceeds, down regulate GATA expression in proximal nonneural ectoderm. The most posterior ectoderm which experiences the longest exposure to posteriorizing signals during epiboly and gastrulation becomes GATA free (Zone III, Fig. 8A) whilst in more anterior regions GATA expression is permitted (Zones I and II). The differential extents of expression of GATA-2 and 3 in the antero-posterior axis could additionally reflect graded suppression starting from different maximal activation levels (Figs. 2-7 –expression levels of GATA-2 are always higher than GATA-3). At low levels of FGF and Wnts both GATA-2 and 3 are expressed (zone I), at intermediate levels only GATA-2 is seen (zone II) and at the highest levels (zoneIII) neither GATA factor can be detected.

Materials and Methods

Embryos and dissections

Production and dissections of *Xenopus* and zebrafish embryos were as described (Walmsley *et al.*, 1994; Neave *et al.*, 1995). For blastula caps, the top one third of the animal pole ectoderm was removed at stage 8-8.5 and cultured to appropriate stages for molecular analysis. Gastrula cap ectoderm was dissected at stage 10.5 biasing the dissection towards the ventral side. Caps were then transferred to 1xMBS containing 0.5% BSA and 0, 20, 50 or 100 ng/ml bFGF and cultured to stage 11 for measurement of GATA-2 and 3 and *Xbra* RNA or stage 21 for measurement of *NCAM* and *HoxA7* RNA.

Whole-mount in situ hybridization

For *Xenopus*, double and single whole-mount *in situ* hybridizations were carried out as described (Bertwistle *et al.*, 1996). For details of DIG and fluorescein labeled probes see:

GATA-2 (Walmsley *et al.*, 1994), GATA-3 (Bertwistle *et al.*, 1996), *Otx2* (Pannese *et al.*, 1995), *XKrox20* (Bradley *et al.*, 1992), HoxB9 (Wright *et al.*, 1990; Godsave *et al.*, 1994), *Xbra* (Smith *et al.*, 1991), *slug* (Mayor *et al.*, 1995).

Zebrafish whole-mount *in situ* hybridizations were performed as described (Neave *et al.*, 1995) and double whole-mount *in situ* hybridizations were performed as in (Hauptman and Gerster, 1994). The *gta2* probe used was transcribed from a full length cDNA cloned from a zebrafish neurula library (kind gift of David Grunwald) using zinc fingers of *Xenopus* GATA-2a as probe. Other probes were: *gta3* (Neave *et al.*, 1995), *ntl* (Schulte-Merker *et al.*, 1992), *dlx3* (Akimenko *et al.*, 1994), *pax2* (Krauss *et al.*, 1991).

RNA preparation and ribonuclease protection assays

Preparation of RNA and ribonuclease protection assays were as described (Walmsley *et al.*, 1994). The protection probe for XGATA-3 was prepared from a 150bp MspI fragment from the 5' end of GATA-3 cDNA inserted in to the ClaI site of pGEM7. Transcription with T7 polymerase gives antisense RNA. Other probes have been described: GATA-2, *NCAM* and *EF-1α* (Walmsley *et al.*, 1994), *Xbra* (Smith *et al.*, 1991), *HoxA7* (Condie and Harland, 1987).

Preparation of in vitro transcribed RNA for injection

In vitro transcribed RNA was prepared using a Megascript kit (Ambion) as per the manufacturer's instructions except that capped GTP was used at a lower concentration of 5 mM. For *Xenopus* embryos, RNA was injected in a volume of 4 nl in to the centre of the animal pole ectoderm at the single cell stage. eFGF RNA was supplemented with carrier tRNA (10 pg/nl final) to avoid losses at the low end of the titration range. For zebrafish embryos, RNA was prepared as in (Cornell and Kimelman, 1994) using Megascript kit transcribing for 4 h with 4:1 cap:GTP for eFGF and 2:1 cap:GTP for XFD and d50. RNA was injected in a volume of approximately 200pl into one cell of a 1-4 cell stage embryo.

Signaling molecules

Human recombinant bFGF was purchased from Gibco. Activin was a tissue culture supernatant (gift from Jim Smith, NIMR, Mill Hill). Noggin was expressed post MBT by injection of the plasmid pCSKA nog and into the animal cap of single cell or four cell stage embryos respectively (Walmsley *et al.*, 1994). Templates for transcription of *in vitro* RNA for eFGF, the dominant negative BMP-4 and FGF receptors, XWnt3A and d50, a mutant FGF receptor control, were prepared as described (Amaya *et al.*, 1991; Isaacs *et al.*, 1994; McGrew *et al.*, 1995; Northrop *et al.*, 1995). All-*trans* RA was purchased from Sigma.

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