Development 126, 3067-3078 (1999)
Printed in Great Britain © The Company of Biologists Limited 1999
DEV1433

Induction of the mesendoderm in the zebrafish germ ring by yolk cell-derived TGF-β family signals and discrimination of mesoderm and endoderm by FGF

Adam Rodaway[1], Hiroyuki Takeda[2,*], Sumito Koshida[2], Joanne Broadbent[1], Brenda Price[3], James C. Smith[3], Roger Patient[1,‡] and Nigel Holder[1,§]

[1]Developmental Biology Research Centre, Randall Institute, King's College London, 26-29 Drury Lane, London, WC2B 5RL, UK
[2]Division of Biological Science, Graduate School of Science, Nagoya University, Chikusa-Ku, Nagoya, Japan 464-8602
[3]Division of Developmental Biology, National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, UK
This paper is dedicated to the memory of Nigel Holder who died tragically as this work was being written up for publication
*Present address: Division of Early Embryology, National Institute of Genetics, 1111 Yata, Shizuoka, 411-8540, Japan
§Author's subsequent address: Department of Anatomy and Developmental Biology, University College, Gower Street, London, WC1E 6BT, UK
‡Author for correspondence (e-mail: roger.patient@kcl.ac.uk)

Accepted 30 April; published on WWW 21 June 1999

SUMMARY

The endoderm forms the gut and associated organs, and develops from a layer of cells which emerges during gastrula stages in the vertebrate embryo. In comparison to mesoderm and ectoderm, little is known about the signals which induce the endoderm. The origin of the endoderm is intimately linked with that of mesoderm, both by their position in the embryo, and by the molecules that can induce them. We characterised a gene, zebrafish *gata5*, which is expressed in the endoderm from blastula stages and show that its transcription is induced by signals originating from the yolk cell. These signals also induce the mesoderm-expressed transcription factor *no tail* (*ntl*), whose initial expression coincides with *gata5* in the cells closest to the blastoderm margin, then spreads to encompass the germ ring. We have characterised the induction of these genes and show that ectopic expression of activin induces *gata5* and *ntl* in a pattern which mimics the endogenous expression, while expression of a dominant negative activin receptor abolishes *ntl* and *gata5* expression. Injection of RNA encoding a constitutively active activin receptor leads to ectopic expression of *gata5* and *ntl*. *gata5* is activated cell-autonomously, whereas *ntl* is induced in cells distant from those which have received the RNA, showing that although expression of both genes is induced by a TGF-β signal, expression of *ntl* then spreads by a relay mechanism. Expression of a fibroblast growth factor (eFGF) or a dominant negatively acting FGF receptor shows that *ntl* but not *gata5* is regulated by FGF signalling, implying that this may be the relay signal leading to the spread of *ntl* expression. In embryos lacking both *squint* and *cyclops*, members of the nodal group of TGF-β related molecules, *gata5* expression in the blastoderm is abolished, making these factors primary candidates for the endogenous TGF-β signal inducing *gata5*.

Key words: GATA-5, GATA transcription factors, Heart, Gut development, TGF-β, *squint*, *cyclops*, *nodal*, *activin*, *no tail*

INTRODUCTION

The endoderm is the germ layer which forms the gut lining and contributes to organs forming as outgrowths of the gut, including the lungs, pancreas and liver. Of the three germ layers that emerge during gastrulation, the induction and patterning of the mesoderm and ectoderm have been much studied. In contrast, little is known about these processes in the endoderm, largely due to a lack of markers of cell differentiation during the early stages of endoderm formation. According to fate maps of *Xenopus* and zebrafish, cells of the future endoderm are located at the vegetal and lateral margin of the embryo prior to gastrulation. In *Xenopus* these are the large yolky cells of the vegetal hemisphere and the superficial layer of the involuting marginal zone (Keller, 1975, 1976; Dale and Slack, 1987; Moody, 1987; Minsuk and Keller, 1997). In zebrafish the endoderm is derived from cells closest to the blastoderm margin, adjacent to the vegetal yolk cell (Kimmel et al., 1990; Warga and Nusslein-Volhard, 1999).

Amongst vertebrates, endoderm specification has been best studied in *Xenopus*. In early studies, whole vegetal pole pieces were cultured (Holtfreter, 1938; Okada, 1960; Takata, 1960) or single blastomeres transplanted (Heasman et al., 1984; Wylie et al., 1987). These showed that endodermal commitment occurs gradually from blastula to early gastrula stages, implying that genes necessary for this commitment have been induced at late blastula stages and that further cell interactions are not necessary to maintain expression. More recent studies addressed the signals involved in mesendoderm specification. These imply that a TGF-β signal can induce endoderm (Gamer and Wright, 1995; Henry et al., 1996, Jones et al., 1993). Activin or processed Vg-1 are capable of inducing the markers

4G6, *XlHbox8* and IFABP, however, since these markers are expressed relatively late in endoderm formation, it is unclear whether their induction is direct. Xsox17α and β, transcription factors important early in specification of endoderm in *Xenopus*, are induced by activin (Hudson et al., 1997).

In *Xenopus* it is unclear whether activin is the endogenous mesoderm inducer (Dyson and Gurdon, 1997; Schulte-Merker et al., 1994), however in teleosts, there is evidence, from injection of RNA encoding interfering variants of activin, that it is involved in mesoderm specification (Wittbrodt and Rosa, 1994). If activin is also involved in endoderm induction in zebrafish, it is likely that it acts in concert with other members of the TGF-β family, since loss of function of two members of the nodal-related subfamily *cyclops* (*cyc*) and *squint* (*sqt*) leads to loss of endoderm (Feldman et al., 1998).

The role of FGF in endoderm induction is unclear. Studies using animal caps and/or vegetal explants by Jones et al. (1993) and Henry et al. (1996) showed that FGF can induce some endoderm-expressed genes, whereas Gamer and Wright (1995) demonstrated that FGF inhibits expression of *XlHbox8*, while it is not involved in induction of endodermin (Sasai et al., 1996). Low levels of FGF can induce mesoderm-specific gene expression in vegetal blastomeres implying that FGF is not normally active in these cells (Cornell and Kimmelman, 1995). It appears likely that a TGF-β family member is required for the induction of both endoderm and mesoderm while FGF signalling plays a role in the maintenance of the mesoderm in *Xenopus* (Isaacs et al., 1994, Schulte-Merker and Smith, 1995) and zebrafish (Griffin et al., 1995). This conclusion is supported by the observation that the early endoderm marker, Xsox17α, is induced by activin but not FGF (Hudson et al., 1997).

In order to analyse endoderm induction in zebrafish we have cloned *gata5* cDNA. We show that *gata5* is expressed in cells with an endodermal fate from the earliest stages of the induction process. In the blastula *gata5* is coexpressed with the mesodermally expressed gene *no tail* (*ntl*), the zebrafish brachyury homologue (Schulte-Merker et al., 1994). However by the start of gastrulation, *ntl* expression has spread to encompass the germ-ring, while *gata5* remains confined to the marginal blastomeres. Transplants show that *gata5*, as well as *ntl* (Mizuno et al. 1996), is induced by a signal from the yolk cell. It is likely that this signal involves TGF-β family members, since a dominant negative activin receptor blocks expression of both genes in the germ ring, and injection of activin RNA results in

A

```
GGACGTTGACAAGGTTTTTAACTAGCCTACAATCTGGGCGAATTTTGAGATTTCGAAATC 60
TAGCTTATTCACTGGATATATCATGTATTCGAGCCTGGCTTTATCTTCCAACCCGTCCCC 120
                    M  Y  S  S  L  A  L  S  S  N  P  S  P  I  13
CTACGCACACGATTCCGGGAACTACATCCATCCGTCTGCCAGCTCACCTGTGTATGTACC 180
 Y  A  H  D  S  G  N  Y  I  H  P  S  A  S  S  P  V  Y  V  P  33
CACCACTCGTGTACCAGCCATGCTCCAGACCTTGCCCTACCTACAGACTTGCGGGTTCAG 240
 T  T  R  V  P  A  M  L  Q  T  L  P  Y  L  Q  T  C  G  F  S  53
CCACCAAGCCCATGGGATCAGCAGTCACCACGCCTGGCCACAGACTGGCATCGATAACTC 300
 H  Q  A  H  G  I  S  S  H  H  A  W  P  Q  T  G  I  D  N  S  73
TTCGTTCAACCCCGGTAGCCCTCATCCACCGCCCGGTTTCTCTTATTCGCACAGTCCGCC 360
 S  F  N  P  G  S  P  H  P  P  P  G  F  S  Y  S  H  S  P  P  93
AGTGAGCAGCAGCACCGGCAGGGACGCCGCGTACCAGAACCCGTTAATGTTAAGTAACGG 420
 V  S  S  S  T  G  R  D  A  A  Y  Q  N  P  L  M  L  S  N  G  113
CGGCCGTGCGGATCAATATGGAAGTGCCCTGGTGCGCTCCGTCGGTGGCTCTTATTCCAG 480
 G  R  A  D  Q  Y  G  S  A  L  V  R  S  V  G  G  S  Y  S  S  133
CCCGTACGCGGCCTACATGAGCCCGGAGATGGCAACCTCATGGACGCCTGGGCCCTTTGA 540
 P  Y  A  A  Y  M  S  P  E  M  A  T  S  W  T  P  G  P  F  D  153
TGGCGGCATGATCGGTCTTCAGGGACGCCAGGGAACTCTACCGGGAAGGAGGTCCAGTAT 600
 G  G  M  I  G  L  Q  G  R  Q  G  T  L  P  G  R  R  S  S  I  173
AGACATGCTGGATGACCTGCCGTGTGAAGGGCGCGAGTGTGTGAACTGCGGCTCAATCTC 660
 D  M  L  D  D  L  P  C  E  G  R  E  C  V  N  C  G  S  I  S  193
AACGCCGCTGTGGAGAAGAGACGGAACCGGACACTACCTGTGCAACGCGTGCGGCCTTTA 720
 T  P  L  W  R  R  D  G  T  G  H  Y  L  C  N  A  C  G  L  Y  213
CCACAAGATGAACGGCATCAATCGACCTCTTATCAAACCACAGAAACGCCTGCAAAGCAC 780
 H  K  M  N  G  I  N  R  P  L  I  K  P  Q  K  R  L  Q  S  T  233
ATCACGACGAGCAGGTCTGTGTTGCACTAACTGTCATACTAGTACGACAACACTGTGGAG 840
 S  R  R  A  G  L  C  C  T  N  C  H  T  S  T  T  T  L  W  R  253
GAGAAACGCAGAGGGAGAGCCAGTCTGCAATGCATGTGGACTTTACATGAAGCTTCATGG 900
 R  N  A  E  G  E  P  V  C  N  A  C  G  L  Y  M  K  L  H  G  273
GGTACCAAGGCCATTAGCTATGAAAAAAGAAAGCATTCAGACAAGGAAACGCAAACCAAA 960
 V  P  R  P  L  A  M  K  K  E  S  I  Q  T  R  K  R  K  P  K  293
GATGCCCAAAACCAAATCATCCTCAGGGTCAACAGTGTCTGGCGCTACGTCTCCCACTTC 1020
 M  P  K  T  K  S  S  S  G  S  T  V  S  G  A  T  S  P  T  S  313
ACTGCCTGTGTCAGAAAACGCCTCTACAATAAAAAGTGAACCTAGTATCGCAGCTTTCCA 1080
 L  P  V  S  E  N  A  S  T  I  K  S  E  P  S  I  A  A  F  H  333
TATGCAGGCCAAACCGTTGTCTCGGTCACACAGGCATCGACACAGTTGGATAGTGCCAGT 1140
 M  Q  A  K  P  L  S  R  S  H  R  H  R  H  S  W  I  V  P  V  353
TCTGCCCATGTGGACATCAAATATGAAGACTACACATACACCCCGACAGTCGATCGCCCC 1200
 L  P  M  W  T  S  N  M  K  T  T  H  T  P  R  Q  S  I  A  P  373
ACAGAACTCCTGGTGTGCTCTGTCTCAAGCGTGATCCGAGACCTCCACTGAAACCTCTCT 1260
 Q  N  S  W  C  A  L  S  Q  A  *                              383
GGCCTAGGGAGAAAAGACTTTTAACTGAATCTACCTAATAGGACTTTTACACAAATGTAG 1320
ACAGACGGTGCTTAGGAAAGCAAGACATTGACAGTAATTTGTGCTACTTTTCCTCCTTGA 1380
TTTATGGTGGCATATCTATGATGTCACGGCCAATTCATGCAATTCCACCAAAAATATGGT 1440
GGATGACAAGCAGCGCCTCTGTAACAGTTCCAATGATCGGGAGGACAAAAAACATGCTTT 1500
TTATGGCTACTGAACGTTTCATGGCTTAGTTGTTTATGATTACCTTTTAAGATACATTAT 1560
TTATGTTGAGATAAGAATGGTGGAGGCACACAAGTCTCTCTCCCATAATTCCCTTTATCC 1620
TTCAGGGAATGCGTCTTTATGGTCGTTATGACTTGAGATCGCATACTTTCCCCTGGATGA 1680
GGCCTTTTAAAAGTTTCCTTGTTGTCTTTGCTGATTCCAGACTCTGTCAATGTTGTATTG 1740
TATTCCTCCGATGATCTGAAGTGCAATGACCCGCGCCGCTTTTCAGCCGATTTTGAAAAG 1800
GGACACGTTTATATTAATTGTTTCGAAATTCTGTGTTTTGTTTTTTGTCAGAATATGTTA 1860
ACCTAAATTTATTTGATGTATTTTGTTGATATATATTGGAATTATATCGTTT          1912
```

B

```
xgata5a MYPSLALTANHAQPAYSHDTPNFLHSTG.SPPVYVPTSRMPAMLQSLPYLQSCDTAHQGH
cgata5  MYQGLALAPNHGQSAYSHDSGNFLHSSA.GSPVYVPTTRVPSVLQTLPYLQSCE.PHQS.
zgata5  MYSSLALSSN..PSPYAHDSGNYIHPSA.SSPVYVPTTRVPAMLQTLPYLQTCGFSHQAH
mgata5  MYQSLALAQSPGQGTYA.DSGAFLHSSGTGSPVFVAPTRMPSM...LPYLPSCEPGSQAP

xgata5a HLANHPGWAQT.A.ESHAFNASSPHT....PTG...FSYSHSPPVGNSSARDGGYQSPLI
cgata5  HLGNPPGWAQS.SGETTAFNAGSPHP....PSG...FSYPHSPRAAAPRGVDGAYQGPLL
zgata5  GISSHHAWPQT.GIDNSSFNPGSPHP....PPG...FSYSHSPPVSSSTGRDAAYQNPLM
mgata5  ALAAHSSWTQTVAADSSAFGSGSPHPAAHPPGATTFPFAHSPPGSGSGGSAGVRDGGAF

xgata5a MGGGAR.DQYGNTLVRT..GSYPSPY.SYVGADWPPSMAAGHFEGSMLHSLQGRQ.SLSG
cgata5  LGGGGR.EQYGNALVRSVNGSYSSPYPAYVTPELPPSWTAGHFESSVLHSLQTRQAALPG
zgata5  LSNGGRADQYGSALVRSVGGSYSSPYAAYMSPEMATSWTPGPFDGGMI.GLQGRQGTLPG
mgata5  QGALLAREQYPTPLGRPMGASYPTTYPAYMSSDVAPSWTSGAFDSSILHGLQARPGGLPG

xgata5a RRSSL..EFLEEFPGEGRECVNCGAMSTPLWRRDGTGHYLCNACGLYHKMNGMNRPLIKP
cgata5  RRSTF..EYLEEFPGDGRECVNCGAMSTPLWRKDGTGHYLCNACGLYHKMNGINRPL.KP
zgata5  RRSSI..DMLDDLPGEGRECVNCGSTSTPLWRRDGTGHYLCNACGLYHKMNGINRPLIKP
mgata5  RRTSFVPDFLEEFPGEGRECVNCGALSTPLWRRDGTGHYLCNACGLYHKMNGVNRPLVRP

xgata5a QKRL.SSSRRAGLCCTNCHTSTTTLWRRNSEGEPVCNACGLYMKLHGVPRPLAMKKESIQ
cgata5  QKRL.SSSRRAGLCCTNCHTTNTTLWRRNAEGEPVCNACGLYMKLHGVPRPLAMKKESIQ
zgata5  QKRLQSTSRRAGLCCTNCHTSTTTLWRRNAEGEPVCNACGLYMKLHGVPRPLAMKKESIQ
mgata5  QKRL.SSSRRSGLCCSNCHTATTTLWRRNSEGEPVCNACGLYMKLHGVPRPLAMKKESIQ

xgata5a TRKRKPKNIGKGKTSTGSSTSAN.NSPSSVTNSDPTPVLKTEPNITSQYSGQAIVPVSQG
cgata5  TRKRKPKNITKGKTSTGSTTSAT.NSPSSITNSDSTVTLKSEPSTTSQYPGQGIVSVSQA
zgata5  TRKRKPK.MPKTKSSSGSTVSGA.TSPTSLPVSENASTIKSEPSIAA.FHMQA.KPLSRS
mgata5  TRKRKPENPAKIKGSSGSTANTTASSPTLLNSESSATTLKAESSLASPVCA.GPTITSQA

xgata5a HSQTDDLVNGSH.ELKFMPDEYTYSPTALSQQSGLSVPLRQESWCALALA*
cgata5  QSQSDEALAGG..EFKFEPEDYPFSPSSMAPQPGLSVPLRQDSWCALALA*
zgata5  HRHRHSWI.....VPVLPMWTSNMKTTHTPRQSIA.P..QNSWCALSQA*
mgata5  SSPADESLASSHLEFKFEPEDFAFTSSSMSPQAGLSGVLRQETWCALALA*
```

Fig. 1. (A) cDNA sequence and translation for zebrafish *gata5*. The ATG beginning the long open reading frame is in bold, as is an upstream, in-frame termination codon. Within the translation, the conserved cysteine residues of the 'zinc fingers' are in bold and underlined. The sequence 3′ to base 1912 is not shown since the partial cDNAs which run beyond this diverge in sequence. (B) Alignment of the predicted sequence of gata5 protein with GATA5 from *Xenopus* (x) (Kelley et al., 1993), chicken (c) (Laverriere et al., 1994) and mouse (m) (Morrisey et al., 1997). Residues identical in two or more sequences are boxed in black, and those where conservative substitutions are present in two or more sequences are boxed in grey. Alignment was performed using the programme Pileup (GCG) and shaded using Boxshade.

ectopic induction of these genes in a distribution which mimics their endogenous arrangement. Injection of constitutively active activin receptor RNA induces both genes. At higher levels of injected RNA, *gata5* is expressed cell autonomously, while *ntl* is expressed in cells distant to those receiving the RNA, implying a relay signal. The relay is likely to be FGF since this signal is necessary for *ntl* expression in all but the most marginal blastomeres, while *gata5* is unresponsive to perturbation of FGF signalling. Fish doubly mutant for *squint* and *cyclops* do not express *gata5* in the marginal blastomeres, implying that the TGF-β signals include these nodal-related factors.

MATERIALS AND METHODS

Cloning of zebrafish *gata5*

A partial cDNA for *gata5* was cloned from a zebrafish neurula library (gift of D. J. Grunwald) by hybridisation with a fragment encoding the conserved DNA binding domain of *Xenopus* GATA-2A as described by Neave et al. (1995). This cDNA was used to probe, at high stringency, a random primed zebrafish 3- to 16-hour library (gift from J. Campos-Ortega) and a further 6 overlapping partial cDNAs were obtained. Since none of the partial cDNAs extended to the translation start site, a 'Marathon' cDNA synthesis kit (Clontech) was used to obtain three separate 5′RACE clones. The cDNA clones from the libraries and the 5′RACE clones were sequenced by a combination of manual (Sequenase, Amersham), and automated fluorescent cycle sequencing (ABI). The sequence of zebrafish *gata5* has the accession number AJ242515.

Cloning of PCR fragments encoding zebrafish activins A and B

PCR fragments encoding zebrafish activins βA and βB were obtained using primers identical to those described by Thomsen et al. (1990). Amplification was performed on 0.5 μg of boiled zebrafish genomic DNA and products of 365 bp were ligated into pSP73 and sequenced.

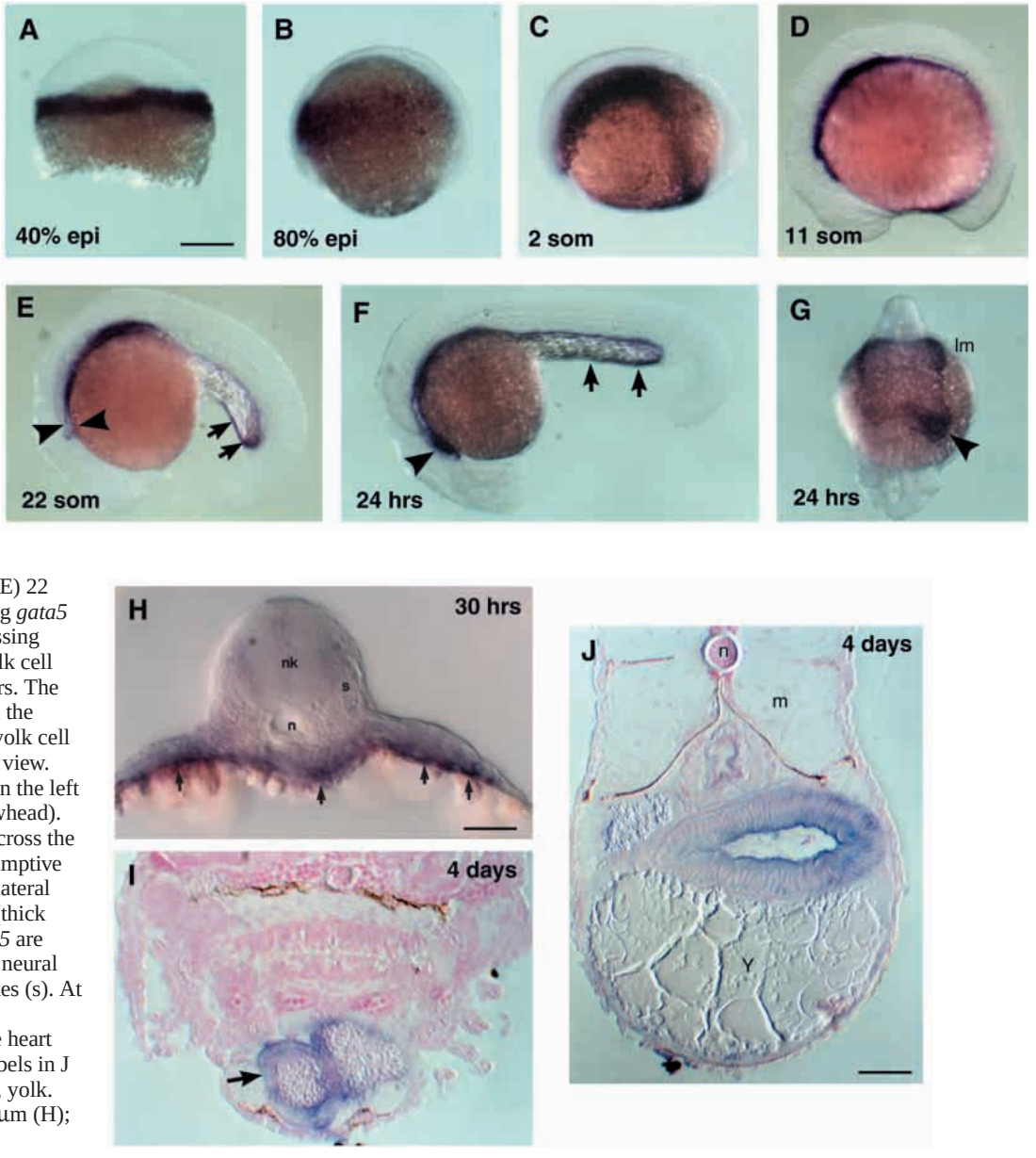

Fig. 2. Expression of zebrafish *gata5* detected by in situ hybridisation. Stages according to Kimmel et al. (1995). In A and B the animal pole is up and in C-F anterior is to the left and dorsal is up. (A) 40% epiboly. *gata5* expression in the germ ring. (B) 80% epiboly. *gata5*-expressing cells have involuted and dispersed towards the animal pole. (C) 2 somites. *gata5*-expressing cells are in the lateral mesoderm anteriorly, and in presumptive endoderm cells converging towards the midline. (D) 11 somites. The anterior extent of the expressing cells is beneath the midbrain. (E) 22 somites. The heart is expressing *gata5* (arrowheads). Caudally, expressing cells are present around the yolk cell extension (arrows). (F) 24 hours. The arrowhead marks the heart and the arrows expression around the yolk cell extension. (G) 24 hour, frontal view. *gata5* expression in the heart on the left hand side of the embryo (arrowhead). More caudally, expression is across the midline in a thin layer of presumptive endoderm, and in the anterior lateral mesoderm (lm). (H) 30 hours (thick section). Cells expressing *gata5* are close to the yolk cell (arrows). neural keel (nk); notochord (n); somites (s). At 4 days (10 μm sections) *gata5* expression is maintained in the heart (arrow in I) and the gut (J). Labels in J are n, notochord; m, muscle; y, yolk. Scale bars, 250 μm (A-G); 50 μm (H); 25 μm (I,J).

The sequences of the activin βA and βB clones have accession numbers AJ238980 and AJ238981 respectively.

Whole-mount in situ hybridisation

Antisense probes for *gata5* (digoxigenin labelled) and *ntl* (fluorescein labelled) were synthesised and single-colour and two-colour whole-mount in situ hybridisations were performed essentially as previously described (Gering et al., 1998). The signal for *gata5* was developed using BM-Purple, and that for *ntl* using Fast Red (both Boehringer Mannheim). Single-colour in situs were sectioned after mounting in JB4 methacrylate resin (Agar Scientific), and counterstained using Nuclear Fast Red (Vector). Two-colour in situs were mounted in agarose, frozen and sectioned using a cryostat. To more clearly distinguish overlapping gene expression, we developed a technique that allowed the separate detection of two genes on the same section. Briefly, after detection using Fast Red (Gering et al., 1998), the embryos were photographed before developing with BM-purple. Finally, the Fast Red stain was removed by washing (4×5 minutes) in methanol, allowing the BM-Purple signal to be seen alone.

Immunohistochemistry

Rabbit polyclonal antiserum directed against zebrafish Fkd2 was a gift from Rachel Warga (Warga and Nusslein-Volhard, 1999). Embryos stained by whole-mount in situ hybridisation for *gata5* message were re-fixed in 4% paraformaldehyde then washed into MABTw (0.1 M maleic acid, 0.15 M NaCl, pH7.5 using NaOH, 0.1% Tween 20). After blocking (2 hours RT, 2% Boehringer Block in MABTw), embryos were incubated (overnight, 4°C) with anti-Fkd2 antiserum (1:200 in 2% Boehringer Block in MABTw). After washing, detection was carried out with the ABC system using DAB substrate (Vector Laboratories). Embryos were sectioned (14 μm) using a cryostat.

Preparation of synthetic RNA and RNA injections

RNA for injection was synthesised from templates linearised with the appropriate restriction enzyme, using the MEGAscript kit (Ambion Inc.) according to the manufacturer's instructions, except that 1.25 μl of 40 mM Cap analogue [$m^7G(5')ppp(5')G$] and 1 μl of 75 mM GTP were used in place of 2 μl of 75 mM GTP. Embryos were injected with 200-400 pl of RNA solution in deionised water into the yolk-free animal-pole cytoplasm at the 2-4 cell or 16-32 cell stages (see below).

The template for synthesis of *Xenopus* eFGF (Isaacs et al., 1992) was as described by Griffin et al. (1995); pSP64T-Z7 (zebrafish activin βB) was a gift from Jochen Wittbrodt (Wittbrodt and Rosa, 1994). Dominant negative FGF receptor, XFD was a gift from Enrique Amaya (Amaya et al., 1992). Dominant negative *Xenopus* activin receptor dnXAR was a gift of Dan Mahony and is essentially the same as ΔXAR1 (Hemmati-Brivanlou and Melton, 1992) except that it is cloned in pBscRN3 (Lemaire et al., 1995). Constitutively active murine Type I activin receptors, ALK-2* and ALK-4* (ten Dijke et al., 1993), contain a single amino acid substitution in the juxtamembrane activation domain (Weiser, et al., 1995), and can be used to assess function of signalling through the pathways normally activated by ligand binding (Armes and Smith, 1997).

Yolk cell transplantation

The method used was that described by Mizuno et al. (1996). Briefly, the blastoderm was removed from a yolk cell at the high to sphere stages (staged according to Kimmel et al., 1995). The donor yolk cell was then transplanted onto the animal pole region of a recipient embryo of approximately the same stage. Grafted embryos were allowed to develop until 50-60% epiboly before fixation and in situ hybridisation using a *gata5* probe.

RESULTS

Features of the zebrafish *gata5* cDNA and protein

We screened a zebrafish neurula cDNA library using a fragment of the *Xenopus* GATA-2A cDNA (Zon et al., 1991) encoding the conserved zinc-finger DNA-binding domain. One clone appeared to be a partial cDNA encoding zebrafish *gata5*. We used this to rescreen a random-primed cDNA library, obtaining six overlapping clones. Since the initiating methionine codon was absent, we used 5′ RACE to extend the cDNA sequence. The largest contig obtained was 2.6 kb, however since the two cDNAs containing the 3′ end diverge in sequence beyond base 1912, we only show sequence 5′ to this in Fig. 1A. The first ATG, which is in-frame with a termination codon 60 bp upstream, begins an open reading frame encoding 383 amino acids. The predicted M_r 41.5×10^3 protein was shown by database searching to be a member of the 4/5/6 subfamily of GATA factors, and by pairwise comparison with these proteins that it was considerably more similar to GATA-5s from other species. We therefore propose that we have cloned zebrafish *gata5*. Use of a zebrafish '*GATA-5*' gene to mark the developing pancreas has been described (Pack et al., 1996), however it is now clear that this was in fact *gata6* (J. Reiter and D. Y. Stainier, personal communication).

Fig. 1B shows a translation alignment between *gata5* and GATA-5 cDNAs cloned from other species. The most highly conserved region is the DNA-binding domain, containing two zinc fingers, characteristic of vertebrate GATA factors. The sequence C-terminal to this region is considerably less conserved than that N-terminal, which has recently been shown to contain transcriptional activation domains conserved amongst members of the GATA-4/5/6 subfamily (Morrisey et al., 1997). The amino-acid residues identified as being vital in the function of these domains in mGATA-4 are either conserved or, in the case of Y21 (F26 in mGATA-4) and D117 (E149 in mGATA-4), conservatively substituted.

gata5 expression in the embryo occurs in gut and heart cells

gata5 transcripts are first detected in the blastula, at 30% epiboly, in cells around the blastoderm margin. This expression is initially symmetrical and at 40% epiboly is in a 3-4 cell deep rim around the blastoderm close to the yolk cell (Fig. 2A). At the onset of gastrulation (50% epiboly) *gata5* is expressed in cells that are amongst the first to involute. By mid-gastrula stages (80% epiboly; Fig. 2B), expression can be seen in the hypoblast, more strongly on the ventral side of the embryo, probably representing precursors of the later expression in the anterior lateral mesoderm. By the end of gastrulation (2 somites; Fig. 2C), the *gata5* expression has begun to move towards the dorsal midline. Expression is stronger in the anterior half of the embryo. At 11 somites (Fig. 2D), this anterior expression is in the anterior mesoderm lateral to the mid- and hindbrain, including the cells that will fuse to form the heart ventral to the midbrain. In addition to this mesodermal expression, a thin layer of *gata5*-positive cells begins to form between the embryo and the yolk cell, running from the midbrain posteriorly to the tail-bud, likely representing the endoderm. At 22 somites (Fig. 2E), *gata5* expression can be seen in the fusing heart-tubes (arrowheads), and in a layer of cells between the embryo and the yolk cell which now extends to surround the posterior end of the yolk-plug extension (arrows). This expression pattern is maintained at 24 hours post fertilisation (hpf). At this stage (Fig. 2F,G) the heart is beginning to circulate the blood and has moved to lie

on the left side of the embryo (Fig. 2G), while *gata5* expression is still also present in the anterior lateral mesoderm (lm). Expression of *gata5* in the endoderm between the embryo proper and the yolk cell can be seen in Fig. 2G, but is more obvious in a transverse section (Fig. 2H) where a monolayer of cells lies across the width of the embryo (arrows). *gata5* expression is maintained in the hatched larva (4 days) in both the heart (Fig. 2I arrow), and in the epithelium of the gut (Fig. 2J).

The *gata5*-expressing cells in the blastoderm margin are the first cells to involute during gastrulation and lineage labelling studies indicate that these cells give rise to the endoderm (Kimmel et al., 1990; Warga and Nusslein-Volhard, 1999). Two additional findings indicate that the *gata5*-expressing cells in gastrulae include endoderm precursors. Firstly, examination of the cells expressing *gata5* at 80% epiboly shows them to be relatively large, flat cells located close to the yolk cell surface (Fig. 3E, white arrow), which is characteristic of gut precursors (Warga and Nusslein-Volhard, 1999). Secondly, in dorsal and lateral regions of the germ ring, *gata5* coexpresses with the endoderm-expressed protein forkhead-2 (fkd2; Warga and Nusslein-Volhard, 1999) (Fig. 3A,B,D). Ventrally (Fig. 3C), a proportion of *gata5*-expressing cells, those closest to the yolk cell surface, coexpress fkd2 (arrow), whereas other, more superficial *gata5*-expressing cells, possibly fated to contribute to the anterior lateral mesoderm and heart, do not.

gata5-positive cells coexpress *no tail* at the onset of gastrulation

In zebrafish fate maps, endoderm precursors lie at the margin of the blastoderm. This region also gives rise to some mesodermal derivatives, while the germ-ring more distant from the margin gives rise to mesoderm alone (Kimmel, et al., 1990; Warga and Nusslein-Volhard, 1999). To define these mesendoderm cells in terms of gene expression we compared the expression of *gata5* with the mesodermal regulatory gene *no tail* (*ntl*; Schulte-Merker et al., 1994). As determined by whole-mount double in situ hybridisation at 50% epiboly, *gata5* is expressed in a marginal subset of *ntl*-expressing cells (Fig. 4A). To more precisely map the spatial relationship of these genes, we developed a technique for sequential visualisation of the *ntl* and *gata5* signals on the same section (see Materials and methods). *gata5* is expressed approximately 3-4 cell diameters from the blastoderm margin, whereas *ntl* expression extends 8-10 cells from the margin (Fig. 4C,E). Thus, by the beginning of gastrulation (50% epiboly), the fate of the cells in the germ-ring, is mirrored by the genes they express. Earlier in development (at 30% epiboly), *gata5* and *ntl* are coexpressed to a depth of 2-3 blastomeres from the blastoderm margin, adjacent to the yolk cell (Fig. 4B,D). *gata5*, but not *ntl*, is also expressed in the yolk syncitial layer (YSL) underlying the blastoderm margin. Thus at blastula stages there may be no distinction between cells fated to form endoderm or mesoderm.

gata5 expression in the blastoderm is induced by signals from the yolk cell

Since *ntl* is induced by signals from the yolk cell (Mizuno et al., 1996), and the expression of *gata5* overlaps with *ntl* in the margin of the blastoderm, we investigated whether *gata5* is also induced by signals from the same source. We performed 33 successful yolk cell transplantations at high to sphere stages. Embryos were fixed between 50 and 60% epiboly. In 31 of the transplanted embryos ectopic *gata5* expression was detected (Fig. 5A). In sections of these embryos the normal *gata5* domain lies adjacent to the host yolk cell (hyc) and a second localised band of expressing cells abuts the grafted, biotin-labelled yolk cell (dyc, Fig. 5B). This domain is similar in position and dimensions to the normal *gata5* expression domain. This result shows that, as for *ntl*, the yolk cell is the origin of inducing signals for *gata5* in the blastoderm.

Ectopic activin induces *gata5* and *ntl* in the blastula in distinct patterns

TGF-β signals have been implicated in endoderm induction and are therefore candidates for the yolk cell signal (Gamer and Wright, 1995; Henry et al., 1996; Jones et al., 1993). To assess the role of the TGF-β family of signalling proteins in induction of mesendodermal gene expression in zebrafish, we used activin and a dominant negative activin receptor. To generate a local, ectopic source of activin, we injected activin RNA (10 μg/ml) apically into 16-32 cell embryos (Fig. 6A-D). RNA (100 μg/ml) coding for a nuclear form of β-galactosidase (nlsβ-gal) was coinjected as a lineage label. Embryos were fixed at 50% epiboly and the location of the injected RNA detected by staining for nuclear β-gal activity (turquoise spots). The cytoplasmic in situ signal for *ntl* (red) was developed and photographed followed by staining for cytoplasmic *gata5* (blue-purple). *ntl* is ectopically induced in activin-injected embryos in a region that overlaps the staining for β-gal and extends 4-8 cell diameters beyond the injected cells (Fig. 6A,C). Sometimes *ntl* expression is absent from some of the β-gal stained cells, probably as a result of suppression of *ntl* expression by high levels of activin signalling, as is seen with *Xbra* in *Xenopus* (Green et al., 1992). Ectopic *gata5* expression is induced in the injected cells and spreads beyond them by, at most, 2-3 cell diameters (Fig. 6B,D). This results in a 'bull's eye' appearance, with the central *gata5*- and *ntl*-expressing cells surrounded by a halo of cells expressing *ntl* but not *gata5*. This mimics the expression of these genes in the germ ring, where the cells closest to the source of the endogenous signal (the yolk cell) express both *gata5* and *ntl*, while more distant cells express only *ntl*.

To investigate further the possibility that endogenous induction of *ntl* and *gata5* involved a TGF-β family member we injected embryos with RNA encoding a dominant negative activin receptor, dnXAR, which blocks signalling by a number of TGF-β family members including activin and Vg-1 (Schulte-Merker et al., 1994). Expression of dnXAR results in inhibition of the germ-ring expression of both *gata5* and *ntl* coinciding with the expression of the coinjected β-gal tracer (Fig. 6E-G).

Differential expression of *gata5* and *ntl* in response to constitutively active activin receptor; evidence for a relay signal for *ntl*

There are two possible interpretations of the pattern of *ntl* and *gata5* expression induced by ectopic activin expression: (1) the cells expressing activin act as the high point of an activin diffusion gradient, and that *ntl* has a lower concentration threshold for induction by activin, and so is induced further from the activin source than *gata5*, or (2) that the activin signal

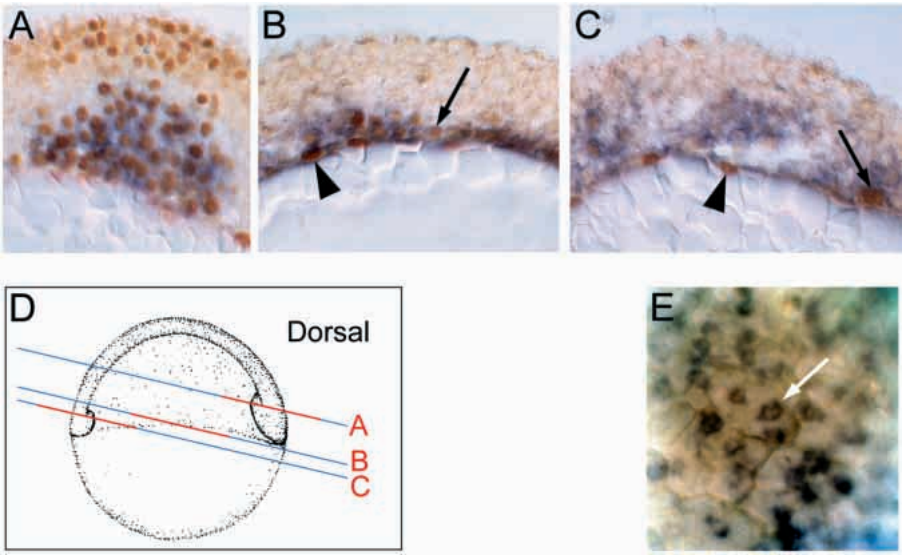

Fig. 3. *gata5* is expressed in endodermal cells in the gastrula embryo. (A-C) Cryostat sections of a 60% epiboly embryo double stained for *gata5* message (in situ hybridisation; blue cytoplasmic staining) and fkd2 protein (antibody; brown nuclear staining). (A) Dorsal view. Coexpression of *gata5* and fkd2 in the mesendoderm of the prechordal plate. (B) Lateral view. Coexpression of *gata5* and fkd2 in endodermal precursors adjacent to the yolk cell, in the innermost layer of the hypoblast (arrow). fkd2 also expressed in the YSL (arrowhead) which weakly expresses *gata5*. (C) Ventral view. fkd2 is expressed predominantly in the YSL (arrowhead) with only the occasional hypoblast cell, coexpressing *gata5* (arrow). *gata5* is expressed in several layers of hypoblast cells without coexpression of fkd2. These may include precursors of the anterior lateral mesoderm. (D) Diagram showing the plane of sections (blue lines) and position in sections (red lines) in parts A-C. (E) High power lateral view of 80% epiboly embryo whole-mount focused immediately above the yolk cell. *gata5* is expressed in cells (arrowed) having the characteristic position (adjacent to the yolk cell) and shape (large, flat cells) of endodermal precursors (Warga and Nusslein-Volhard, 1999).

is able to cause cells to produce a second 'relay' signal which is able to induce *ntl* but not *gata5*, so resulting in the spread of *ntl* expression beyond the cells that directly receive the activin signal. To distinguish between these hypotheses, we injected RNA encoding constitutively active forms of the type I activin receptor, ALK-4 (ALK-4*) and the type I BMP2/7 receptor ALK-2 (ALK-2*) (Jones et al., 1996, Weiser et al., 1995). Such receptors activate the intracellular signals normally driven by their ligands, but work in a cell-autonomous manner.

RNA for ALK-4* was injected at three different concentrations, 1, 10 and 100 μg/ml, and different patterns of gene expression were observed at 50% epiboly. At the lowest concentration (Fig. 7A), gene expression was little affected except where the injected RNA (localised by β-gal staining) overlapped the germ-ring, resulting in slight spreading of *ntl* and *gata5* expression away from the margin. At 10 μg/ml (Fig. 7B), ectopic expression of both genes was clearly induced. This often resembled the expression induced by injection of activin RNA, with the patch of injected cells expressing *ntl* and *gata5* fringed by cells expressing *ntl*. These *ntl*-expressing cells generally did not contain β-gal staining. This is more clearly seen at the highest concentration of injected ALK-4*, where *ntl* expression is suppressed in a proportion of the injected cells

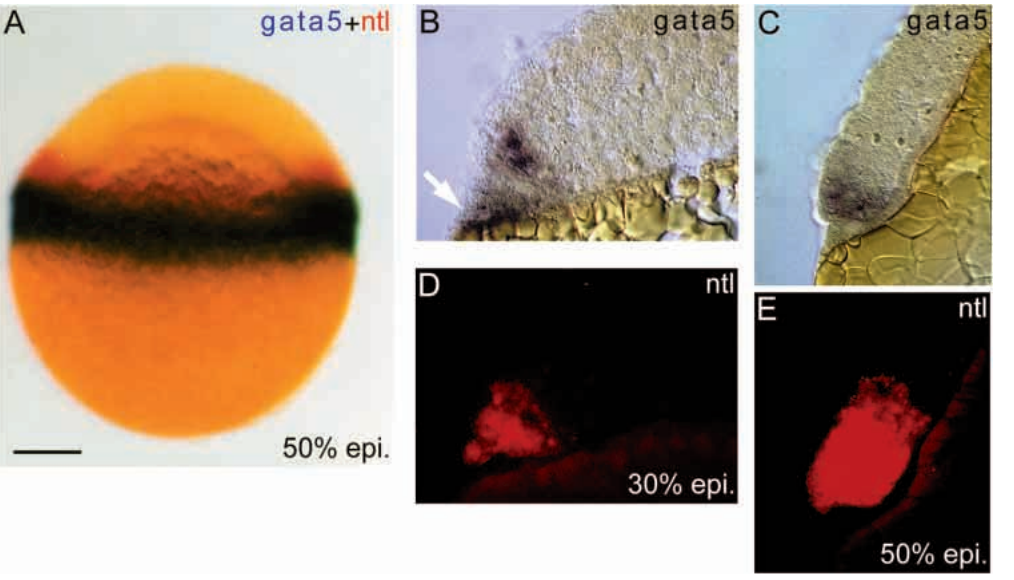

Fig. 4. *gata5* and *ntl* coexpress in the blastoderm margin. (A) Two-colour whole-mount in situ of a 50% epiboly embryo showing *gata5* (blue) expression in the margin of the blastoderm, and wider expression of *ntl* (red). Scale bar,150 μm. (B-E) sequential in situ detection on the same sections of *gata5* (blue; B and C) and *ntl* (red fluorescence; D and E), at 30% epiboly (B,D) and 50% epiboly (C,E). (B) The initial expression of *gata5* in the blastoderm extends 2-3 cell diameters from the blastoderm margin. Expression is also detected in the YSL (white arrow). (C) At 50% epiboly, *gata5* expresses for approximately the same distance from the blastoderm margin, which now represents 3-4 cell diameters. (D) Initially *ntl* expresses in the same blastomeres as *gata5*, but not in the YSL. (E) 50% epiboly. *ntl* expression has spread to encompass blastomeres within 8-10 cell diameters of the margin. Thus, by the beginning of gastrulation, *gata5*-expressing cells are a marginal subset of those expressing *ntl*.

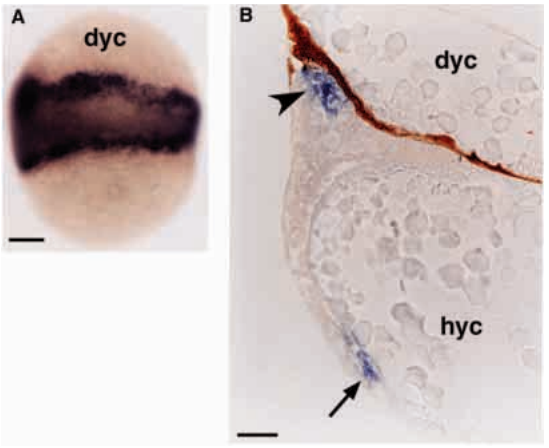

Fig. 5. Induction of *gata5* by a signal from the yolk cell. (A) Embryo at 60% epiboly with a grafted second yolk cell. *gata5* is expressed in cells in association with both the host and the donor yolk cell (dyc). (B) A section through such a grafted embryo showing the additional layer of *gata5*-expressing cells (arrowhead). Normal *gata5* expression in the host embryo is arrowed. The dark material in the donor yolk cell is dye injected prior to grafting to distinguish it from the host yolk cell (hyc). Scale bars, 150 μm (A); 40 μm (B).

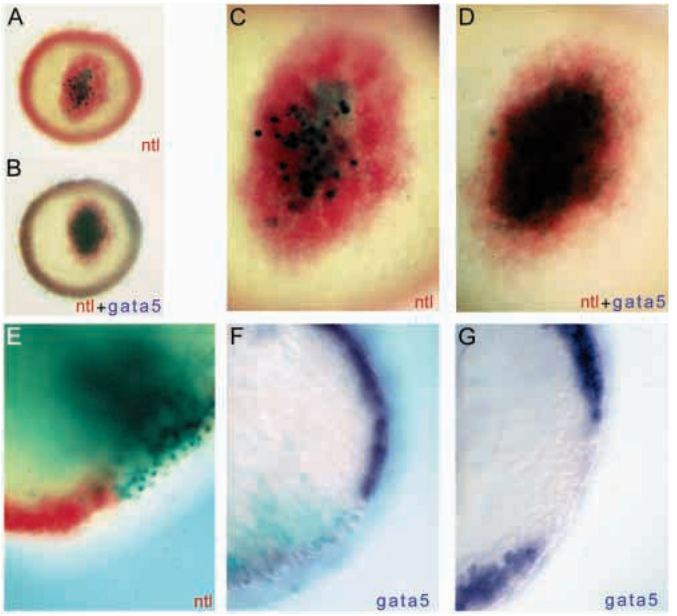

Fig. 6. Patterned response of *gata5* and *ntl* expression to activin; dependence of both genes on TGF-β signalling. (A-D) 50% epiboly embryo coinjected at 16-32 cell stage with activin (10 μg/ml) and nls-β-gal (100 μg/ml) RNA. β-gal detected with X-Gal (turquoise nuclear staining). The embryo was photographed after staining for *ntl* (red) and again after detection of *gata5* (blue; B,D). (A,B) Low magnification, animal pole views of the whole embryo. (C,D) Higher magnification views of the patch of ectopic expression. (C) *ntl* expression overlaps with the injected cells and extends 4-8 cell diameters beyond the cells injected. (D) *gata5* is induced in injected cells and up to 2-3 cell diameters beyond them. A ring of cells expressing *ntl* alone extends for about 4 cell diameters beyond those coexpressing *ntl* and *gata5*, mimicking the normal pattern of expression of these genes. (E-G) Blastoderm margin, 50% epiboly embryos coinjected at 2-cell stage with 1 μg/μl dnXAR and 100 ng/μl nls-β-gal RNA. (E) Stained for *ntl* expression (red); (F) stained for *gata5* expression (blue) after staining for β-gal activity and (G) *gata5* stain without staining for β-gal to show more clearly the gap in *gata5* expression. Expression of both genes is eliminated by inhibiting TGF-β signalling.

while neighbouring patches of uninjected cells strongly express *ntl* (Fig. 7C,D white arrows and arrowhead, respectively). In contrast, *gata5* is expressed cell-autonomously only in the cells that have received the ALK-4* message.

These data imply that cells responding to a TGF-β signal are able to produce a secondary relay signal, and that it is this signal which induces *ntl* expression in cells distant from the source of the TGF-β signal. In contrast, the induction of *gata5* is restricted to cells expressing the constitutively active receptor, indicating that the spread of expression seen with activin RNA injections is due to activin diffusion.

Injection of RNA encoding ALK-2* (BMP-2/7 receptor) at a concentration of 100 μg/ml did not perturb expression of *gata5* or *ntl* at the onset of gastrulation (Fig. 6E). The resulting embryos were, however, severely abnormal showing that the receptor was active.

Unlike *ntl*, *gata5* expression in the blastoderm is not regulated by FGF

Expression of the *Xenopus* homologue of *ntl*, Xbra, is maintained by a positive feed back loop involving FGF during blastula and gastrula stages (Isaacs et al., 1994; Schulte-Merker and Smith, 1995). A similar mechanism acts on zebrafish *ntl* (Griffin et al., 1995). We therefore examined whether *gata5* is regulated by FGF by injecting RNA encoding either eFGF or the dominant negative FGF receptor, XFD (Amaya et al., 1992).

Unlike *ntl* (Fig. 8C), *gata5* is not ectopically expressed in response to injection of *eFGF* RNA (Fig. 8D), while expression of XFD does not downregulate *gata5* expression in the germ ring (Fig. 8F). XFD does cause localised loss of *ntl* expression in the germ ring (Fig. 8E) in cells containing this dominant negative FGFR RNA. However in the presence of XFD, a depth of 1-2 cells at the blastoderm margin continue to express *ntl* prior to involution (Fig. 8E arrow) implying that an endogenous non-FGF signal induces the most marginal *ntl* expression at this stage, but that FGF is required for the spread of *ntl* expression. The FGF independence of *gata5* expression is further confirmed by the fact that it is unaltered in *no tail* mutant embryos in which functional *ntl* protein is absent (data not shown).

Nodal-related TGF-β family signals are required for expression of *gata5* in the blastoderm margin

Since ectopic activin is able to induce expression of *gata5* and *ntl*, we were interested to determine whether activin could be the endogenous yolk cell-derived signal inducing these genes. We isolated partial cDNAs to activin βA and βB, and RNAse protection assays indicated that activin βA was not detectable until 5 hpf (data not shown), too late to be the endogenous inducing signal for *gata5* expression which is detectable by 3 hpf. In contrast, βB RNA is present at low levels maternally, and increases soon after MBT, a result we have confirmed by semi-quantitative RT-PCR (data not shown), however we have

not been able to reproducibly detect localised activin βB expression in the zebrafish blastula by in situ hybridisation or immunohistochemistry (Bartlett, 1995), while RT-PCR on dissected blastulae does not indicate that activin RNA is concentrated in the yolk cell relative to the blastomeres (data not shown). There is therefore currently insufficient evidence to establish a role for activin in endoderm induction in zebrafish.

Recently, it has been shown that the zebrafish mutants *cyclops* (*cyc*) and *squint* (*sqt*) result from mutations in the nodal-related TGF-β family members *znr1* and *znr2* respectively (Sampath et al., 1998; Feldman et al., 1998; Rebagliati et al., 1998; Erter et al., 1998). Significantly, while the loss of either of these genes predominantly affects the development of the prechordal plate mesendoderm and the ventral midline of the nervous system, embryos lacking the function of both these genes have essentially no mesoderm or endoderm (Feldman et al., 1998). We therefore examined the effect of these mutations on the induction of *gata5* expression. In crosses between carriers of the *cyc* mutation, we could detect no effect on *gata5* expression between 30% epiboly and shield stages (data not shown), with normal expression in the germ ring and YSL (see Fig. 9A,D open and black arrowheads respectively). In *sqt* crosses however, approximately one quarter of the embryos showed reduced *gata5* expression: at 30% epiboly, expression in the blastoderm margin was weaker and sometimes patchy, while at 50% epiboly and shield stages, gaps in the ring of expression were clear (data not shown, but see Fig. 9B,E with black arrows indicating weak expression and white arrowheads delineating gaps). In a cross between *sqt*;*cyc* double heterozygotes (a gift from Will Talbot), embryos showing the phenotype for absence of *sqt* were seen as expected (Fig. 9B,E). In embryos lacking both *sqt* and *cyc*, expression of *gata5* was completely absent from the blastoderm (Fig. 9C,F), with only some expression in the remaining YSL (Fig. 9C, black arrowhead). This shows that full induction of *gata5* in the blastoderm margin is dependent on the overlapping activities of *sqt* and *cyc*.

DISCUSSION

gata5 is expressed in the endoderm and its precursors

To study the induction of endoderm in zebrafish, we have cloned zebrafish *gata5*, and conclude, for the following reasons, that it is expressed in endoderm and its precursors from 30% epiboly. (1) *gata5* is expressed in the margin of the blastula, which contains the cells fated to be endoderm (Kimmel et al., 1990; Warga and Nusslein-Volhard, 1999). (2) During gastrulation *gata5* is coexpressed with the endoderm-expressed nuclear protein, fkd2 (Fig. 3A-D). (3) During gastrulation *gata5* is expressed in cells with the characteristics of endoderm (large, flat cells adjacent to the yolk cell; Fig. 3E). (4) Later, *gata5* expression is found where endoderm forms in the embryo (Fig. 2D-H,J). (5) Eventually, *gata5*-expressing cells are located in the differentiating gut (Fig. 2J), consistent with the expression pattern of GATA-4/5/6 genes in other species (Kelley et al., 1993; Laverriere et al., 1994).

The expression pattern of *gata5* and *ntl* in the blastoderm margin reflects cell fate

Fate maps show that the mesoderm and endoderm of zebrafish derive from cells near the blastoderm margin (Kimmel et al., 1990). Even in the late blastula, cells can give rise to clones which contribute to both germ layers (Warga and Nusslein-Vollhard, 1999). Within the blastoderm margin the cell fates are to some extent segregated, in that cells within 4 cell diameters of the margin can give rise to endoderm and/or mesoderm, while cells further than this only generate mesodermal clones (Warga and Nusslein-Vollhard, 1999). The expression of *gata5* and *ntl* at the beginning of gastrulation (Fig. 4) mirrors this fate restriction: cells destined to form mesoderm alone express *ntl* alone, while cells in the region that gives rise to both mesoderm and endoderm express both *ntl* and *gata5*. This is, however, a simplification, in that endodermal structures derive more often from the dorsal and lateral regions of the blastoderm margin, whereas *gata5* expression is symmetrical. The expression domain of fkd2 includes endoderm precursors from gastrula stages, and this protein is coexpressed with *gata5* in dorsal and lateral regions of the hypoblast, while ventrally only a few of the *gata5*-expressing cells surface coexpress fkd2 (Fig. 3). It may be that some of the ventral *gata5*-expressing cells contribute to the anterior lateral mesoderm and heart, which express *gata5* at later stages. Our studies do not address how $gata5^{+}$ endoderm and $gata5^{+}$ heart cells are differentiated, however there is a ventral to dorsal gradient of nkx2.5 in the germ ring (Chen and Fishman, 1996) which is known in other systems to cooperate with GATA factors to activate heart gene expression (Durocher et al., 1997). Stronger *gata5* expression becomes apparent on the ventral side of the embryo at mid-gastrula stages, and may indicate *gata5* expression in additional cells which will give rise to anterior lateral plate mesoderm and the heart.

gata5 and *ntl* are induced by a TGF-β-like signal derived from the yolk cell

As has been previously demonstrated for *ntl* and *gsc* (Mizuno et al., 1996), transplantation experiments indicate that the signal to induce expression of *gata5* derives from the yolk cell. Like *ntl* and *gsc*, *gata5* induction occurs only at the margin of the blastoderm: cells contacting most of the transplanted yolk cell surface do not express *gata5*. It is likely that this is due to the endogenous signal emanating only from the yolk cell where it contacts the blastoderm margin, since the response to ectopically expressed activin demonstrates that blastomeres in other locations are competent to respond by expressing *gata5* and *ntl*.

The signal emanating from the yolk cell is likely to be a member of the TGF-β family of growth factors. Expression of a dominant negative activin receptor (which is able to block signalling by activin, Vg1 and possibly other TGF-β family members (Schulte-Merker et al., 1994)) prevents expression of both *ntl* and *gata5* in the margin of the blastoderm. Ectopic expression of the TGF-β family member, activin, is able to induce expression of these genes in a pattern that mimics their endogenous expression: *gata5* and *ntl* are coexpressed close to the source of the signal, and *ntl* alone is expressed 3-6 cell diameters beyond the expression domain of *gata5*.

Different responses to a relay signal account for the patterned expression of *gata5* and *ntl* in the blastoderm margin

The patterned expression of *ntl* and *gata5* after ectopic

expression of activin raised the possibility that this represented a differential response of these genes to an activin gradient, however it was also possible that activin was acting via a second, relay signal. The relative importance of diffusion and relay mechanisms in mesoderm induction is an area of considerable debate. It has been shown that activin can act as a morphogen by diffusing within explants and differentially inducing gene expression in distant cells (Jones et al., 1996; McDowell et al., 1997). These studies also provided evidence that the effect of activin on distant cells did not depend on a secondary relay signal. However, it has also been shown that *Xenopus* animal caps exposed to activin are able to induce mesoderm in untreated caps with which they are combined (Cooke et al., 1987), and Reilly and Melton (1996) showed that cells induced to form mesoderm by coinjection of TGF-β1 and its receptor were able to induce muscle differentiation in uninjected animal cap cells. Both of these studies therefore imply the presence of a relay signal in mesoderm induction. Our studies support this conclusion: expression of a constitutively active activin receptor (ALK-4*), which activates intracellular responses to activin signalling, showed that while *gata5* appears to respond cell-autonomously to such signalling, *ntl* is induced in distant, uninjected cells showing that it is induced by a relay signal.

This result and that of Reilly and Melton (1996) appear to contradict the findings of Jones et al. (1996) and McDowell et al. (1997). However, this might result from the different experimental techniques used. In Jones et al. and McDowell et al., the response to TGF-β and potential relay signalling was assayed in late blastula animal caps, whereas in Reilly and Melton and in our study, the cells that produce the relay signal were present in the responding tissue throughout the early development of the embryo. It is therefore possible that the spread of the relay signal requires longer contact between signalling and responding tissue than was allowed in the experiments of Jones et al. and McDowell et al.

The identity of the relay signal as FGF was implied by studies which showed positive feedback between FGF signals and *ntl/Xbra* expression (Griffin et al., 1995; Isaacs et al., 1994; Schulte-Merker et al., 1995). In this study we confirm the induction of *ntl* by FGF and show that the spread of *ntl* expression beyond the most marginal 1-2 tiers of blastomeres depends on FGF signalling (Fig. 8E). Previously we had assayed *ntl* expression in XFD-injected embryos at early gastrula stages (Griffin et al., 1995), at which stage the most marginal cells have involuted and downregulated *ntl*, and so had not detected this residual expression of *ntl*. This expression likely represents a direct response to the endogenous yolk cell-derived signal and may correspond to the transient expression of Xbra detected in *Xenopus* when FGF signalling is blocked (Schulte-Merker and Smith, 1995).

Unlike *ntl*, *gata5* in the blastula is not induced by FGF, nor does it require FGF signalling. We therefore propose that the patterned expression of *ntl* and *gata5* in the blastoderm margin results from differential responsiveness to TGF-β and FGF signalling. Thus, initial TGF-β signals derived from the yolk cell induce both genes in the most marginal tiers of blastomeres (cf. Fig. 4B,D). This also induces (either directly, or via *ntl*) expression of an FGF, which can induce *ntl* but not *gata5*. *ntl*, but not *gata5*, expression is then able to spread beyond those cells directly exposed to the yolk cell TGF-β, either by means of the diffusion of FGF which has recently been shown to occur (Christen and Slack, 1999), or by a relay working through positive feedback between *ntl* and FGF. Thus by means of its responsiveness to FGF, *ntl* can spread to occupy the whole depth of the germ-ring, while *gata5* is confined to the marginal 3-4 tiers.

This is consistent with results in *Xenopus* where the endoderm-specific genes *Xlhbox8* and *Xsox17* are not induced by FGF (Hudson et al., 1997; Gamer and Wright, 1995) (but see Henry et al. (1996) who show that expression of Xlhbox8 in mid-tailbud stages can be blocked by injection of XFD). It is also consistent with the model put forward by Cornell et al. (1995) where FGF signalling is involved in inducing or maintaining mesoderm in the equatorial region of the *Xenopus* embryo, and is not active in the vegetal region. It is possible that lack of a requirement for FGF signalling for induction of gene expression is a characteristic of endoderm.

The inducing signal for *gata5* involves the nodal-related proteins *squint* and *cyclops*

The mesendodermal-inducing TGF-β signal has been much investigated in *Xenopus*, however its identity is still unclear. Candidates proposed include activin (Asashima et al., 1990; Green and Smith, 1990), Vg1 (Dale et al., 1993) and nodal related proteins (Jones et al., 1995). This controversy is in part due to the unsuitability of *X. laevis* for genetic study. Here we have used mutants in nodal-related candidate signals which are available in zebrafish to define a role for these factors in the induction of germ ring expression of *gata5*. Feldman et al. (1998) showed that fish mutant for both *sqt* and *cyc* had essentially no mesoderm or endoderm. We therefore tested whether these factors were necessary for the induction of *gata5* in the blastoderm margin. In embryos lacking *cyc*, *gata5* expression appeared normal, whereas in those lacking *sqt*, the early expression was weak and patchy, and by the beginning of gastrulation gaps were present in the ring of *gata5* expression. Despite the apparent dispensability of *cyc*, a role for this molecule was shown by the phenotype of the double *sqt*;*cyc* mutants. In these embryos, all expression of *gata5* was lost from the blastoderm, with only some residual expression in the YSL remaining. These data show that a combination of *sqt* and *cyc* is necessary for the full induction of *gata5* in the zebrafish blastoderm (see also Warga and Nusslein-Vollhard, 1999).

sqt is expressed in the YSL from the earliest stages of zygotic transcription, while *cyc* does not seem to be expressed in the YSL. *sqt* is therefore likely to be yolk cell-derived signal detected in yolk cell transplants. *sqt* and *cyc* are later expressed in the margin of the blastoderm and it is probable that this expression is responsible for maintaining *gata5* expression. Interestingly, in *sqt*;*cyc* double mutants *ntl* expression is only lost dorsally. This may imply that another TGF-β signal is present which is able to induce expression of this mesodermally expressed gene, but our data clearly show that *gata5* cannot be fully induced without both *sqt* and *cyc*.

Is there a role for activin in mesendodermal induction?

That the absence of *sqt* and *cyc* prevents induction of *gata5* in the zebrafish does not preclude a role for activin in *gata5*

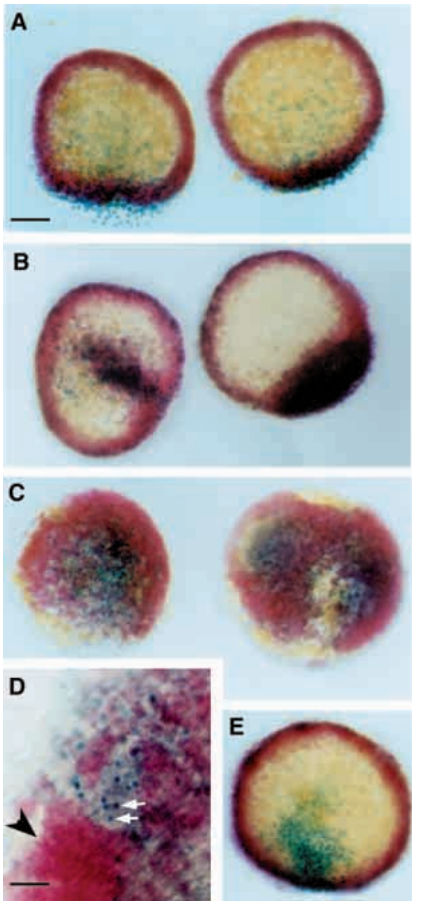

Fig. 7. Induction of *gata5* and *ntl* in embryos injected with RNA encoding constitutively active activin receptor, Alk4* (A-D) or BMP2/7 receptor, Alk2* (E), animal pole views, 50% epiboly. *ntl*, (red); *gata5*, (blue); β-gal, (turquoise). (A) 1 μg/ml Alk-4*. There is little alteration to the expression of *ntl* and *gata5*. (B) 10 μg/ml Alk4*. The expression of *ntl* and *gata5* is ectopic in the location of cells expressing β-gal. *gata5* ectopic expression occurs within the wider area of *ntl* expression. (C) 100 μg/ml of Alk4*. *ntl* expression is clearly in regions of the blastula not expressing β-gal whilst *gata5* expression remains in the β-gal-expressing cells. This is shown in higher magnification in D. Black arrowhead indicates *ntl*-expressing cells without nuclear β-gal. *gata5* expression is seen as blue cytoplasm in cells with turquoise nuclei (white arrows). (E) 100 μg/ml Alk2*. Normal expression of *ntl* and *gata5*.

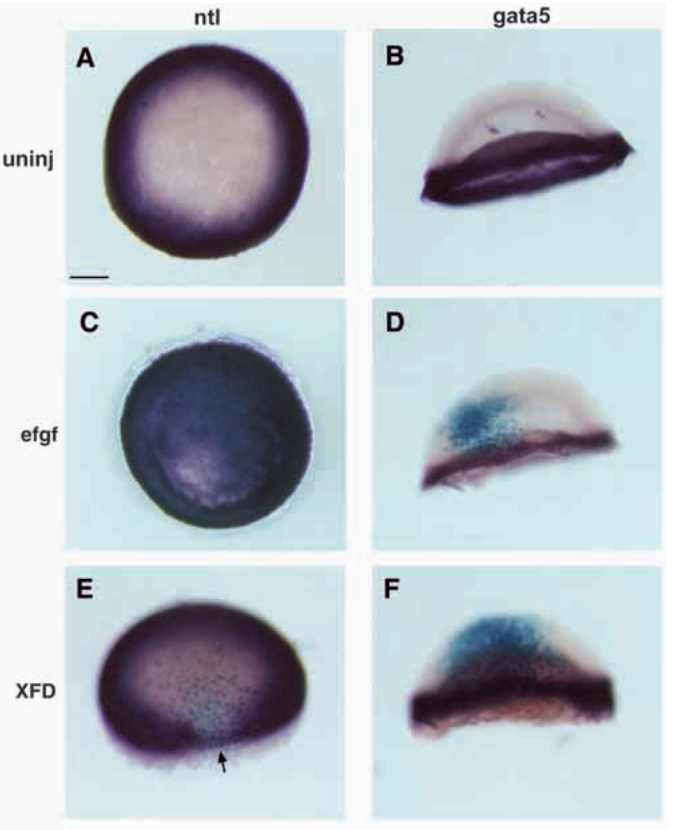

Fig. 8. *Ntl* but not *gata5* is positively regulated by FGF. A,C,E are 50% epiboly embryos hybridised with a probe to *ntl*. (A) Uninjected (uninj) embryo. (C) Injection of eFGF RNA causes widespread expression of *ntl*. (E) Injection of RNA encoding dominant negative FGF receptor, XFD. Where the β-gal tracer overlaps the germ ring, *ntl* expression is abolished in all but a single tier of cells closest to the blastoderm margin. B,D,F are embryos hybridised to *gata5* probe. (B) uninjected embryo, (D) injected with eFGF, (F) injected with XFD. Neither eFGF nor XFD affect *gata5* expression. Scale bar, 150 μm.

induction. Activin and the activated activin receptor ALK-4* are able to induce *gata5*, and activin βB message is present at the appropriate time, albeit apparently not localised to the yolk cell. Experiments in medaka using an interfering mutant of activin imply that activin signalling is necessary in mesoderm induction (Wittbrodt and Rosa, 1994). In *Xenopus*, expression of the extracellular domain of activin receptor IIB, which appears to block activin but not other TGF-βs tested, interfered with induction of *Xbra* (Dyson and Gurdon, 1997). Furthermore, activin is able to diffuse and activate genes at a distance from its source, whereas BMP-4 and the nodal-related Xnr-2 cannot (Jones et al., 1996; McDowell et al., 1997). There is, however, also evidence that activin is not the endogenous mesodermal inducing signal in *Xenopus* (Schulte-Merker et al., 1994). Mice disrupted at the activin βB locus are viable (Schrewe et al., 1994), however the role of *cyc* in inducing *gata5* is only evident in the absence of *sqt*, so the role of activin βB in these mice may be similarly masked.

It is therefore still possible that activin could be playing a role in the induction of mesendoderm in zebrafish. Even if activin is not localised within the blastula, activin signalling may be required to potentiate signalling by nodal related local signals in a similar manner to the synergism of the BMP-related signalling molecules DPP and SCW in patterning *Drosophila* (Nguyen et al., 1998). A better understanding of the induction of the mesendoderm will require further investigation of the signalling pathways involved; for example it is not clear which receptors act to transduce the nodal-related signals nor how the downstream signalling events link with those triggered by other TGF-β family signals. The role of activin may be revealed by the isolation of fish mutated in these genes and the investigation of the interactions of these mutations with *sqt* and *cyc*.

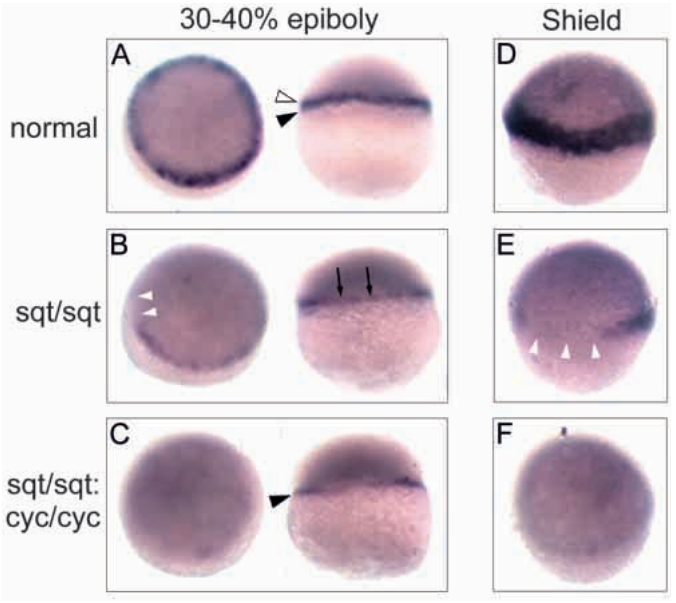

Fig. 9. *gata5* expression phenotypes obtained from a cross of fish doubly heterozygotic for *squint* (*sqt* ^{cz35}) and *cyclops* (*cyc*^{m294}). Expression was assayed at 30-40% epiboly (A-C, left embryos oblique animal pole view, right embryos lateral view) and shield stage (D-F, animal-lateral view). A and D show the normal expression pattern seen in most embryos. (A) At 30-40% epiboly, expression is in the margin of the blastoderm (open arrowhead) and the underlying YSL (black arrowhead). At shield stage (D) *gata5* is expressed in the newly involuted hypoblast. One abnormal phenotype (B and E) was the same as that found in embryos from crosses of *sqt* single heterozygotes. At 30-40% epiboly (B), expression of *gata5* in the blastoderm margin was generally weaker (black arrows) and in some embryos expression was absent from part of the blastoderm margin (white arrowheads). At shield stage (E), expression was absent from typically one quarter of the hypoblast (white arrowheads), and reduced in the remainder. In the second abnormal phenotype, representing embryos homozygous for both sqt^{cz35} and cyc^{m294}, *gata5* was completely absent from blastomeres at both stages (C and F). Some remaining expression could be seen in the YSL (C, black arrowhead).

We are pleased to thank the members of all four laboratories for valuable contributions during the course of this work, Steve Wilson for critically reading the manuscript and Mike Jones for stimulating discussions. Thanks to Rachel Warga and Didier Stainier for sending unpublished data, and Will Talbot for embryos. The work was funded by The Wellcome Trust (R. P, A. R.), MRC (J. C. S., N. H. and R. P.), a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sport and Culture of Japan (H. T.), HFSP (J. C. S. and N. H.) and BBSRC and EC (N. H.).

REFERENCES

Amaya, E., Musci, T. J. and Kirschner, M. W. (1992). Expression of a dominant negative mutant of the FGF receptor disrupts mesoderm formation in *Xenopus* embryos. *Cell* **66**, 257-270.

Armes, N. and Smith, J. (1997). The ALK-2 and ALK-4 activin receptors transduce distinct mesoderm-inducing signals during early *Xenopus* development but do not co-operate to establish thresholds. *Development* **124**, 3797-3804.

Asashima, M., Nakano, H., Shimada, K., Kinoshita, K., Ishii, K., Shibai, H. and Ueno, N. (1990). Mesodermal induction in early amphibian embryos by activin A (erythroid differentiation factor). *Roux's Arch. Dev. Biol.* **198**, 330-335.

Bartlett, S. R. (1995). Activin in early development. PhD Thesis, University of London.

Chen, J.-N. and Fishman, M. C. (1996). Zebrafish tinman homolog demarcates the heart field and initiates myocardial differentiation. *Development* **122**, 3809-3816.

Christen, B. and Slack, J. M. W. (1999). Spatial response to fibroblast growth factor signalling in *Xenopus* embryos. *Development* **126**, 119-125.

Cooke, J., Smith, J. C., Smith, E. J. and Yaqoob, M. (1987). The organization of mesodermal pattern in *Xenopus laevis*: Experiments using a *Xenopus* mesoderm inducing factor. *Development* **101,** 893-908.

Cornell, R. and Kimmelman, D. (1995). FGF is a prospective competence factor for early activin-type signals in *Xenopus* mesoderm induction. *Development* **121**, 2429-2437.

Dale, L. and Slack, J. (1987). Fate map of the 32-cell stage of *Xenopus laevis*. *Development* **99**, 527-551.

Dale, L., Matthews, G. and Colman, A. (1993). Secretion and mesoderm-inducing activity of the TGF-β-related domain of *Xenopus* Vg1. *EMBO J.* **12**, 4471-4480.

Durocher, D., Charron, F., Schwartz, R. J., Warren, R. and Nemer, M. (1997). The cardiac transcription factors Nkx2-5 and GATA-4 are mutual cofactors. *EMBO J.* **16**, 5687-5696.

Dyson, S. and Gurdon, J. (1997). Activin signalling has a necessary function in Xenopus early development. *Curr. Biol.* **7**, 81-84.

Erter, C. E., Solnica-Krezel, L. and Wright, C. V. E. (1998). Zebrafish nodal-related 2 encodes an early mesendodermal inducer signaling from the yolk syncytial layer. *Dev. Biol.* **204**, 361-372.

Feldman, B., Gates, M. A., Egan, E. S., Dougan, S. T., Rennebeck, G., Sirotkin, H. I., Schier, A. F. and Talbot, W. S. (1998). Zebrafish organiser development and germ layer formation require nodal-related signals. *Nature* **395**, 181-185.

Gamer, L. and Wright, C. (1995). Autonomous endodermal determination in Xenopus: regulation of expression of the pancreatic gene XlHbox 8. *Dev. Biol.* **171**, 240-251.

Gering M., Rodaway, A. R. F., Gottgens, B., Patient, R. K., and Green, A. R. (1998). The SCL gene specifies haemangioblast development from early mesoderm. *EMBO J.* **17**, 4029-4045.

Green, J. B. A. and Smith, J. C. (1990). Graded changes in dose of a Xenopus Activin homologue elicit stepwise transformations in embryonic cell fate. *Nature* **347**, 391-394.

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

Griffin, K., Patient, R. and Holder, N. (1995). Analysis of FGF function in normal and no tail zebrafish embryos reveals separate mechanisms for formation of the trunk and tail. *Development* **121**, 2983-2994.

Heasman, J., Wylie, C., Hausen, P. and Smith, J. (1984). Fates and states of determination of single vegetal pole blastomeres of Xenopus laevis. *Cell* **37**, 185-194.

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

Henry, G., Brivanlou, I., Kessler, D., Hemmati-Brivanlou, A. and Melton, D. (1996). TGF-β signals and a prepattern in *Xenopus laevis* endodermal development. *Development* **122**, 1007-1015.

Holtfreter, J. (1938). Differenzierungspotenzen isolierter Teile der Urodelengastrula. *Wilhelm Roux Arch. Entwmech. Org.* **138**, 522-656.

Hudson, C., Clements, D., Friday, R., Stott, D. and Woodland, H. (1997). Xsox17α and β mediate endoderm formation in Xenopus. *Cell* **91**, 397-405.

Isaacs, H., Pownal, M. and Slack, J. (1994). eFGF regulates Xbra expression during Xenopus gastrulation. *EMBO J.* **13**, 4469-4481.

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

Jones, C. M., Armes, N. and Smith, J. C. (1996). Signalling by TGF-β family members: short-range effects of Xnr-2 and BMP-4 contrast with the long-range effects of activin. *Curr. Biol.* **6**, 1468-1475.

Jones, C. M., Kuehn, M., Hogan, B., Smith, J. and Wright, C. (1995). Nodal-related signals induce axial mesoderm and dorsalize mesoderm during gastrulation. *Development* **121**, 3651-3662.

Jones, E., Abel, M. and Woodland, H. (1993). The possible role of mesodermal growth factors in the formation of the endoderm in *Xenopus laevis*. *Roux Arch. Dev. Biol.* **202**, 233-239.

Keller, R. (1975). Vital dye mapping of the gastrula and neurula of Xenopus laevis, I. Prospective areas and morphogenetic movements of the superficial layer. *Dev. Biol.* **42**, 222-241.

Keller, R. (1976). Vital dye mapping of the gastrula and neurula of Xenopus laevis., II. Prospective areas and morphogenetic movements of the deep layer. *Dev. Biol.* **51**, 118-137.

Kelley, C., Blumberg, H., Zon, L. and Evans, T. (1993). GATA 4 is a novel transcription factor expressed in endocardium of the developing heart. *Development* **118**, 817-827.

Kimmel, C. B., Ballard, W. W., Kimmel, S. R., Ullmann, B. and Schilling, T. F. (1995). Stages of embryonic development of the zebrafish. *Dev. Dyn.* **203**, 253-310.

Kimmel, C. B., Warga, R. M. and Schilling, T. F. (1990). Origin and organisation of the zebrafish fate map. *Development* **108**, 581-594.

Laverriere, A., MacNeil, C., Mueller, C., Poelmann, R., Burch, J. and Evans, T. (1994). GATA-4/5/6, a subfamily of three transcription factors transcribed in developing heart and gut. *J. Biol. Chem.* **269**, 23177-23184.

Lemaire, P., Garrett, N. and Gurdon, J. (1995). Expression cloning of Siamois, a Xenopus homeobox gene expressed in dorso-vegetal cells of the blastulae and able to induce a complete secondary axis. *Cell* **81**, 85-94.

McDowell, N., Zorn, A. M., Crease, D. J. and Gurdon, J. B. (1997). Activin has direct long-range signalling activity and can form a concentration gradient by diffusion. *Curr. Biol.* **7**, 671-681.

Minsuk, S. B. and ., R. E. (1997). Surface mesoderm in Xenopus: a revision of the stage 10 fate map. *Dev. Genes Evol.* **207**, 389-401

Mizuno, T., Yamaha, E., Wakahara, M., Kuroiwa, M. and Takeda, H. (1996). Mesoderm induction in zebrafish. *Nature* **383**, 131-132.

Moody, S. (1987). Fates of the blastomeres of the 32 cell stage Xenopus embryo. *Dev. Biol.* **122**, 300-319.

Morrisey, E., Ip, H., Tang, Z., Lu, M. and Parmacek, M. (1997). GATA-5: a transcriptional activator expressed in a novel temporally and spatially restricted pattern during embryonic development. *Dev. Biol.* **183**, 21-36.

Morrisey, E., Ip, H., Tang, Z. and Parmacek, M. (1997). GATA-4 activates transcription via two novel domains that are conserved within the GATA-4/5/6 subfamily. *J. Biol. Chem.* **272**, 8515-8524.

Neave, B., Rodaway, A., Wilson, S. W., Patient, R. and Holder, N. (1995) Expression of zebrafish GATA 3 (*gta3*) during gastrulation and neurulation suggests a role in the specification of cell fate. *Mech. Dev*. **51**, 169-182

Nguyen, M., Park, S., Marques, G. and Arora, K. (1998). Interpretation of a BMP activity gradient in Drosophila embryos depends on synergistic signalling by two type I receptors, SAX and TKV. *Cell* **95**, 495-506.

Okada, T. (1960). Epithelio-mesenchymal relationships in the regional differentiation of the digestive tract in the amphibian embryo. *Roux Archive Dev. Biol.* **152**, 1-21.

Pack, M., Solnica-Krezel, L., Malicki, J., Neuhauss, S., Schier, A., Stemple, D., Driever, W. and Fishman, M. (1996). Mutations affecting development of the zebrafish digestive organs. *Development* **123**, 321-328.

Rebagliati, M. R., Toyama, R., Haffter, P. and Dawid, I. B. (1998). cyclops encodes a nodal related factor involved in midline signalling. *Proc. Natl. Acad. Sci. USA* **95**, 9932-9937.

Reilly, K. M., and Melton, D. A. (1996). Short-range signaling by candidate morphogens of the TGFβ family and evidence for a relay mechanism of induction. *Cell* **86,** 743-754.

Sampath, K., Rubinstein, A. L., Cheng, A. M. S., Liang, J. O., Fekany, K., Solnica-Krezel, L., Korzh, V., Halpern, M. E. and Wright, C. V. E. (1998). Induction of the zebrafish ventral brain and floorplate requires cyclops/nodal signalling. *Nature* **395**, 185-189.

Sasai, Y., Lu, B., Piccolo, S. and De Robertis, E. (1996). Endoderm induction by the organiser-secreted factors chordin and noggin in Xenopus animal caps. *EMBO J.* **15**, 4547-4555.

Schrewe, H., Gendron-Maguire, M., Harbison, M. L. and Gridley, T. (1994). Mice homozygous for a null mutation of activin βB are viable and fertile. *Mech. Dev.* **47**, 43-51.

Schulte-Merker, S., Smith, J. and Dale, L. (1994). Effects of truncated activin and FGF receptors and of follistatin on the inducing activities of BVg1 and activin: does activin play a role in mesoderm formation? *EMBO J.* **13**, 3533-3541.

Schulte-Merker, S. and Smith, J. C. (1995). Mesoderm formation in response to Brachyury requires FGF signalling. *Curr. Biol.* **5**, 62-67.

Schulte-Merker, S., Van Eeden, F., Halpern, M. E., Kimmel, C. B. and Nüsslein-Volhard, C. (1994). *no tail (ntl)* is the zebrafish homolog of the mouse *T (Brachyury)* gene. *Development* **124**, 1009-1015.

Takata, C. (1960). The differentiation in vitro of the isolated endoderm under the influence of the mesoderm in *Triturus pyrogaster*. *Embryologia* **5**, 38-70.

ten Dijke, P., Ichijo, H., Franzen, P., Schulz, P., Saras, J., Toyashima, H., Heldin, C.-H. and Miyazono, K. (1993). Activin receptor-like kinases: a novel class of cell-surface receptors with predicted serine/threonine kinase activity. *Oncogene* **8**, 2879-2887.

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

Warga, R. M., and Nusslein-Volhard, C. (1999). Origin and development of the zebrafish endoderm. *Development* **126**, 827-838.

Weiser, R., Wrana, J. and Massague, J. (1995). GS domain mutations that constitutively activate TβR-1, the downstream signalling component in the TGFβ complex. *EMBO J.* **14**, 2199-2208.

Westerfield, M. (1993) *The Zebrafish Book: A guide for the laboratory use of zebrafish*. University of Oregon Press.

Wittbrodt, J. and Rosa, F. (1994). Disruption of mesoderm and axis formation in fish by ectopic expression of activin variants: the role of maternal activin. *Genes Dev.* **8**, 1448-1462.

Wylie, C., Snape, A., Heasman, J. and Smith, J. (1987). Vegetal pole cells and commitment to form endoderm in Xenopus laevis. *Dev. Biol.* **119**, 496-502.

Zon, L. I., Mather, C., Burgess, S., Bolce, M. E., Harland, R. M. and Orkin, S. H. (1991). Expression of GATA-binding proteins during embryonic development in *Xenopus laevis. Proc. Nat. Acad. Sci. USA* **88**, 10642-10646.