THE ROLE OF FGFR1 SIGNALLING IN THE SPECIFICATION AND MORPHOGENESIS OF MESODERM DURING MOUSE GASTRULATION

by

Brian Garrett Ciruna

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Graduate Department of Molecular and Medical Genetics
and the Collaborative Program in Developmental Biology
University of Toronto

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The Role of FGFR1 Signalling in the Morphogenesis and Specification of Mesoderm During Mouse Gastrulation

By Brian Garrett Ciruna

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy in the Graduate Department of Molecular and Medical Genetics and Collaborative Program in Developmental Biology at the University of Toronto, 2001.

ABSTRACT

Fibroblast growth factor (FGF) signalling plays an integral role in the morphogenesis and patterning of mesoderm at gastrulation. In the mouse, however, the downstream targets of FGF activity at the primitive streak have remained elusive - despite mutational analyses of FGF signalling pathway components. In an attempt to better understand the cellular and molecular mechanisms by which FGF signalling regulates the movement and specification of mesoderm progenitor cells at gastrulation, I have performed a series of mutant and chimeric analyses of FGF Receptor-1 (FGFR1) function.

In chimeric embryos, Fgfr1-/- cells show defects in migration through the primitive streak: as a result, Fgfr1-/- cells are deficient in populating mesodermal and endodermal lineages. Observed morphogenetic defects include failures in both the epithelial to mesenchymal transition (EMT) and movement of mesodermal cells away from the primitive streak - results are consistent with abnormalities in Fgfr1 -/- cell adhesion and/or cell migration. However, general deficiencies in Fgfr1 -/- cell migration were not observed in mesoderm explant cultures. Rather, I have implicated abnormal intercellular adhesion in the failed EMT and aberrant morphogenesis observed at gastrulation. I demonstrate that Snail expression is down-regulated, and that E-cadherin is ectopically expressed at the primitive streak of Fgfr1-/- embryos.

I also show that FGFR1 signalling functions in mesoderm cell fate specification by positively regulating Brachyury and Tbx6 expression. I propose that down-regulation of T and Tbx6 can therefore explain the absence of posterior and paraxial mesoderm
formation in Fgfrl mutants, and the formation of ectopic neural tubes in Fgfrl -/- chimeric embryos. Furthermore, I demonstrate that FGFR1 indirectly regulates Wnt signalling activity at the primitive streak. I argue that ectopically expressed E-cadherin in Fgfrl-/- progenitor cells sequesters free β-catenin from its intracellular signalling pool and thus attenuates Wnt signal transduction. I show that β-catenin remains co-localized with E-cadherin in Fgfrl -/- gastrula, and that forced down-regulation of E-cadherin expression can restore Wnt signalling in Fgfrl -/- embryonic explants. Results suggest a molecular link between FGF and Wnt signalling pathways at the primitive streak, and underscore the interdependent nature of morphogenesis and patterning at gastrulation.
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LIST OF ABBREVIATIONS

AER  apical ectodermal ridge
AP   anteroposterior
CAM  cell adhesion molecule
Dof  Downstream of FGF
Dpp  Decapentaplegic
ECM  extracellular matrix
EGF  epidermal growth factor
EGFP enhanced green fluorescence protein
EMT  epithelial to mesenchymal transition
EnR  Engrailed repressor domain
Eomes Eomesoderm
ES   cell embryonic stem cell
FAK  focal adhesion kinase
FGF  fibroblast growth factor
FGFR FGF receptor
dnFGFR dominant negative FGFR
FRS2 FGF receptor substrate 2
GFP  green fluorescence protein
GSK3β glycogen synthase kinase 3β
HSPG heparan sulfate proteoglycan
Htl  Heartless
ICM  inner cell mass
Ig   immunoglobulin
MAPK mitogen activated protein kinase
MBT  mid-blastula transition
NT   neural tube
Ntl  No tail
PAPC paraxial protocadherin
PLCγ phospholipase C-gamma
PTB  phosphotyrosine-binding
PZ   progress zone
RTK  receptor tyrosine kinase
SH2  Src homology 2
Spry Sprouty
Spt  Spadetail
T   Brachyury
TGFβ transforming growth factor-beta
VE   visceral endoderm
WT   wild-type
Xbra Xenopus Brachyury
General Introduction

The mechanisms underlying the induction, morphogenesis and patterning of embryonic mesoderm represent fundamental issues in vertebrate developmental biology. Mesoderm is generated during gastrulation - an integrated process of cellular proliferation, differentiation and morphogenetic movements which serve to transform a single epithelial layer into the three definitive germ layers of the vertebrate body plan: endoderm, mesoderm and ectoderm. An understanding of gastrulation requires identification of the molecular mechanisms which underlie cell fate specification, cell migration, and the changes in cellular adhesion which accompany mesoderm formation and morphogenesis. Several lines of evidence suggest that fibroblast growth factors (FGFs) may regulate one or more of these processes. This review will attempt to summarize the roles that FGFs play in the patterning and morphogenesis of mesoderm at gastrulation, as identified by genetic and embryological studies performed in *Xenopus*, zebrafish, *Drosophila* and the mouse.

**Fibroblast Growth Factors:**

The term “fibroblast growth factor” was first used in the early 1970s to describe a novel cationic polypeptide purified from bovine pituitary and brain extracts, which could stimulate the growth of NIH-3T3 cells. A second, anionic FGF was subsequently isolated from brain extracts, and it was demonstrated to have potent mitogenic activity when added to endothelial cells (reviewed in Burgess and Maciag, 1989; Basilico and Moscatelli, 1992). These FGFs, called basic FGF (bFGF) and acidic FGF (aFGF), respectively, were the founding members of what is now a large family of structurally related polypeptide growth factors that share protein sequence similarities within a core of 120 amino acids. FGFs have been cloned from many species, including mammals, birds, fish, amphibians, fruit flies and worms. In the mammalian genome alone, 23 *Fgf* genes have been identified (Venter et al., 2001).
FGFs thus constitute one of the largest known families of peptide growth factors. Although initially identified by their effects on cell replication and angiogenesis, FGFs are multipotent in activity and regulate a variety of fundamental cellular activities including cell survival, apoptosis, adhesion, migration, chemotaxis and differentiation (reviewed in Szebenyi and Fallon, 1999, and references therein). The biological effect invoked by a FGF often depends on the nature of the target cell. Indeed, cells may display alternate, sometimes opposite responses to FGFs depending on their states of differentiation, biochemical status, and cellular environment. In addition, different FGFs may elicit different biological activities, and alternative splicing within individual Fgf mRNAs can generate multiple variants of each FGF that may also differ in their biological activity (Szebenyi and Fallon, 1999).

A common feature of all FGFs, however, is a strong affinity for the glycosaminoglycan heparin, and for heparan sulfate proteoglycans (HSPGs). HSPGs are associated with the cell surface, basement membranes, and the extracellular matrix (ECM) of embryonic and adult tissues, and interactions between FGFs and HSPGs have been shown to stabilize FGFs to thermal denaturation and to protect them from proteolysis (reviewed in Ornitz, 2000). Thus, HSPGs serve as a stable reservoir for FGFs in vivo, and may also function to limit the diffusion of FGFs upon cell secretion. A large body of biochemical and cellular evidence also points to a direct role for heparin/HSPGs in promoting the high affinity binding of FGFs to their cognate FGF receptors (FGFRs) (Ornitz, 2000).

FGF Receptors:

The high affinity receptors for FGFs are members of the receptor tyrosine kinase (RTK) superfamily. To date, four distinct FGFR cDNAs have been cloned in mammals and are classified as FGFR1-4. Fgfrs have also been found in fruit flies, worms and sea urchins (in addition to vertebrates), as might be expected from the prevalence of FGFs across species. FGFRs share a common structural plan (Figure 1). They are single transmembrane proteins with an extracellular ligand binding domain that consists of a number of immunoglobulin-like motifs (Ig loops). Most vertebrate FGFRs contain three Ig-loops, although Ig-loop I is the least conserved region among FGFRs and is not
required for ligand binding. The FGF binding site is contained within a 139 amino acid stretch that includes part of Ig-loops II and III. Ligand selectivity is determined by the Ig III loop, and alternative splicing in Ig III dramatically changes the specificity of the FGFR for certain FGFs. Alternative splicing of fgr mRNA generates a multitude of isoforms of cell-bound or secreted receptors, some of which have distinct ligand-binding specificities and signalling properties. The isoforms generated by alternative splicing are tissue specific, and developmentally regulated (see Givol and Yayon, 1992). Overall, however, FGFRs have overlapping recognition and redundant specificity, whereby one receptor may bind with a similar affinity to several FGFs and one FGF may bind similarly to several distinct receptors (Ornitz et al., 1996).

Other FGFR extracellular domains include a stretch of acidic amino acids between Ig loops I and II (acid box), a heparin-binding domain, and a cell adhesion molecule (CAM) homology domain (Figure 1). The CAM homology region has been shown to be a binding site for L1, N-CAM and N-cadherin, and it has been suggested that these cell adhesion molecules may promiscuously activate FGFRs (Green et al., 1996). The acid box acts to bind divalent cations, which are thought to be required for the high-affinity interactions between FGFRs and HSPGs. In the current model of FGFR activation, interactions between heparin, FGF and FGFR augment the affinity of FGFs for the FGFR, and also promote the dimerization of two FGF-FGFR complexes (Schlessinger et al., 2000).

The intracellular region of FGFRs includes a juxtamembrane region, two conserved kinase domains, a kinase insert region, and a carboxy tail that has several potential autophosphorylation sites (reviewed in Szebenyi and Fallon, 1999). The intracellular domains of FGFRs are structurally similar to other transmembrane RTKs. Upon binding to their receptors, FGFs (and associated HSPGs) induce receptor dimerization and activation, resulting in the rapid autophosphorylation of a number of intracellular tyrosine residues. The kinase domains and several of the phosphorylation sites in FGFRs are required for FGF-initiated signal transduction.
Figure 1. The structure of a generic FGFR protein. A schematic diagram showing the major structural features of an FGFR protein, including the acid box, cell adhesion molecule (CAM) and heparin-binding domains, immunoglobulin (Ig) loops, transmembrane domain, juxtamembrane (JM) region, and the split tyrosine kinase domain. See text for details.
Figure 1:

- Signal peptide
- Acid box
- CAM- and heparin-binding domains
- Alternatively spliced (ligand specificity)
- Transmembrane domain
- Split tyrosine kinase domain
- Kinase insert

Ig Loop:

- Ligand binding domain
- JM region

NH2 to COOH
FGF Signal Transduction:

FGFR1 contains seven autophosphorylation sites, two of which are located within the catalytic domain (Y653 and Y654) and are essential for stimulation of kinase activity (Mohammadi et al., 1996). Autophosphorylation sites located outside of the catalytic domain are usually involved in the recruitment of cellular target proteins, by serving as docking sites for the Src homology 2 (SH2) or phosphotyrosine-binding (PTB) domains of cytoplasmic effector proteins (Cohen et al., 1995; Pawson, 1995). One phosphotyrosine in the C-terminal tail of FGFR1 (Y766) functions as a high affinity binding site for the SH2 domain of phospholipase C-gamma (PLCγ) (Mohammadi et al., 1991). Activation of PLCγ, via phosphorylation of Y766, couples FGFR1 signalling to phosphatidylinositol hydrolysis, activation of protein kinase C (PKC) and the mobilization intracellular Ca2+ in several cell types. However, in vitro functional assays using a Y766F mutant FGFR1 have failed to show a requirement for this pathway for any FGFR1 induced biological responses (Mohammadi et al., 1992; Peters et al., 1992).

Another consequence of FGFR autophosphorylation is the activation of the Ras/mitogen activated protein kinase (MAPK) pathway. It is well established that the adapter protein Grb2 (Clark et al., 1992; Lowenstein et al., 1992) links RTKs to the Ras signalling pathway by binding to both the guanine nucleotide-releasing factor Sos through its SH3 domains, and to tyrosine-phosphorylated receptors or docking molecules via its SH2 domain (Schlessinger, 1994). However, Grb2 does not bind directly to FGF receptors. Instead, FGF receptors utilize intermediate (docking) proteins to indirectly recruit the Grb2-Sos complex to the cell surface, a step essential for activation of the Ras/MAPK signalling cascade.

SHC and FRS2 both function as docking molecules for FGFR1. Shc, however, appears to be a promiscuous target for tyrosine phosphorylation downstream of the activation of a broad range of receptor tyrosine kinases. FRS2 (for FGF Receptor Substrate 2), is tyrosine phosphorylated by only a limited repertoire of RTKs, and it was proposed that this docking protein may participate more specifically in the control of FGF-induced signalling responses (Kouhara et al., 1997). Two members of the FRS2 family (FRS2α and FRS2β) have been identified, and both are structurally and functionally very similar (Kouhara et al., 1997; Xu et al., 1998a; Ong et al., 2000).
Analysis of FRS2 sequence revealed four potential Grb2 binding sites, as well as both a consensus myristylation sequence and a putative PTB domain in its amino-terminus (Kouhara et al., 1997). The PTB domain of FRS2 binds directly and constitutively to the juxtamembrane region of FGFR1, independent of ligand stimulation and tyrosine phosphorylation (Ong et al., 2000). In vitro mutational analysis of FRS2 has revealed that myristylation is essential for its membrane localization, tyrosine phosphorylation, Grb2/Sos recruitment, and MAPK activation (Kouhara et al., 1997). It was also shown that FRS2 acts upstream of Ras as a link between activated FGFRs and the Ras/MAPK cascade.

FRS2 also indirectly recruits Grb2 molecules via Shp2 – a protein tyrosine phosphatase that associates with FRS2 in response to FGF signalling (Hadari et al., 1998). Protein tyrosine phosphatases have been shown to play both positive and negative roles downstream of RTK signalling (Tonks and Neel, 1996); however, biochemical studies have indicated that Shp2 plays a positive role in the control of FGF induced MAP kinase activation, cell growth, and differentiation (Tang et al., 1995; Saxton et al., 1997; Hadari et al., 1998). FGF receptors do not engage Shp2 directly; rather, two phosphotyrosine binding sites for Shp2 have been identified on FRS2 (Hadari et al., 1998). The interaction between Shp2 and FRS2 leads to tyrosine phosphorylation of Shp2, and results in the recruitment of Grb2/Sos complexes. In vitro analyses of catalytically deficient Shp2 mutants, and of FRS2 mutants deficient in Shp2 binding, demonstrate that both the recruitment of Shp2 by FRS2 and the catalytic activity of Shp2 are crucial for sustained MAP kinase activity in response to FGFs, and for FGF-induced cell biological responses (Hadari et al., 1998).

A role for FGF signalling in mesoderm induction?

In the vertebrate embryo, the activities of FGFs are required from the earliest stages of development through to the detailed patterning of organ systems (reviewed in Szebenyi and Fallon, 1999, and references therein). Analysis of the expression patterns and activities of FGFs during embryonic development have provided further insight into their normal biological functions. Perhaps the best characterized activity for FGFs during early development, is their ability to induce mesoderm formation from naïve ectodermal
tissue explanted from *Xenopus* blastulae (Slack et al., 1987; Kimelman and Kirschner, 1987).

In normal development, cells from the animal pole of the *Xenopus* blastula will form ectodermal derivatives. This has been demonstrated using explantation and self-differentiation experiments where tissue from the animal pole, when explanted and cultured in isolation, will differentiate as a ball of ciliated epidermis (Dale and Slack, 1987). Commitment studies have also demonstrated that explants from the vegetal hemisphere will form endodermal tissues (Dale and Slack, 1987). Although animal hemisphere explants will form epidermis when left to differentiate in isolation, they also have the competence to form mesodermal and endodermal tissues in response to inductive signals. Indeed, the formation of the basic body plan during early amphibian development is believed to result from a series of inductive interactions between different regions of the embryo (Nieuwkoop, 1985; Smith et al., 1985). The first of these interactions is termed mesoderm induction, an interaction between vegetal and animal tissues which leads to the formation of a ring of mesodermal tissue around the equatorial marginal zone of the blastula. This interaction was first demonstrated by combining isolated explants from animal and vegetal pole regions Nieuwkoop, 1969. On their own, neither piece forms any mesoderm whereas in combination the animal pole explant produces abundant mesodermal tissue. Further studies using cell lineage labels, antibodies and molecular probes have repeatedly confirmed the mesoderm inducing properties of the original “Nieuwkoop combination” experiments (Dale et al., 1985; Gurdon et al., 1985).

The fact that FGFs could induce mesoderm formation in animal cap explants made them strong candidates for the endogenous mesoderm inducing factors (MIFs) produced by vegetal hemisphere cells. Moreover, it was shown that stimulation of the MAPK signal transduction pathway downstream of FGF signalling was both necessary and sufficient for mesoderm induction in *Xenopus*. Overexpression of MAP Kinase Phosphatase (MKP-1; LaBonne et al., 1995; Gotoh et al., 1995; Umbhauer et al., 1995), and of dominant-inhibitory mutants of p21ras (Whitman and Melton, 1992), raf-1 (MacNicol et al., 1993), Grb2 (Gupta and Mayer, 1998), or the protein tyrosine phosphatase Shp-2 (Tang et al., 1995), will all prevent the induction of mesodermal markers in response to FGF treatment, and will cause severe defects in gastrulation and
posterior mesoderm formation. Conversely, overexpression of constitutively activated mutants of p21ras, raf-1, MEK, or MAP Kinase is sufficient to induce mesodermal markers in animal cap explants (Whitman and Melton, 1992; LaBonne et al., 1995; LaBonne and Whitman, 1994; Gotoh et al., 1995; Umbhauer et al., 1995). Thus, the Ras/MAPK signalling pathway plays a critical role in transducing the mesoderm-inducing signal provided by FGF stimulation.

However, the expression patterns of the known Xenopus FGFs do not fit with the theory that they are the signals responsible for mesoderm induction in vivo. bFgf, eFgf and Fgf9 are expressed maternally in the animal cap and marginal zone of Xenopus blastulae (Song and Slack, 1994; Isaacs et al., 1992; Song and Slack, 1996), and therefore, they are produced in the cells that would normally respond (and not produce) mesoderm inducing factors. Zygotic expression of Xenopus bFgf, eFgf, Fgf3, Fgf8, Fgf9 and Fgf20 all commence at late blastula/early gastrula stages, with strongest expression around the blastopore (Song and Slack, 1994; Isaacs et al., 1992; Song and Slack, 1996; Tannahill et al., 1992; Christen and Slack, 1997; Koga et al., 1999). Zygotic expression would therefore be consistent with a later role for FGF signalling in mesodermal patterning at gastrulation (see below).

**Current models for vertebrate mesoderm induction**

More recent genetic and embryological studies have demonstrated that members of the Nodal family of transforming growth factor-ß (TGFß) signals play an essential role in the formation of mesoderm across vertebrate species. In Xenopus, it has been demonstrated that a maternally expressed, vegetally localized T-box transcription factor – VegT - activates zygotic signals that induce mesoderm as well as endoderm formation (Zhang et al., 1998; Kimelman and Griffin, 1998). These zygotic signals include at least four members of the TGFß superfamily: Derriere and the Nodal-related genes Xnr1, Xnr2 and Xnr4 (Clements et al., 1999; Kofron et al., 1999; Yasuo and Lemaire, 1999; Agius et al., 2000; Hyde and Old, 2000). Furthermore, although the depletion of maternal VegT mRNA results in the abnormal specification of mesodermal and endodermal germ layers, injection of Xnr1, Xnr2, Xnr4 and derriere mRNA into the vegetal cells of VegT-depleted embryos will rescue mesoderm induction, blastopore formation and the
generation of a wild-type (WT) body axis (Kofron et al., 1999). Moreover, injection of eGFImRNA fails to rescue any aspect of the VegT-depletion phenotype. This establishes that zygotic TGFβ growth factors downstream of VegT are responsible for mesoderm induction by the vegetal mass. The maternal TGFβ factor VgI may also be required for the expression of Nodal-related factors (Agius et al., 2000), and inhibition of VgI with a dominant-negative mutant leads to defects in dorsal mesoderm formation (Joseph and Melton, 1998).

Nodal-related factors also play essential roles in mesoderm induction in zebrafish. Mutant analysis of the zebrafish Nodal-related genes cyclops and squint, and of the extracellular factor one-eyed-pinhead (oep; which is an essential co-factor for Nodal signalling) have shown that Nodal signalling is required for dorsal mesoderm and endoderm induction (Schier and Shen, 2000). Although nodal signalling is not required for ventral mesoderm induction (Feldman et al., 1998; Gritsman et al., 1999), Nodal signalling is essential for the morphogenesis of mesendoderm at gastrulation; this accounts for the absence of ventral mesodermal derivatives in cyclops;squint double mutants and in maternal zygotic oep (Mzoep) mutant embryos. Ventral mesoderm induction depends upon a non-Nodal TGFβ signal from the yolk syncitial layer, the activity of which is inhibited by high levels of the TGFβ antagonist antivin (Thisse and Thisse, 1999; Chen and Kimelman, 2000).

Nodal signalling has also been demonstrated to be essential for mesoderm formation in the mouse. Mouse nodal mutants lack a primitive streak and most mesoderm, and display only sporadic formation of some posterior mesoderm (Conlon et al., 1994). Similarly, mouse Smad2 and ActRIB mutants as well as ActRIIA;ActRIIB double mutants (which disrupt the Nodal signal transduction pathway) display gastrulation-defective phenotypes very similar to that of nodal mutants (Waldrip et al., 1998; Weinstein et al., 1998; Nomura and Li, 1998; Gu et al., 1998; Song et al., 1999). Thus, Nodal-related TGFβ factors appear to play a conserved role in mesoderm formation and gastrulation across vertebrate species.
FGFs as competence factors in mesoderm induction

Although FGFs do not act as vegetally localized mesoderm inducing factors, it has been shown that a functional FGFR signalling pathway is necessary for the induction of the *Xenopus Brachyury* (Xbra) gene, and of a number of other early mesodermal markers in response to activin treatment (Cornell and Kimelman, 1994; LaBonne and Whitman, 1994). Thus, FGFs may play an accessory role in TGFβ-mediated mesoderm induction *in vivo*. Furthermore, as discussed, a functional Ras/MAPK signal transduction pathway is also required for the induction of a full spectrum of mesodermal genes in response to MIFs (LaBonne and Whitman, 1994; LaBonne et al., 1995). However, activin and BMP4 do not activate MAP kinase when injected into *Xenopus* blastula. Moreover, overexpression of a dominant negative activin receptor, which has been shown to inhibit all mesoderm formation (Hemmatti-Brivanlou and Melton, 1992; Schulte-Merker et al., 1994a), does not block endogenous MAP kinase activation in the developing embryo (Christen and Slack, 1999). Therefore, despite the requirement for a functional Ras/MAPK signalling pathway for TGFβ-mediated mesoderm induction, TGFβ signals do not directly stimulate the MAP kinase signalling cascade.

There is growing evidence that all observed MAPK activity in the early *Xenopus* embryo can be attributed to stimulation of the FGF signalling pathway. In animal caps expressing a dominant negative FGF receptor (dnFGFR) construct, the phosphorylation of MAP kinase is greatly diminished. This indicates that normal MAP kinase activity is generated by endogenous FGF signalling (LaBonne et al., 1995). *In vivo*, the activation of MAPK has been examined immunohistochemically using an antibody specific for the dually phosphorylated (activated) form of MAPK (Gabay et al., 1997). Although MAPK is a potential target of many receptor tyrosine kinases (RTKs), all normal domains of MAPK activation in young *Xenopus* embryos were abolished upon expression of the dnFGFR construct (Christen and Slack, 1999; Curran and Grainger, 2000). Therefore, the expression of activated MAPK is a very useful marker of active FGF signalling during early *Xenopus* development.

In whole embryos, a low level of cytoplasmic MAP kinase activity is observed throughout the animal cap and marginal zones of the early blastula (Curran and Grainger, 2000). This would suggest that FGF signalling does take place in these regions of the
embryo. However, the levels of maternal FGF in the animal hemisphere must be sub-threshold because isolated animal cap explants do not mesodermalize. Since the induction of \textit{Xbra} expression in animal caps by mesoderm inducing signals does not require protein synthesis (Smith et al., 1991), then maternally supplied FGFs must provide the necessary MAPK activity for the induction of a full spectrum of mesodermal genes (LaBonne et al., 1995). It has been proposed, therefore, that endogenous MAP kinase activation by maternal FGFs plays a role in establishing the responsiveness or competence of embryonic tissues to respond to mesoderm inducing signals (Cornell et al., 1995).

High levels of activated MAP kinase are later seen in the dorsal marginal zone of \textit{Xenopus} embryos prior to blastopore formation; MAP kinase then remains active during gastrulation in a ring around the blastopore corresponding to the domain of newly forming mesoderm (Christen and Slack, 1999; Curran and Grainger, 2000). This spatial and temporal pattern of MAP kinase activation in the early \textit{Xenopus} gastrula is very similar to the zygotic expression patterns of the known \textit{Fgfs} (Christen and Slack, 1997; Isaacs et al., 1995; Tannahill et al., 1992; Song and Slack, 1996). This would be consistent with a role for zygotic FGF signalling in patterning the newly formed mesoderm.

\section*{FGF signalling and mesodermal patterning}

To investigate the roles for FGF signalling \textit{in vivo}, dominant negative FGFR constructs were injected into \textit{Xenopus} and zebrafish embryos (Amaya et al., 1991; Amaya et al., 1993; Griffin et al., 1995). Expression of the dnFGFR construct, which encodes for a truncated receptor that lacks an intracellular kinase domain, has been shown to inhibit all WT FGFR function. In both zebrafish and \textit{Xenopus} embryos, dnFGFR expression caused defects in gastrulation and resulted in the reduction of posterior and lateral mesoderm formation, although head structures developed normally (Amaya et al., 1991; Amaya et al., 1993; Griffin et al., 1995). There was a considerable reduction in the amount of blood, somitic muscle and notochord formed, indicating that the differentiation of both ventral and dorsal tissues was affected by the lack of FGFR signalling (Amaya et al., 1991). In dnFGFR injected embryos, expression of \textit{Xbra} and \textit{no tail} (\textit{ntl}, the zebrafish \textit{Brachyury} homolog) were lost throughout the marginal zone. This
suggested that FGF function is required for the correct regulation of a subset of genes that are expressed in the newly formed mesoderm (Amaya et al., 1993; Griffin et al., 1995). However, since gross abnormalities in dnFGFR expressing embryos only appeared after extensive morphogenesis, it was difficult to specify whether the defects were due to inappropriate inductive events or abnormal gastrulation movements, which are inherently linked to mesodermal patterning (see below).

In mouse, zebrafish, *Xenopus* and the chick, *Brachyury* is expressed transiently throughout the presumptive mesoderm, with transcripts persisting in the tailbud and notochord (Herrmann, 1991; Smith et al., 1991; Schulte-Merker et al., 1992; Kispert et al., 1995). In these vertebrates, *Brachyury* has been shown to play a conserved role in the morphogenesis and patterning of mesoderm at gastrulation, especially in the specification and maintenance of axial and posterior mesoderm populations (Herrmann et al., 1990; Halpern et al., 1993; Schulte-Merker et al., 1994b; Conlon et al., 1996; Conlon and Smith, 1999). In particular, zebrafish *ntl* mutant embryos develop without a notochord and tail structures (Halpern et al., 1993). In *Xenopus*, the function of *Xbra* has been inhibited through expression of an *Xbra-EnR* construct, in which the activation domain of *Xbra* has replaced by the Engrailed repressor domain (EnR) (Conlon et al., 1996; Conlon and Smith, 1999). *Xenopus* embryos expressing the *Xbra-EnR* fusion protein do not gastrulate normally and also fail to form notochord and tail structures (Conlon et al., 1996). Indeed, the phenotype of *ntl* mutant and *Xbra-EnR* expressing embryos closely resembles that of embryos injected with a dominant negative FGF receptor. This, and the fact that *Brachyury* expression was lost in dnFGFR expressing embryos, suggested that *Xbra* and *Ntl* may act downstream of FGF signalling.

In *Xenopus*, it was demonstrated that animal caps expressing the *Xbra-EnR* construct failed to form mesoderm in response to FGF treatment (Conlon and Smith, 1999). Since *Xbra* expression is an early response to mesoderm induction (Smith et al., 1991), and since both *Xbra* and FGF induce ventro-posterior mesoderm from animal cap tissue (Cunliffe and Smith, 1992; Ruiz i Altaba and Melton, 1989a), data further suggested that *Xbra* may act downstream of FGF signalling. The zygotic expression of eFGF was also inhibited by dnFGFR expression in *Xenopus* embryos (Isaacs et al., 1994). Since eFGF and *Xbra* are co-expressed in the dorsal mesoderm of the blastopore (Isaacs et al., 1992; Smith et al., 1991), it was suggested that eFGF may be required to
maintain *Xbra* expression in this region of the embryo (Isaacs et al., 1994). Indeed, a requirement for cell-cell signalling in the maintenance of *Xbra* expression was demonstrated by the fact that explants taken from the blastopore region rapidly down-regulate *Xbra* expression when dissociated into single cells (Isaacs et al., 1994). The addition of eFGF to these dissociated cell cultures was found to maintain *Xbra* expression (Isaacs et al., 1994; Schulte-Merker and Smith, 1995).

*Xbra* is also able to activate the zygotic transcription of *eFGF* (Isaacs et al., 1994; Schulte-Merker and Smith, 1995), and more recent experiments show that the activation of *eFGF* expression by *Xbra* is direct (Casey et al., 1998). This suggests that the continued expression of *Xbra* and *eFGF* during gastrulation is co-dependent and forms a positive-feedback regulatory loop (Isaacs et al., 1994; Schulte-Merker and Smith, 1995). Thus, after its induction by TGFβ mesoderm inducing signals, *Xbra* is able to activate the zygotic transcription of *eFgf*, which is then necessary to maintain the expression of a subset of mesodermal genes, including *Xbra*, in the blastopore region (Isaacs et al., 1994). This initiates a period of autocatalytic activation of *eFgf* and *Xbra* transcription within the forming mesoderm of the marginal zone. Since *Xbra* activity has been shown to play an essential role in the convergent extension movements of gastrulation, and in the maintenance of posterior and ventral cell types (Conlon and Smith, 1999), the FGF-*Xbra* autoregulatory loop is likely to play a pivotal role in regulating the morphogenesis and specification of mesoderm at gastrulation.

**T-box genes downstream of FGF signalling:**

Studies in *Xenopus* and zebrafish have thus demonstrated that *Brachyury* expression at gastrulation requires a functional FGF signalling pathway, and that many of the abnormalities associated with loss of FGF signalling can be phenocopied by *Xbra-Enr* expression or *ntl* mutations. However, careful comparison of dnFGFR expressing embryos with *ntl* mutant embryos has revealed that activation of *Brachyury* expression alone cannot be the sole function of FGF signalling at gastrulation (Griffin et al., 1995). Recent studies in zebrafish have established a critical role for a number of T-box transcription factors as key orchestrators of convergent extension movements and posterior mesoderm differentiation downstream of FGF activity.
Griffin et al. (1995) studied the effect of injecting mRNAs for either eFGF or a dominant negative FGF receptor into zebrafish embryos (Griffin et al., 1995). As discussed, expression of a dnFGFR led to a loss of ntl expression and a complete loss of trunk and tail structures; however, the expression of the mesodermal marker snail (the zebrafish homologue of Drosophila snail; Hammerschmidt and Nusslein-Volhard, 1993) was unaltered in blastula stage embryos (Griffin et al., 1995). Since snail expression is also a general marker of mesoderm induction (Hammerschmidt and Nusslein-Volhard, 1993), this suggested that loss of ntl expression and posterior mesoderm formation in dnFGFR injected embryos was not due to general failures in mesoderm induction, but rather to abnormal mesodermal patterning or morphogenesis (Griffin et al., 1995).

Overexpression of eFGF in zebrafish embryos resulted in abnormal morphogenesis from gastrulation onwards, as manifested by a thickening of the germ ring and hypoblast, as well as the formation of multiple foci of convergent extension movements (Griffin et al., 1995). Overexpression of eFGF also resulted in the loss of head structures; this indicated an additional role for FGF signalling in the regulation of anteroposterior patterning (Griffin et al., 1995). After injection of eFGF, ntl expression was induced throughout the epiblast prior to gastrulation (Griffin et al., 1995). However, the phenotypes associated with eFGF overexpression were not completely dependent upon ntl, since ntl mutant embryos injected with eFGF also developed a thickened germ ring and showed abnormal gastrulation movements and morphogenesis (Griffin et al., 1995). Therefore, ntl function is not required for all of the observed phenotypes associated with eFGF over-expression.

Furthermore, expression of the dominant negative FGFR resulted in loss of trunk and tail, with severely affected embryos lacking the body axis from the level of the first somite (Griffin et al., 1995). Zebrafish ntl mutants, however, only lack notochord and the caudal body axis posterior to somite 17-19 but have an otherwise normal trunk (Halpern et al., 1993). The difference between these two phenotypes suggested that FGF signalling was essential for the development of a larger domain of the embryo than that which could be explained by the loss of ntl function alone. This indicated that another gene or set of genes, also dependent upon FGF signalling, are required for the development of the trunk (Griffin et al., 1995). It was later demonstrated that the spadetail locus encoded for a T-
box transcription factor, and that spadetail was likely the key mediator of FGF signalling in the trunk mesoderm (Griffin et al., 1998).

*Spadetail (spt)* is expressed ubiquitously throughout the zebrafish blastula, but becomes rapidly restricted to marginal cells in late blastula- and early gastrula-staged embryos (Griffin et al., 1998). *spt* is expressed in both epiblast and hypoblast cells of the lateral and ventral germ ring, but is not expressed in dorsal marginal cells which are fated to become notochord (Griffin et al., 1998). In *spt* mutants, somites do not form in the trunk region although the tail develops relatively normally except for a characteristic bulge of excess mesodermal cells at the end of the tail (Kimmel et al., 1989). Cell labeling studies have shown that lateral margin cells, which normally involute during gastrulation and then converge towards the dorsal side of the embryo, fail to converge properly in *spt* mutants; instead, *spt* mutant cells move abnormally towards the vegetal pole, which will become the tail bud (Kimmel et al., 1989). Cell transplantation experiments have shown that *spt* is required cell autonomously for the convergence of lateral margin cells into the dorsal axis during gastrulation (Ho and Kane, 1990).

*Spt* homologues have been identified in both *Xenopus* (Xombi- Lustig et al., 1996; *Antipodean*- Stennard et al., 1996; *VegT*- Zhang and King, 1996; *BraT*- Horb and Thomsen, 1997) and in the chick (*ChTbx6L*- Knezevic et al., 1997). As discussed, maternally expressed *VegT*, the *Xenopus spt* homologue, is involved in the specification of the mesoderm and endodermal germ layers (Zhang et al., 1998; Kofron et al., 1999). Zygotic expression of *VegT* occurs soon after the midblastula transition in the dorsal marginal zone, and later becomes restricted to posterior paraxial and lateral mesodermal layers (Lustig et al., 1996; Stennard et al., 1996; Zhang and King, 1996; Horb and Thomsen, 1997); there is also evidence that zygotic *VegT* may be involved in blastopore lip formation (Lustig et al., 1996). *ChTbx6L* is also expressed in mesodermal progenitors prior to gastrulation, and is later expressed in the paraxial mesoderm lineage (Knezevic et al., 1997). The expression of both *Xenopus* and chick *spt* homologues have been shown to be regulated by FGF signalling (Lustig et al., 1996; Horb and Thomsen, 1997; Knezevic et al., 1997).

In zebrafish, studies using dominant negative FGF receptor constructs have also demonstrated that *spt* expression depends on a functional FGF signalling pathway (Griffin et al., 1998). Although FGF signalling does not activate *spt* expression, it is
required for the maintenance of spt expression from the mid-gastrula stage onward. To determine whether spt expression was regulated by ntl function downstream of FGF signalling, Griffin et al (1998) examined spt expression in ntl mutant embryos: spt expression appeared normal through gastrulation up until the 4 somite stage but spt expression was subsequently lost in the tail bud (Griffin et al., 1998). This indicated that during gastrulation, when trunk mesoderm is forming, spt expression is independent of ntl function. Therefore, FGF signalling must regulate ntl and spt expression independently of one another (Griffin et al., 1998). It was suggested, therefore, that the requirement for FGF activity in trunk and tail formation can be accounted for by the combined complementary functions of two targets of FGF signalling: spt and ntl (Griffin et al., 1995). Indeed, ntl;spt double mutants lack all trunk and tail mesoderm and resemble the dominant negative FGF receptor phenotype (Sharon L. Amacher and Charles B. Kimmel, unpublished data).

A third zebrafish T-box gene that may play a role in trunk and tail mesodermal patterning is Tbx6 (Hug et al., 1997). Tbx6 expression appears almost identical to that of spt, and both spt and tbx6 are co-expressed with ntl in the ventral and lateral germ ring (Griffin et al., 1998). It has been demonstrated that tbx6 expression in the trunk is regulated by spt function (Griffin et al., 1998), and that tbx6 expression in the tail is regulated by ntl (Hug et al., 1997). Although the function of zebrafish tbx6 is unknown, targeted mutation of tbx6 in the mouse has demonstrated a major role for the T-box gene in paraxial mesoderm specification and trunk somite formation (Chapman and Papaioannou, 1998). Therefore, T-box genes appear to play pivotal roles downstream of FGF signalling, in both specifying trunk and tail mesoderm, and in regulating the morphogenetic movements of gastrulation.

FGF signalling and anteroposterior patterning:

Although T-box genes play important roles as “selector” genes of trunk and tail development, they are not the only targets of FGF signalling. There is growing evidence that FGFs have additional roles in specifying and patterning the anteroposterior (AP) axis. During gastrulation, several secreted FGFs are expressed around the dorsal lip and blastopore region of the gastrulating Xenopus embryo (Tannahill et al., 1992; Isaacs et
al., 1994; Christen and Slack, 1997; Song and Slack, 1996; Koga et al., 1999). This region is considered the posterior end of the forming AP axis, and the potential source of the “posteriorizing” signal required for patterning of the developing AP axis (reviewed in Slack and Tannahill, 1992; see below).

As already discussed, dominant negative FGF receptor experiments have demonstrated that *Xenopus* and zebrafish embryos require functional FGF activity for the development of the posterior body axis (Amaya et al., 1991; Griffin et al., 1995). Griffin et al (1995) examined the effects of FGFR inhibition on the expression of members of the *caudal* and *even-skipped* gene families. *Cad1* (Joly et al., 1992) and *evel* (Joly et al., 1993) are the zebrafish homologues of the *Drosophila caudal* and *even-skipped* genes, respectively - homeodomain transcription factors which have been implicated in the specification of posterior fates and in the suppression of anterior ones (Ruiz i Altaba and Melton, 1989b; Mlodzik et al., 1990; Joly et al., 1993; Barro et al., 1994). Both *cad1* and *evel* expression were found to be dependent upon a functional FGF signalling pathway (Griffin et al., 1995). Furthermore, after over-expression of *eFGF*, *cad1* and *evel* were expressed ectopically throughout the entire epiblast of the early and late gastrula (Griffin et al., 1995). In zebrafish therefore, FGF signalling regulates the expression of two genes with posteriorizing activity.

Similarly, it has been demonstrated that inhibition of the FGF signal transduction pathway in *Xenopus* embryos results in down-regulated expression of both *Xcad3*, a *Xenopus caudal* family member, and the posterior Hox gene *HoxA7* (Northrop and Kimelman, 1994; Isaacs et al., 1994). Again, this suggests an important role for FGFs in establishing AP pattern during gastrulation. Injection of *Xcad3* mRNA can rescue the expression of *HoxA7* in the absence of FGF signalling (Pownall et al., 1996). This supports the view that members of the vertebrate caudal gene family lie downstream of FGF signalling, but are upstream activators of *Hox* gene expression (Subramanian et al., 1995).

The injection of *eFGF* mRNA into *Xenopus* or zebrafish embryos results in grossly abnormal gastrulation movements and very little morphological differentiation, thus making it difficult to study the effects of FGF overexpression later in development (Thompson and Slack, 1992; Griffin et al., 1995). However, in *Xenopus* embryos injected with *eFGF* DNA constructs, mRNA does not accumulate until after mid-blastula
transition (MBT) in the late blastula and early gastrula stages, and the majority of embryos gastrulate normally (Isaacs et al., 1994). Later in development, these embryos show a posteriorized phenotype with loss of eyes and forebrain, coincident with an enlarged proctodeum at the posterior of the embryo (Isaacs et al., 1994). Consistent with this posteriorized phenotype, overexpression of eFGF either as an injected plasmid or as protein applied on a bead, has been demonstrated to cause the up-regulation and anterior expansion of a number of posteriorly expressed genes including Xcad3 and the posterior Hox genes HoxA7 and HoxB9 (Isaacs et al., 1994; Pownall et al., 1996).

Similar anterior truncations in Xenopus embryos are caused by the dorsal overexpression of Xcad3 or HoxA7 (Pownall et al., 1996; Isaacs et al., 1998); this indicates that the posteriorized phenotype observed upon eFGF overexpression could be caused by the ectopic activation of posterior genes in anterior regions. Conversely, the overexpression of an Xcad3 repressor mutant (Xcad-EnR), which potently blocks the activity of wild-type Xcad3, inhibits the expression of HoxA7 and HoxB9 and leads to a dramatic disruption of posterior development with a reduction or complete loss of trunk and tail (Isaacs et al., 1998). It has also been demonstrated that Xcad3 is required for the activation of Hox genes by FGFs in animal cap experiments, and that Xcad3 is an immediate early target of the FGF signalling pathway (Isaacs et al., 1998). These data provide strong evidence that the initial expression of posterior Hox genes, and thus the formation of the trunk and tail, is dependent on FGF signalling and that this regulation is likely mediated by the activation of Xcad3.

**FGF signalling in morphogenesis:**

In the fruit fly Drosophila melanogaster, FGF signalling plays an essential role in the morphogenesis and directed migration of several different cell types. Two Drosophila FGF receptors (DFGFRs), Breathless and Heartless, have been identified by sequence similarity to vertebrate FGFR homologues (Glazer and Shilo, 1991; Shishido et al., 1993). Breathless is expressed in cells of the developing tracheal system where it is required for proper cell migration and tracheal branch morphogenesis (Jarecki et al., 1999; Klambt et al., 1992; Reichman-Fried et al., 1994). Heartless is expressed in the embryonic mesoderm and plays an essential role in the spreading and migration of
nascent mesodermal cells at gastrulation (Beiman et al., 1996; Gisselbrecht et al., 1996; Shishido et al., 1993; Shishido et al., 1997). Genetic and embryological analyses of FGF function in Drosophila have uncovered novel downstream components of the FGF signalling pathway, and have provided many insights into the role and mechanisms of FGF signalling in regulating cell migration and morphogenesis.

Branching morphogenesis:

The DFGFR Breathless was found to be expressed during embryogenesis in cells of the developing trachea (Glazer and Shilo, 1991; Klambt et al., 1992). The tracheal system develops from ten clusters of approximately 80 ectodermal cells on either side of the embryo which invaginate into sac-like structures called tracheal pits, or placodes. Subsequently, cells from each placode undergo directed cell migrations to form a stereotypical array of primary and secondary branches. Mutations in the breathless gene result in the failure of tracheal cell migration from invaginated placodes, and therefore in the loss of primary and secondary tracheal branch formation (Klambt et al., 1992). The Drosophila FGF homolog Branchless has been identified as the ligand for Breathless. In branchless mutants the placode invaginates but primary branches fail to form, just as is observed in mutants that lack the Breathless receptor (Sutherland et al., 1996).

Branchless is expressed in a very dynamic pattern that is complementary to breathless expression, and which prefigures the patterning of primary tracheal branches. It is first seen in six patches of cells outside the tracheal placode adjacent to the points at which primary tracheal branches are about to form; branchless expression is then downregulated as the primary branches extend outward and reach the original branchless-expressing cells (Sutherland et al., 1996). These observations, and the fact that localized misexpression of branchless can direct ectopic primary branch migration, has led to the suggestion that Branchless acts as a chemotactic signal which directs tracheal cell migration and patterns the tracheal tree (Sutherland et al., 1996).

Branchless/Breathless signalling has also been shown to play a role in cytoneme formation. Cytonemes are fine cellular processes which can be extended over the distance of many cell diameters, and appear to function in long-range cell-cell communication (Ramirez-Weber and Kornberg, 1999). In Drosophila wing imaginal disc cells, the
projection of cytonemes towards the anterior-posterior compartment boundary appears to require Branchless signals (Ramirez-Weber and Kornberg, 1999). However, it is unclear whether Branchless simply plays a permissive role in cytoneme formation, or whether it is involved in the directional growth of cytonemes towards a signalling source. Mouse limb bud cells have also been shown to extend cytonemes in response to FGF, and it has been suggested that cytonemes may play a conserved role across vertebrate and invertebrate species in allowing cells to make physical contact with distant morphogen sources (Ramirez-Weber and Kornberg, 1999). It is possible, therefore, that cytoneme formation may play a critical role in the FGF-mediated chemotaxis of tracheal cells during Drosophila development (Zelzer and Shilo, 2000).

Downstream of Breathless activation, the gene sprouty was identified as a negative regulator of FGFR function. Normally, signalling through the Breathless FGFR results in secondary branch formation at the tips of primary branch structures. In sprouty mutants however, supernumerary secondary branches are formed in non-tip cells—a phenotype similar to that observed when FGF signalling is hyperactive, suggesting that Sprouty acts to inhibit FGFR activity (Hacohen et al., 1998). Overexpression of sprouty during primary branch outgrowth causes the opposite effect, inhibiting the FGF inductive pathway and blocking all secondary branching. Thus, Sprouty antagonizes FGF signalling in the developing trachea. Moreover, expression of sprouty is induced by FGF signalling; therefore, the FGF pathway regulates the expression of its own antagonist (Hacohen et al., 1998).

While sprouty is expressed in tip cells in response to Branchless signalling, its inhibitory activity is eventually manifest in neighboring cells. It was proposed therefore, that Sprouty is a secreted antagonist to FGF, limiting the range of FGF responsiveness in the developing tracheal system (Hacohen et al., 1998). Subsequent genetic screens have revealed a broader function for Sprouty in inhibiting a variety of RTKs such as the Drosophila EGF receptor, Torso and sevenless receptors (Casci et al., 1999; Kramer et al., 1999; Reich et al., 1999), Although the biochemical mechanism by which Sprouty antagonizes receptor tyrosine kinase signalling is not fully understood, further genetic studies suggest that instead of acting extracellularly, Sprouty acts intracellularly to inhibit the Ras/MAPK pathway (Casci et al., 1999; Kramer et al., 1999; Reich et al., 1999).
Four Sprouty homologues have been identified in mouse and humans; gene expression patterns suggest that they are also induced by FGF signalling and act to regulate lung and vascular branching morphogenesis (de Maximy et al., 1999; Tefft et al., 1999; Minowada et al., 1999). Although targeted mutations of the mSpry genes have yet to be reported, it has been demonstrated that mSpry2 antisense oligonucleotides will increase branching when added to mouse embryonic lung cultures (Tefft et al., 1999), and that in vivo overexpression of mSpry2 results in low levels of lung branching morphogenesis (Mailleux et al., 2001). Thus, mSpry2 functions as a negative regulator of embryonic lung morphogenesis and growth, a process that depends on FGF signalling (see below). Similarly, mSpry may also negatively regulate angiogenesis, another form of branching morphogenesis. Overexpression of mSpry4 inhibits the branching and sprouting of small blood vessels in whole mouse embryo cultures (Lee et al., 2000). mSpry4 also inhibits FGF- and VEGF- induced cell proliferation, cell migration and MAPK phosphorylation in in vitro endothelial cell cultures. These phenotypes are rescued by a constitutively activated mutant Ras (Lee et al., 2000), suggesting that in the mouse, Sprouty also inhibits branching morphogenesis by antagonizing the FGFR/Ras/MAPK pathway upstream of Ras activation.

Dof (Downstream of FGF) was also identified in Drosophila as an intracellular protein essential for FGFR signal transduction (Vincent et al., 1998; Michelson et al., 1998a; Imam et al., 1999). Unlike Sprouty, which functions downstream of several other Drosophila receptor tyrosine kinases, Dof appears to act exclusively downstream of FGFR signalling. Dof is only expressed in cells that express the Heartless or Breathless FGF receptors, and Dof mutants show the combination of tracheal morphogenesis and mesoderm migration phenotypes associated with the breathless and heartless mutants, respectively (Vincent et al., 1998; Michelson et al., 1998a; Imam et al., 1999). Dof is a cytoplasmic protein that is essential for MAPK activation downstream of FGF receptors, and upstream of Ras (Vincent et al., 1998). The protein contains an ankyrin repeat, a coiled-coil structure and many tyrosines within peptide environments that suggest that, if phosphorylated, they could act as binding sites for the SH2 domains of proteins such as Grb2/drk, Csw/Shp2, Ras-GAP and P13-kinase (Vincent et al., 1998). Therefore Dof may act as an adapter molecule which functions to recruit signalling components specifically to the FGFR. To date, other proteins with significant homology to Dof have not been
identified, although the vertebrate adapter protein FRS2 may serve an analogous function (Kouhara et al., 1997).

**Mesoderm morphogenesis:**

In *Drosophila* embryogenesis, mesoderm is formed from a broad band of epithelial cells on the ventral side of the blastoderm (reviewed in Leptin et al., 1992). The beginning of mesoderm development is marked by the formation of an indentation in the ventral blastoderm called the ventral furrow. Ventral furrow formation is the first morphogenetic event of *Drosophila* gastrulation. The mesoderm then continues to invaginate as an intact epithelial layer. This process is driven by cell shape changes alone, and is under the control of the transcription factors Twist and Snail which are expressed in the ventral blastoderm prior to furrow formation. After the mesoderm has fully internalized it loses its epithelial structure; individual cells disperse, migrate and spread out along the underlying ectoderm to form the mesodermal germ layer.

Heartless is the second *Drosophila* FGFR homologue; it is expressed throughout the early embryonic mesoderm as it invaginates at the ventral furrow, and it is required immediately after mesoderm internalization (Beiman et al., 1996; Gisselbrecht et al., 1996). In *heartless* mutants, the invaginated mesoderm primordium fails to make contact with the ectoderm and to spread out as a single-cell layer; instead, cells remain adhering to one another in a multi-layered cluster near the site of invagination (Beiman et al., 1996; Gisselbrecht et al., 1996). Although current models of FGFR signalling suggest that an FGF should be produced at gastrulation to induce mesoderm spreading, no candidate ligand for the Heartless receptor has yet been identified. In *heartless* mutants, mesodermal cells only begin to disperse along the underlying ectoderm after a long delay, and as a result, mesodermal patterning is abnormal: dorsal mesoderm elements such as the heart and visceral mesoderm are missing, and dorsal somatic muscles are reduced and irregularly arranged (Beiman et al., 1996; Gisselbrecht et al., 1996). Decapentaplegic (Dpp) is a TGFβ homologue that is expressed in the dorsal ectoderm; in WT embryos, Dpp signals to the underlying mesoderm to specify dorsal mesodermal fates such as the heart, visceral mesoderm and dorsal somatic muscles. It is believed that patterning defects observed in *heartless* mutants result in part from defective morphogenesis:
mesodermal cells fail to reach the dorsal ectoderm and thus fail to receive Dpp inductive cues (Michelson et al., 1998b; Beiman et al., 1996; Shishido et al., 1997).

In both *Xenopus* and zebrafish, one of the main consequences of inhibiting FGF signalling is also a disruption in the morphogenesis of newly induced mesodermal cells at the marginal zone (Isaacs et al., 1994; Griffin et al., 1995). In *Xenopus* embryos injected with a dominant negative FGF receptor construct, the blastopore fails to close at the end of gastrulation, and dorsal mesoderm spreads laterally around the blastopore instead of extending anteriorly to form a normal axis (Isaacs et al., 1994). Interestingly, a low dose injection of the dnFGF receptor into the ventral side of a *Xenopus* embryo down-regulates *Xbra* expression but has very little effect of development (Isaacs et al., 1994). However, a similar dose injected into the dorsal side recapitulates the full posterior truncated phenotype as described by Amaya et al (1991), as a result of abnormal cell movements by the dorsal mesoderm (Isaacs et al., 1994). This demonstrates that the most sensitive requirement for FGF and Xbra activity is on the dorsal side of the embryo, where convergent extension activity of the involuting mesoderm is greatest (Keller and Danilchik, 1988). The phenotype after eFGF over-expression in zebrafish is also consistent with a role for FGF signalling in regulating morphogenesis (Griffin et al., 1995). In these embryos the hypoblast no longer converges onto the dorsal side, but instead convergence extension movement is hyper-activated and multiple foci of convergence extension develop throughout the embryo.

Given the intimate relationship between morphogenesis and patterning during gastrulation, it is tempting to suggest that the reduction of trunk and tail structures in dnFGFR expressing embryos is a direct consequence of abnormal mesodermal cell movements. For example, transplantation experiments in the zebrafish have shown that margin cells just prior to gastrulation are not yet committed to a mesodermal germ-layer fate, even though they express the mesodermal marker *ntl* (Schulte-Merker et al., 1994b), and have been fate mapped to give rise to mesodermal and endodermal derivatives (Warga and Kimmel, 1990). Margin cells transplanted to the animal pole region, which gives rise exclusively to ectodermal derivatives, will take on the fate of their new position (Ho, 1992). Thus, the position or environment that a cell is ‘allocated’ to, as a result of the morphogenetic movements that occur during gastrulation, ultimately plays a very important role in cell fate determination. Presumably, any defect that interferes with the
normal gastrulation movements would have drastic effects upon patterning of the embryo.

As discussed, it has been demonstrated that the T-box genes *spadetail* and *Brachyury* play pivotal roles downstream of FGF signalling, in regulating the convergence extension movements of mesodermal cells at gastrulation (Kimmel et al., 1989; Ho and Kane, 1990; Conlon et al., 1996; Conlon and Smith, 1999). In particular, *spt* function is required very specifically for the convergence movements of prospective trunk paraxial mesodermal cells towards the zebrafish midline (Kimmel et al., 1989; Ho and Kane, 1990). During convergence and extension, mesodermal cells associate closely with their neighbors: cell attachments must be formed and subsequently released as intercalating cells move past each other in directional movements towards the dorsal midline (Keller et al., 1992). The regulation of intercellular adhesion is thus likely to play an important role in mesoderm cell behaviour. In particular, the expression of the cell adhesion molecule Paraxial Protocadherin (PAPC) is believed to function downstream of Spadetail during zebrafish gastrulation: the patterns of *PAPC* and *spt* expression within paraxial mesoderm precursors are almost indistinguishable, and PAPC expression in the trunk depends on *spt* activity (Yamamoto et al., 1998). Moreover, PAPC may mediate the effects of *spt* on cell movements, as embryos expressing a dominant negative PAPC construct phenocopy *spt* mutants to some degree, and demonstrate inhibited convergence movements of paraxial mesoderm cells towards the dorsal midline (Yamamoto et al., 1998). In Drosophila gastrulation, it is also believed that a switch in intercellular adhesion molecules, from E-cadherin to N-cadherin, might play a role at the ventral furrow in the transition to a mesenchymal cell type (Oda et al., 1998).

**Mouse gastrulation and mesoderm formation:**

In the mouse, less is known about the morphogenetic processes or inductive interactions that regulate the recruitment, patterning and movement of mesoderm progenitor cells at gastrulation. However, comparisons of the fate maps of *Xenopus*, zebrafish, mouse and chick gastrulae show many similarities in the regionalization of progenitor populations and organizing centers (see Tam and Behringer, 1997). This
suggests that the molecular mechanisms which pattern and shape that gastrulating embryo may be conserved across vertebrate species.

Prior to gastrulation, the embryonic portion of the mouse conceptus exists as a cup-shaped undifferentiated columnar epithelial sheet known as the primitive ectoderm, or epiblast (Figure 2). At this stage of development, called the egg cylinder, the epiblast surrounds an embryonic space known as the proamniotic cavity, and a single layer of cells known as the visceral endoderm covers the outer surface of the epiblast. The visceral endoderm is not destined to form part of the fetus, but rather will contribute to extraembryonic membranes. Similarly, the polar trophectoderm derived- extraembryonic ectoderm layer, which develops at the proximal end of the egg cylinder adjacent to the epiblast, will also contribute to extraembryonic lineages. At some point prior to the onset of gastrulation, the anterior-posterior (A-P) axis of the embryo is specified. Although morphologically indiscernible, A-P specification is apparent on a molecular level via the expression of a number of genes within the visceral endoderm and epiblast of the egg cylinder. Hex and HesX1 expression, for example, are localized to the anterior visceral endoderm, while Evx1, Fgf8, Goosecoid and nodal are expressed in the posterior epiblast prior to gastrulation (Thomas and Beddington, 1996; Thomas et al., 1998; Dush and Martin, 1992; Crossley and Martin, 1995; Varlet et al., 1997). However, the location (or existence) of a Nieuwkoop center equivalent in the mouse, and the mechanisms by which A-P axis specification occurs remain poorly understood.

Gastrulation begins around embryonic day 6.5 (E6.5), as manifested by the formation of the primitive streak. Concerted cell growth and proliferation within the epiblast layer progressively displaces epiblast cells towards the posterior end of the embryo (Lawson et al., 1991; Lawson and Pedersen, 1992), resulting in an accumulation of cells in the proximal posterior epiblast. This thickening in the posterior epiblast layer is called the primitive streak - a transient embryonic structure that extends along the posterior midline of the embryo (Figure 2). At the primitive streak, recruited epiblast cells undergo an epithelial to mesenchymal transition (EMT) and then ingress between the epiblast and visceral endoderm to become incorporated into either the mesoderm or the definitive endodermal germ layers. As cells located more distally in the epiblast move into the streak, the primitive streak lengthens until ultimately, it occupies the entire proximal-distal length of the embryo. As mesoderm progenitor cells exit the streak, some
move proximally and cross the boundary between the epiblast and the extraembryonic region, where they develop into the extraembryonic mesoderm. The remainder of the mesodermal cells spread laterally towards the anterior end of the embryo, between the epiblast basal surface and the visceral endoderm, forming what are referred to as “mesodermal wings”. Cells that become embryonic endoderm incorporate into and replace the outer visceral endoderm layer. Finally, cells that remain in the epiblast are fated to become neuroectoderm and epidermis (Lawson and Pedersen, 1987; Tam, 1989; Lawson et al., 1991).

Fate mapping studies of the late primitive streak demonstrate that the site of progenitor-cell ingresson through the streak determines the spatial distribution and the fate of mesodermal cells at gastrulation (Figure 2). Axial mesoderm, which will form the notochord, arises from the node at the anterior end the primitive streak; paraxial mesoderm, which comprises cephalic mesoderm anteriorly and somitic mesoderm throughout the trunk and tail, arises from the anterior segment of the late primitive streak; lateral mesoderm, which will form intermediate and lateral plate mesodermal populations, arises from the mid-segment of the streak; and finally extraembryonic mesoderm, which will contribute to extra-embryonic membranes, arises from the most posterior segment of the late primitive streak (Tam and Beddington, 1987; Lawson et al., 1991; Lawson and Pedersen, 1992). Similarly, the temporal order of progenitor cell ingresson through the primitive streak directly influences the allocation of mesodermal cells within the embryo. Mapping of the developing mesoderm reveals that cells destined to form the extraembryonic mesoderm are the major constituent of the nascent mesodermal wings; that precursors for cranial and heart mesoderm are present in the mesodermal layer of the mid-streak stage embryo; and that progenitors of trunk paraxial and lateral mesoderm form the bulk of the embryonic mesoderm in the late-streak embryo (Parameswaran and Tam, 1995; Tam et al., 1997; Kinder et al., 1999).
Figure 2. Gastrulating mouse embryo.

(A) Schematic depiction of a gastrulating mouse embryo from E6.0 through to E7.5 of development. Only the embryonic portion of the conceptus has been shown. The mesodermal germ layer is formed as epiblast cells ingress through the primitive streak (marked in red), which forms at the posterior end of the embryo.

(B) A “flattened” diagram of the late-primitive streak, depicting that the anteroposterior site of progenitor-cell ingestion through the streak will determine the spatial distribution and the fate of mesodermal cells at gastrulation.
Figure 2:
FGF and FGFR expression at gastrulation:

In the mouse, the expression patterns of known Fgfs and Fgfrs are consistent with a potential role for FGF signalling in the recruitment, morphogenesis and patterning of mesoderm at gastrulation. In particular, gene expression studies have demonstrated that transcripts for 5 of the 23 known FGFs (Fgf3, Fgf4, Fgf5, Fgf8 and Fgf17) are expressed in the mouse embryo around gastrulation. Fgf3 is expressed at high levels in mesodermal cells as they exit and migrate away from the primitive streak; Fgf3 is not expressed in the epiblast layer (Wilkinson et al., 1988; Niswander and Martin, 1992). In contrast, Fgf4 is initially expressed throughout the epiblast layer and, just prior to gastrulation, Fgf4 expression decreases in the epiblast and becomes restricted to the forming primitive streak (Niswander and Martin, 1992). Fgf4 expression is found only in the distal 2/3 of the primitive streak and adjacent ectodermal cells, and Fgf4 expression is lost in the nascent mesoderm. Fgf4 is therefore expressed in the region of the streak fated to give rise to axial, paraxial and lateral plate mesoderm (Niswander and Martin, 1992). Fgf5 is expressed prior to gastrulation throughout the epiblast and visceral endoderm layers (Haub and Goldfarb, 1991; Hebert et al., 1991). Fgf5 expression in the embryonic ectoderm and endoderm persists throughout early gastrulation, with intensity of expression increasing in a proximal to distal gradient; Fgf5 is not expressed in mesodermal cells which migrate away from the primitive streak (Haub and Goldfarb, 1991; Hebert et al., 1991). Fgf8 expression is also detected in the epiblast and visceral endoderm prior to gastrulation, and is localized to the side of the embryo where the primitive streak will form, starting from the embryonic-extraembryonic junction and extending distally along approximately two-thirds of the length of the embryo (Crossley and Martin, 1995; Mahmood et al., 1995). Fgf8 continues to be expressed in a similar pattern as gastrulation proceeds, with Fgf8 expression restricted to the epithelial component of the primitive streak and the embryonic ectoderm cells immediately lateral to the streak. Fgf8 expression is more intense in the proximal part of the streak and decreases distally, and Fgf8 expression is lost as mesodermal cells exit the primitive streak (Crossley and Martin, 1995; Mahmood et al., 1995). Late in gastrulation, the proximodistal gradient of Fgf8 expression is lost: Fgf8 becomes expressed throughout the length of the streak, and expression is maintained in the nascent mesoderm. Fgf17 is also
expressed during gastrulation in a similar pattern to that of Fgf8, but at a reduced level (Maruoka et al., 1998).

In situ hybridization studies have also shown that the FGF receptors Fgfr1 and Fgfr2 are expressed in gastrulation-staged embryos (Orr-Urteger et al., 1991; Yamaguchi et al., 1992). Fgfr1 is expressed throughout the primitive ectoderm prior to gastrulation, and as gastrulation proceeds Fgfr1 expression concentrates around the primitive streak. By mid-streak stages, Fgfr1 is expressed predominantly in the posterior mesoderm lateral to the primitive streak. Fgfr1 expression is maintained in the mesodermal wings, but is not detected in extraembryonic mesodermal populations. Headfold stage embryos show strong Fgfr1 expression in both the neuroectoderm and the developing paraxial and pre-somatic mesoderm (Orr-Urteger et al., 1991; Yamaguchi et al., 1992). Fgfr2 expression is confined to the extraembryonic ectoderm both prior to and during early gastrulation. Fgfr2 transcripts are not detected in the embryo proper until late-streak stages when Fgfr2 expression is detected in the forming neuroectoderm (Orr-Urteger et al., 1991). Expression of Fgfr3 and Fgfr4 have not been detected in the gastrulating mouse embryo (Peters et al., 1993; Stark et al., 1991).

FGF and FGFR loss of function phenotypes:

As discussed, studies in both Xenopus and zebrafish indicate that the FGF signalling pathway is essential for the specification and morphogenetic movements of mesoderm at gastrulation. However, such studies have not yet provided a detailed understanding of the roles of individual FGFs and FGFRs in vertebrate development. One approach towards investigating this issue has been to mutate individual Fgf and Fgfr loci through targeted gene disruption in the mouse. Targeted mutation of Fgf3 (Mansour et al., 1993), Fgf5 (Hebert et al., 1994), and Fgf17 (J.Xu and D. Ornitz, unpubl. data as cited in Sun et al., 1999) have revealed that individually, these genes are not required for gastrulation: null mutant homozygotes are viable and fertile. However, mutational analyses have revealed later developmental functions for these genes: targeted disruption of Fgf3 results in the abnormal development of the tail and inner ear (Mansour et al., 1993), and Fgf5 mutant mice present an angora phenotype (Hebert et al., 1994).
Targeted disruption of Fgf4 results in abortive development after blastocyst implantation (Feldman et al., 1995). While this study has implicated a role for FGF4 in the survival and growth of the inner cell mass during implantation development, lethality occurs too early to address the role for FGF4 signalling at gastrulation. Similarly, embryos homozygous for a targeted mutation of the Fgfr2 locus also die a few hours after implantation (Arman et al., 1998). Since Fgf2 is expressed predominantly in the trophectoderm layer of the implanting blastocyst (Arman et al., 1998), it has been suggested that FGF4 signals transmitted by FGFR2 are required for the normal development of the extraembryonic trophoblast lineage (Arman et al., 1999). However, mice which are homozygous for a second, independently targeted mutation of Fgfr2 survive beyond implantation and egg cylinder formation; these embryos die at 10-11 days of gestation because of placental deficiencies (Xu et al., 1998b). These Fgfr2-/- embryos do not form limb buds, and it was demonstrated that loss of FGFR2 specifically blocks an epithelial-mesenchymal regulation loop between FGF8 and FGF10 signalling that is essential for limb induction (Xu et al., 1998b). Interestingly, when FGFR2 function is rescued in the trophectoderm and primitive endoderm lineages (by generating chimeric embryos from Fgfr2-/- embryonic stem cells and WT tetraploid embryos), Fgfr2 mutant embryos survive until term (Arman et al., 1999). This demonstrates that the early lethality of both targeted Fgfr2 mutations was indeed caused by abnormal development of the extraembryonic lineages. However, Fgfr2 tetraploid chimeras develop with a complete absence of limb and lung formation (Arman et al., 1999), a phenotype almost identical to mice homozygous for targeted mutations in Fgf10 (Min et al., 1998; Sekine et al., 1999). Therefore, in the embryo proper, interactions between FGF10 and FGFR2 appear to be essential for normal limb outgrowth and lung branching morphogenesis.

The first indication that FGF signalling played a fundamental role during mouse gastrulation came from the targeted disruption of Fgfr1 (Yamaguchi et al., 1994; Deng et al., 1994). Embryos homozygous for mutations in Fgfr1 die between 7.5 and 9.5 days of development, with defects first manifesting themselves at the onset of gastrulation. Fgfr1-/- embryos are developmentally retarded and misshapen relative to their WT littermates, suggesting an early mitogenic role for FGFR1. Although Fgfr1-/- embryos initiate gastrulation and primitive streak formation occurs relatively normally, epiblast and mesodermal cells accumulate in the streak causing a thickening of the posterior primitive
streak. Less severely affected \textit{Fgfr1} -/- embryos develop further and form abnormal heart tubes and unusually broad anterior midline structures; they also lack somites, have a reduction in cephalic mesenchyme underlying the neural folds, and do not elongate along the anteroposterior axis (Yamaguchi et al., 1994; Deng et al., 1994). Analysis of \textit{mox-1} expression, a marker of posterior paraxial, presomitic and somitic mesoderm (Candia et al., 1992), confirms that somites are not formed in \textit{Fgfr1} mutants and that the paraxial mesoderm population is severely reduced (Yamaguchi et al., 1994). \textit{Brachyury}, a marker of primitive streak, node and axial mesoderm populations (Wilkinson et al., 1990; Herrmann, 1991), shows intense expression in \textit{Fgfr1} mutants which correlates well with the observed expansion of anterior midline structures and of the axial mesoderm population (Yamaguchi et al., 1994; Deng et al., 1994). Thus, results of morphological and molecular analyses of \textit{Fgfr1} -/- embryos suggest that FGFR1 signalling may be playing a role in mesodermal patterning at gastrulation, and in directing the movement of mesodermal cells out of the primitive streak. The function of FGFR1 signalling beyond its role at gastrulation has been studied using a series of hypomorphic \textit{Fgfr1} alleles. Hypomorphic alleles cause posterior truncations and homeotic transformations in the vertebral column, providing genetic evidence for the involvement of FGFR1 signalling in the regulation of Hox gene activity and A-P patterning (Partanen et al., 1998).

Targeted disruption of \textit{Fgf8} results in a similar phenotype to that of \textit{Fgfr1} -/- embryos (Sun et al., 1999). To date, FGF4 and FGF8 are the only known FGFs that are required for early embryonic development. As discussed, \textit{Fgf4} is co-expressed with \textit{Fgf8} in the primitive streak of gastrulating mouse embryos; unfortunately, \textit{Fgf4} -/- embryos die peri-implantation and thus too early to assess a role for FGF4 at gastrulation. However, embryos homozygous for null alleles of \textit{Fgf8} lose expression of \textit{Fgf4} in the streak and in the absence of both FGF8 and FGF4, gastrulation is severely disrupted and embryos die prior to E9.5 (Sun et al., 1999). In these \textit{Fgf8} -/- embryos, epiblast cells move into the primitive streak and undergo an epithelial-to-mesenchymal transition, but most cells then fail to move away from the streak. As a consequence, no embryonic mesoderm- or endoderm-derived tissues develop (Sun et al., 1999). The morphogenesis of extraembryonic tissues occurs normally in \textit{Fgf8} mutant embryos. Interestingly, patterning of the prospective neuroectoderm is greatly perturbed in \textit{Fgf8} -/- embryos, with an expansion in the expression domains of anterior neuroectoderm markers at the
expense of posterior neuroectoderm markers (Sun et al., 1999). Although it has been suggested that neuroectodermal patterning defects in Fgf8 mutant embryos are secondary to defects in node and endoderm morphogenesis (Sun et al., 1999), results would also be consistent with the demonstrated role for FGFR signalling in anteroposterior patterning (Partanen et al., 1998) - a function for FGF signalling conserved among vertebrates (Cox and Hemmati-Brivanlou, 1995; Kengaku and Okamoto, 1995; Lamb and Harland, 1995).

Activation of the Ras/MAPK pathway downstream of RTK signalling is also required for normal gastrulation in the mouse, as demonstrated by targeted mutations of FRS2α and Shp2. Embryos lacking FRS2α die at gastrulation and show severe defects in cell migration through the primitive streak (Hadari and Shlessinger, unpubl. data). This phenotype is very similar to that of Fgf8 mutant embryos (Sun et al., 1999), and is consistent with the hypothesis that FRS2 is essential for the activation of the Ras/MAPK pathway downstream of FGFR signalling, and for the formation and morphogenesis of mesoderm at gastrulation.

Shp2 is also required for embryonic development, as mice homozygous for a mutant Shp2 allele die at mid-gestation (Saxton et al., 1997). Shp2 mutant embryos fail to gastrulate properly and show defects in node and notochord formation as well as axial elongation (Saxton et al., 1997). Since Shp2 -/- gastrulation defects are comparable to those observed with the Fgfr1 mutant phenotype (Yamaguchi et al., 1994; Deng et al., 1994), it has been suggested that the phenotype of Shp2 mutant embryos may result from defects in FGFR signalling (Saxton et al., 1997). Moreover, it was demonstrated that Shp2 is required for full and sustained activation of the MAP kinase pathway following FGF stimulation of Shp2 -/- embryonic fibroblasts (Saxton et al., 1997).

Although targeted mutation of the Sprouty genes have not been reported in the mouse, the addition of exogenous FGF4 to late-primitive streak staged embryos has been shown to induce rapid up-regulation of mSpry2 expression, resulting in gastrulation defects (Davidson et al., 2000). Shortly after FGF treatment, mSpry2 expression, which is normally confined to the primitive streak (Minowada et al., 1999), was observed ectopically throughout the epiblast, and its activity remained elevated after a prolonged period of in vitro development (Davidson et al., 2000). After 24 hours of culture, FGF treated embryos demonstrated aberrant development of the primitive streak and paraxial mesoderm: cells accumulated in the primitive streak, an excess amount of posterior
paraxial and tail bud mesoderm was formed, but there was a deficiency in trunk paraxial mesoderm formation. Also, axial mesoderm appeared broader and more diffuse than in control embryos (Davidson et al., 2000). Since the phenotype of FGF treated embryos closely resembled FGFR1 null mutant embryos, it was proposed that the major phenotypic consequences of FGF treatment on embryonic development may be due to the widespread inhibition FGF signalling as a result of the ectopic activation of mSpry2 (Davidson et al., 2000). Thus, in the mouse, overexpression of mSpry appears to be associated with both mesodermal patterning and morphogenetic defects, consistent with a conserved role for mSpry2 as an antagonist of FGFR signalling.

In conclusion therefore, mutational analyses of the Fgf and Fgfr genes expressed during early mouse embryogenesis suggest that development of the extra-embryonic trophoderm lineages is dependent upon FGF4 signalling through FGFR2, and that in the embryo proper, FGFR2 and FGF10 interactions are essential for limb formation and lung branching morphogenesis. FGFR1, however, appears to be essential for the patterning and morphogenesis of mesoderm at gastrulation, presumably downstream of FGF4 or FGF8 signals at the primitive streak, and upstream of FRS2, Shp2 and activation of the Ras/MAPK signalling cascade. To some degree, the phenotype of Fgfr1 -/- embryos resembles that of Xenopus and zebrafish embryos injected with dnFGFR constructs: Fgfr1 mutant embryos form head fold-like structures yet display posterior patterning defects with reduced paraxial mesoderm formation and truncations of the posterior body axis (Yamaguchi et al., 1994; Deng et al., 1994). However, although inhibition of FGF signalling in Xenopus and zebrafish embryos allows for the formation of relatively normal head structures, the head folds observed in Fgfr1 mutant embryos were abnormally formed with a scarcity of underlying cephalic mesoderm. Indeed, analysis of Fgf8 mutants revealed a requirement for FGF signalling in the morphogenesis of all embryonic mesoderm and endodermal populations (not just trunk and tail structures). Moreover, FGF8/FGFR1 signalling is not absolutely required for Brachyury expression at gastrulation; in fact, Brachyury expression domains appear expanded in Fgfr1 mutants. Thus, while FGF signalling appears to function in the morphogenetic movement of mesoderm progenitors across vertebrate species, a conserved role for FGF signalling in mesodermal patterning remains to be determined.
Outline of Thesis:

The primary focus of my thesis has been to further investigate the role for FGFR1 signalling during mouse gastrulation. As discussed, embryological and genetic studies in the mouse have indicated an important role for FGF8, FGFR1 and components of the FGFR signal transduction pathway in the proper morphogenesis and patterning of mesoderm at the primitive streak. However, relatively little is known about the cellular mechanisms and the downstream targets of FGF signalling which ultimately regulate both the movement of progenitor cells through the primitive streak, and the specification of mesodermal cell fate. To further characterize the role for FGFR1 signalling at gastrulation, I have performed a chimeric analysis of FGFR1 function. A population of homozygous Fgfr1 mutant embryonic stem (ES) cells was established and aggregated with WT diploid morulae to generate chimeric embryos. Careful analysis of the behaviour and distribution of Fgfr1 -/- cells within chimeric embryos revealed a defect in mutant cell migration through the primitive streak; as a result, few Fgfr1 -/- cells contributed to anterior mesoderm and endodermal lineages. Fgfr1 -/- progenitor cells showed defects in undergoing epithelial to mesenchymal transitions and in moving away from the primitive streak. This indicated a potential role for FGFR1 signalling in regulating aspects of cell adhesion and/or cell migration.

I have developed a novel in vitro assay of mesoderm chemotaxis and cell migration that utilizes primary cultures of explanted embryonic tissue; this allowed physiologically relevant investigations into the morphogenetic movements of Fgfr1 -/- mesoderm at gastrulation. Although results indicate a possible chemotactic role for FGFR1 signalling at the streak, general cell migration defects were not observed in explanted Fgfr1 -/- mesoderm cultures. Rather, I demonstrate that FGFR1 signalling regulates both Snail and E-cadherin expression, and implicate abnormal intercellular adhesion in the failed EMT and aberrant morphogenesis observed in Fgfr1 mutant and chimeric analyses. More specifically, I show that Snail, which represses E-cadherin expression and is required for normal EMT at gastrulation (Hemavathy et al., 2000), is down-regulated in the streak of Fgfr1 -/- embryos. Furthermore, I show through immunohistochemical analysis of Fgfr1 mutant and chimeric embryos that E-cadherin expression is maintained in Fgfr1 -/- cells at the primitive streak. I propose that ectopic
E-cadherin expression provides a molecular explanation for the abnormal morphogenesis of Fgfrl -/- progenitor cells at gastrulation.

I also demonstrate that Fgfrl -/- cells, which had accumulated within the primitive streak of chimeric embryos, form ectopic neural tubes. This suggests that, in addition to regulating paraxial and axial mesoderm formation (Yamaguchi et al., 1994; Deng et al., 1994), FGFR1 may play an unexpected role in negatively regulating neural fate determination. I further establish a role for FGF signalling in mesoderm cell fate specification and show that members of the T-box gene family, which have been implicated in the specification of posterior and paraxial mesoderm, are positively regulated by FGFR1. Tbx6, which functions in promoting a paraxial mesoderm over neuroectoderm fate (Chapman and Papaioannou, 1998), is down-regulated in Fgfrl mutants; moreover, chimeric analyses of Tbx6 expression confirm that Tbx6 expression is positively regulated by FGFR1. Similarly, I show that Brachyury expression in the mid-primitive streak is also dependent upon FGFR1 signalling. I propose, therefore, that down-regulation of Brachyury and Tbx6 can explain both the absence of posterior and paraxial mesoderm in Fgfrl mutants, and the formation of ectopic neural tubes in the Fgfrl chimeras. I have also performed a screen for novel T-box genes that function at gastrulation. I describe the cloning and expression pattern of murine Eomesoderm, a new mouse T-box gene which has since been shown to function in trophoblast differentiation, and in the induction and morphogenesis of mesoderm at gastrulation (Russ et al., 2000).

Finally I demonstrate that FGFR1 indirectly regulates Wnt signalling activity at the primitive streak. I argue that ectopically expressed E-cadherin in Fgfrl -/- progenitor cells sequesters free β-catenin from its intracellular signalling pool and thus attenuates Wnt signal transduction. Furthermore, I show that forced down-regulation of E-cadherin in Fgfrl -/- explants can rescue endogenous Wnt signalling at the primitive streak. Results indicate a molecular link between FGF and Wnt signalling pathways at the primitive streak, and underscore the interdependent nature of morphogenesis and patterning at gastrulation.
Chimeric analysis of fibroblast growth factor receptor-1 (Fgfr1) function: a role for FGFR1 in morphogenetic movement through the primitive streak

Portions of this chapter appear in the following publication:

SUMMARY

Fibroblast growth factor (FGF) signalling has been implicated in the patterning of mesoderm and neural lineages during early vertebrate development. In the mouse, FGF receptor-1 (FGFR1) is expressed in an appropriate spatial and temporal manner to be orchestrating these functions. Mouse embryos homozygous for a mutated Fgfr1 allele (Fgfr1/-) die early in development, show abnormal growth and aberrant mesodermal patterning. We have performed a chimeric analysis to further study FGFR1 function in the morphogenesis and patterning of the mesodermal germ layer at gastrulation. At E9.5, Fgfr1/- cells showed a marked deficiency in their ability to contribute to the extra-embryonic, cephalic, heart, axial and paraxial mesoderm, and to the endoderm of chimeric embryos. Analysis at earlier stages of development revealed that Fgfr1/- cells accumulated within the primitive streak of chimeric embryos, and consequently failed to populate the anterior mesoderm and endodermal lineages at their inception. We suggest that the primary defect associated with the Fgfr1 mutation is a deficiency in the ability of epiblast cells to traverse the primitive streak. Fgfr1/- cells that accumulated within the primitive streak of chimeric embryos tended to form secondary neural tubes. These secondary neural tubes were entirely Fgfr1/- cell derived. The adoption of ectopic neural fate suggests that normal morphogenetic movement through the streak is essential not only for proper mesodermal patterning but also for correct determination of mesodermal/neuroectodermal cell fates.
INTRODUCTION

Recently, much attention has been directed toward possible roles for FGF signalling in early vertebrate development, especially with respect to the patterning of early mesodermal and neural lineages. Studies in Xenopus laevis have provided compelling evidence that FGF signalling is directly involved in both of these processes. Exogenous basic FGF, when added to animal cap explants, is able to induce mesoderm from tissue normally fated to become ectoderm (Kimelman and Kirschner, 1987; Paterno et al., 1989; Slack et al., 1989). The introduction of a dominant negative FGF receptor into Xenopus embryos at various stages of development has demonstrated that FGF signalling is required for the expression of several early mesodermal markers, for the induction of posterior and ventral mesoderm, and for proper mesodermal maintenance during gastrulation (Amaya et al., 1991; Amaya et al., 1993; Kroll and Amaya, 1996). Also, overexpression of embryonic FGF (eFGF) in Xenopus embryos has been shown to both upregulate and anteriorly extend the expression domains of posterior homeobox genes (Pownall et al., 1996). These and other recent findings suggest that FGF signalling may be involved in the proper patterning of the developing anterior-posterior axis (Cox and Hemmati-Brivanlou, 1995; Kengaku and Okamoto, 1995; Lamb and Harland, 1995).

In mouse embryogenesis, mesoderm induction and patterning are thought to occur at gastrulation. The beginning of gastrulation is manifested by the formation of the primitive streak at the posterior end of the embryo. In a process of highly integrated cell and tissue movements, cells delaminate through the primitive streak and emerge between the epiblast and visceral endoderm to form the mesodermal germ layer. Fate-mapping of the mouse epiblast suggests that cells emerging from different anterior-posterior levels of the streak adopt different fates (Tam, 1989; Lawson et al., 1991; Beddington et al., 1992; Parameswaran and Tam, 1995). FGFs 3, 4, 5 and 8 are expressed within the primitive streak in spatial and temporal patterns which would be consistent with a role for FGF signalling in mesoderm induction or in regulating processes of fate determination (Wilkinson et al., 1988; Haub and Goldfarb, 1991; Niswander and Martin, 1992; Hebert et al., 1991).

Mutational analyses of Fgf3 and Fgf5 have failed to show defects in early gastrulation (Mansour et al., 1993; Hebert et al., 1994). The apparent "expendable"
nature of FGF3 and FGF5 function in early embryonic development is likely due to redundancy in FGF signalling as there is cross-specificity in ligand-receptor interactions (Ornitz et al., 1996), and local co-expression of other FGFs may therefore compensate for the early losses of function (Givol and Yayon, 1992). Targeted disruption of Fgf4 results in abortive post-implantation development (Feldman et al., 1995). While this implicates a role for FGF4 in the survival and growth of the inner cell mass during post-implantation development, lethality occurs too early to address the role of FGF4 signalling in mesoderm induction and patterning.

Analysis of FGF function at the level of the receptor has proven more informative. Fgfr1, for example, is first expressed throughout the primitive ectoderm. In mid-streak staged embryos, Fgfr1 expression is concentrated in the posterior mesoderm lateral to the primitive streak and is maintained in the migrating mesodermal wings; headfold stage embryos show strong expression in both neuroectoderm and the developing paraxial and pre-somite mesoderm (Orr-Urtreger et al., 1991; Yamaguchi et al., 1992). Thus, Fgfr1 expression is consistent with a role for FGFs in mesoderm induction, neural induction, and somite formation and patterning. Targeted mutation of Fgfr1 results in embryonic lethality between day 7.5 and 9.5 of development, with defects first manifesting themselves at the onset of gastrulation (Yamaguchi et al., 1994; Deng et al., 1994). Retarded development of homozygous Fgfr1 mutant embryos has argued for an early mitogenic role for FGFR1, and thickening of the posterior streak suggested that Fgfr1 mutant cells were defective in migrating from the posterior primitive streak. Paraxial mesoderm of Fgfr1 mutant embryos was much reduced, yet axial mesoderm was still present and possibly expanded, suggesting that FGFR1 may be involved in the allocation of progenitor cells among alternative pathways of mesoderm differentiation (Yamaguchi et al., 1994; Deng et al., 1994). Although it was clear that FGFR1 may play a role in mesodermal patterning and perhaps cell migration away from the posterior streak, the phenotype was complex and it was difficult to distinguish primary defects associated with the Fgfr1 mutation from secondary defects resulting from grossly abnormal morphogenetic movements at gastrulation.

To further elucidate the primary modes of FGFR1 function, a chimeric analysis has been performed. A population of homozygous Fgfr1 mutant embryonic stem (ES) cells was established and aggregated with wild-type (WT) diploid morulae to generate
chimeric embryos. It was expected that WT cell contribution to chimeric embryos would rescue the phenotypic abnormalities and early lethality associated with the \textit{Fgfr1} mutation, thus allowing analysis of FGFR1 function in cell lineages which were abnormal or absent in \textit{Fgfr1-/-} embryos. Cell autonomous roles for FGFR1 in either the ontogeny of a particular tissue, cell proliferation or in cell migration were expected to become manifested by the local absence, under-representation or accumulation of \textit{Fgfr1-/-} cells, respectively. Careful analysis of the behaviour and distribution of \textit{Fgfr1-/-} cells within chimeric embryos revealed that defects in mesodermal patterning may be secondary to an initial deficiency in the ability of mutant cells to traverse the primitive streak. The chimeric analysis also revealed that \textit{Fgfr1} mutant cells that have accumulated in the streak display an inherent tendency to form secondary neural tubes. A role for FGFR1 in regulating the morphogenetic movements that are thought to mediate cell fate determination and lineage restriction at gastrulation, is discussed.

\textit{RESULTS}

\underline{Isolation of \textit{lacZ} marked ES cell lines}

ES cell lines were generated from the ICMs of blastocysts isolated from crosses of \textit{Fgfr1+/-}; ROSA26 \textit{lacZ+/-} animals (Fig. 1A). \textit{lacZ} expression via the ROSA26 transgene is ubiquitous throughout early to mid gestational development (Friedrich and Soriano, 1991), and is therefore a useful marker of mutant cells in chimeric embryos.

From approximately 90 blastocysts cultured, 9 ES cell lines were recovered. It was expected that 3/4 of the isolated cell lines would be positive for \textit{lacZ} expression (as determined by 8-galactosidase staining), and that 1/4 of the cell lines would be homozygous for the \textit{Fgfr1} mutation. Southern blot analysis revealed that 7 ES cell lines were homozygous for the \textit{Fgfr1} mutation, 2 cell lines were \textit{Fgfr1+/-}, and that no \textit{+/-} cell lines were recovered (Fig. 1B, C). Therefore, in deriving ES cell lines from primary blastocyst cultures, there appeared to be a positive selection for \textit{Fgfr1-/-} cell lines. This selection for homozygous \textit{Fgfr1} mutant cells was statistically significant (p<0.005; Fig. 1C).
Figure 1. Generation and characterization of ES cell lines.

(A) Strategy for generating ES cell lines from primary blastocyst cultures. Δtmk refers to the Fgfr1 mutant allele. (B) Southern blot analysis of isolated ES cell lines; 5' and 3' Fgfr1 probes were as previously described (Yamaguchi et al., 1994). (C) Summary of the Fgfr1 genotypes and lacZ phenotypes of isolated ES cell lines.
Figure 1:

A

B

C

- 43 -
129 Fgfrl+/- mice were initially crossed with ROSA26 mice of a mixed background before generating ES cell lines, therefore the Fgfrl mutant allele was carried on a 129-derived chromosome. Since isolation of ES cells is more efficient on a 129 background (McWhir et al., 1996; and references therein), Fgfrl may be linked to a 129-specific polymorphism which promotes the maintenance, and hence derivation of Fgfrl-/- ES cell lines. Another explanation for the positive selection of homozygous Fgfrl mutant lines is that FGFR1 signalling may promote differentiation from a pluripotent state. In this case, Fgfrl-/- ES progenitors would have a selective advantage in maintaining pluripotency and hence in being isolated as an undifferentiated cell line.

Fgfrl-/- ↔ tetraploid CD-1 chimeras were generated using cell lines B2, C5B, C15 and A3, in order to assess the developmental potential of these ES cell lines. Tetraploid chimeras were examined at E8.5 and E9.5 of development. At E8.5, chimeras were developmentally delayed in comparison to similarly staged WT embryos. These embryos showed abnormal thickening of the posterior primitive streak, poorly developed head folds, an absence of somites, and ruffling of the extraembryonic endoderm. At E9.5, chimeras were poorly developed and showed an expanded midline, an absence of somites, small malformed head folds, and kinking of the posterior neural tube. All of these properties are characteristic of homozygous Fgfrl mutant embryos (Yamaguchi et al., 1994), indicating that the developmental potential of the Fgfrl-/- cell lines had not been altered. Furthermore, it has been shown that the trophectoderm and visceral endoderm lineages of tetraploid embryo ↔ ES cell chimeras are strictly embryo-derived (Nagy et al., 1993). Since Fgfrl-/- ↔ tetraploid CD-1 chimeras phenocopy homozygous Fgfrl mutant embryos, then defects associated with the Fgfrl mutation are not caused by abnormal development of these tissues. Therefore, all defects observed in the Fgfrl homozygous mutant embryos can be explained by the abnormal development, morphogenesis and patterning of the extraembryonic mesoderm and definitive germ layers.

Cell lines B2 and C5B (which expressed the ROSA26 lacZ transgene) were used in subsequent diploid chimeric analyses. Since both cell lines produced similar results, data for these analyses have been pooled. Tetraploid aggregations were also performed with ES cell line C16 (Fgfrl+/-, lacZ positive) in order to test its pluripotency. Normal
C16 derived embryos were recovered at E13.5, and therefore this Fgfr1 +/- cell line was used as a positive control in subsequent diploid chimeric analyses.

Whole-mount analysis of β-galactosidase stained chimeric embryos at E9.5 to E10.5

Diploid chimeric embryos were generated by aggregating approximately 8 Fgfr1 +/- or Fgfr1 -/- ES cells with wild-type (WT) CD-1 embryos. At E9.5, Fgfr1 +/- ↔ CD-1 chimeras showed great variations in the extent of ES cell contribution to the embryo, with values ranging from <5% to 100% ES cell-derived, as estimated by β-galactosidase staining (Fig. 2A, C, E). A similar range in ES contribution was observed in E9.5 Fgfr1 -/- ↔ CD-1 chimeras (Fig. 2B, D, F). This argues against a broad requirement for FGFR1 in early mitogenesis, since a major impairment in growth and proliferation would have been expected to reduce the overall contribution of Fgfr1 -/- cells to chimeric embryos.

Some phenotypically normal Fgfr1 -/- ↔ CD-1 chimeras were recovered at E10.5 (Fig. 3), indicating that WT cells could rescue the embryonic lethality and gastrulation defects associated with homozygous Fgfr1 mutant embryos. Viable chimeric embryos were also recovered at E16.5, however a complete analysis at late gestation was beyond the scope of this study. When the overall mutant cell contribution exceeded about 50%, Fgfr1 -/- ↔ CD-1 chimeric embryos developed gross phenotypic defects which ranged from apparent posterior neural tube duplications and ruffling of the dorsal ectoderm/neuroepithelium to posterior truncations, abnormal heart development, and failure in anterior neural tube closure (Fig. 3C-G). Similar defects were not observed in Fgfr1 +/- ↔ CD-1 chimeras (data not shown). Chimeric embryos that were completely Fgfr1 -/- cell-derived phenocopied homozygous Fgfr1 mutant embryos.

Fgfr1 mutant cells show deficiencies in contributing to anterior mesoderm and endoderm lineages

Whole mount β-galactosidase staining of Fgfr1 -/- ↔ CD-1 chimeric embryos revealed striking patterns in mutant cell dispersal: Fgfr1 -/- cells were deficient in contributing to the heart, and showed a strong tendency to accumulate within the tail of
Figure 2. Whole-mount analysis of ES-derived cell contribution to chimeric embryos at E9.5.

Fgfrl-/- or Fgfrl+/+ cells were visualized by β-galactosidase staining. Fgfrl +/- ↔ CD-1 chimeras (A,C,E) and Fgfrl -/- ↔ CD-1 chimeras (B,D,F) show similar ranges in ES-derived cell contribution. Fgfrl -/- ↔ CD-1 chimeras show obvious deficiencies in mutant cell contribution to the heart (arrow in D). Chimeric embryos derived completely from Fgfrl +/- cells appear phenotypically normal (E), while chimeras derived entirely from Fgfrl -/- cells phenocopy homozygous Fgfrl mutant embryos (F). Scale bars, 200 μm.
Figure 2:
Figure 3. Whole mount analysis of β-galactosidase stained Fgfr1-/– ↔ CD-1 chimeras at E10.5.

(A,B) Fgfr1-/– ↔ CD-1 chimeras with very low ES-derived cell contribution show caudal accumulations of Fgfr1-/– cells (arrows) but are otherwise normal. (C,D,E) As the ES-derived cell contribution to chimeric embryos increases, deficiencies in Fgfr1-/– contribution to the heart (arrow in C), apparent duplications of the posterior neural tube (arrowheads in D), and distinct aggregations of Fgfr1-/– cells within nasal placode (arrow in E) become obvious. (F,G) Grossly abnormal embryos obtained when contribution of mutant cells exceed 50%. Scale bars, 200 μm.
Figure 3:
chimeric embryos (Figs 2D, 3A–C). To further investigate the distribution and behaviour of mutant cells within a chimeric background, 22 E9.5-10.5 Fgfr1+/− ↔ CD-1 chimeric embryos, which showed varying degrees of ES-derived contribution, were histologically sectioned. All chimeras chosen appeared phenotypically normal, with the possible exception of posterior neural tube duplications and spina bifida. Figure 4 depicts the level of ES cell contribution to various tissues of individual Fgfr1+/− or Fgfr1−/− ↔ CD-1 chimeric embryos. The anterior neural tube was used as a "standard" for assessing the original ES-derived cell contribution to the epiblast since this tissue does not ingress through the primitive streak.

In Fgfr1−/− ↔ CD-1 chimeric embryos, mutant cells contributed extensively to ectodermally derived tissues without causing gross morphological defects. The neural tube, surface ectoderm, and neural crest derivatives such as the branchial arches and dorsal root ganglia, were equally well populated by mutant cells (Figs. 4 and 5). Interestingly, Fgfr1−/− cells showed a tendency to aggregate into distinct patches in the neuroepithelium and nasal placodes (Fig. 3E).

In contrast, mutant cells were consistently under-represented in anterior mesoderm and endodermal lineages of Fgfr1−/− ↔ CD-1 chimeric embryos (Fig. 4). Few mutant cells were observed in the cephalic, heart, and somitic mesoderm populations (Fig. 5), and mutant cells were almost completely absent from the gut of Fgfr1−/− ↔ CD-1 chimeras (Fig. 4). Fgfr1−/− cells were also under-represented in the notochord of these chimeras, and contributed only to notochord of the posterior trunk and tail. Conversely, Fgfr1−/− cells appeared to accumulate in the mesenchyme of the tail (Figs. 3A,B and 4). Similar discrepancies in ES-derived cell contribution were not observed in Fgfr1+/− ↔ CD-1 chimeras, although there was a slight bias against Fgfr1+/− cell contribution to the endoderm (Fig. 4). This might indicate a dose-dependent requirement for FGFR1 in endoderm formation, or it may reflect an inherent bias against ES-derived contribution to the endoderm of ES cell ↔ embryo chimeras (see Discussion).

The level of mutant cell contribution to the limb bud and branchial arch mesenchyme of Fgfr1−/− ↔ CD-1 chimeras was similar to that found in the anterior neural tube; however, the distribution of Fgfr1−/− cells within these lineages was not random. In the limb bud, Fgfr1−/− cells appeared to be excluded from the mesenchyme cells of the progress zone (PZ), directly beneath the distal ectoderm and apical
Figure 4. Summary of the ES-derived cell contribution observed in various tissues of chimeric embryos.

*ES-derived cell contribution to the notochord is recorded only as present (+) or absent (-) owing to difficulties in scoring small tissue samples.

**The presence of secondary neural tubes was scored as follows: (-) no secondary neural tube observed; (+/-) secondary neural tubes observed which ranged in size from small structures which pinched-off from the primary neural tube, to (+++) structures comparable to the primary neural tube in both size and form which extended caudally from the level of the hind limb bud.

<table>
<thead>
<tr>
<th>Lineage</th>
<th>Notochord</th>
<th>Neural Tube</th>
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<tbody>
<tr>
<td>Fgfr1-/- ↔ CD-1</td>
<td>+</td>
<td>-</td>
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<tr>
<td>Fgfr1+/+ ↔ CD-1</td>
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Chimeras chosen for analysis were phenotypically normal, with the possible exception of duplications in the posterior neural tube. The % ES-derived cell contribution to each lineage was estimated from histological sections on the basis of β-galactosidase staining. Each horizontal row depicts a different chimeric embryo; vertical columns represent the different tissues analyzed.
Figure 4: Results of E9.5-10.5 Chimeric Analysis

Legend:

- 0%
- 0-15%
- 15-30%
- 30-45%
- 45-60%
- >60%
- n.a. not available

% ES-derived cell contribution
Figure 5. Histological analysis of whole-mount β-galactosidase stained chimeric embryos at E9.5.

(A,B) Sections through the head of matched Fgfr1-/- ↔ CD-1 (A) and Fgfr1+/- ↔ CD-1 (B) chimeric embryos show a relative deficiency in Fgfr1-/- cell contribution to cephalic mesenchyme. (C,D) Fgfr1-/- cell contribution to the heart of chimeric embryos (C) is deficient compared to ES-derived contribution to the heart of a matched Fgfr1+/- ↔ CD-1 chimera (D). (E,F) Sections through the trunk of matched Fgfr1-/- ↔ CD-1 (E) and Fgfr1+/- ↔ CD-1 (F) chimeric embryos show strong deficiencies in Fgfr1-/- cell contribution to somitic mesoderm. (G) Fgfr1-/- ↔ CD-1 chimeras commonly form secondary neural tubes through their posterior trunk and tail; secondary neural tubes are entirely Fgfr1-/- cell derived. Neural tube (nt), neural crest (nc), cephalic mesenchyme (c), heart (h), dorsal root ganglion (d), somitic mesoderm (s), secondary neural tube (snt).

Scale bars, 100 µm.
Figure 5:
ectodermal ridge (AER) (Fig. 6). The accumulations of \textit{Fgfr1-/-} cells in the proximal mesenchyme of the limb bud were visible both in whole-mount preparations and histological sections of \textit{Fgfr1-/-} \Leftrightarrow CD-1 chimeric embryos (Fig. 6A,B). \textit{Fgfr1-/-} cells were also excluded from the distal outgrowths of the nasal processes of E10.5 chimeric embryos (Fig. 6C), and from the distal mesenchyme of the developing branchial arch (data not shown).

\textit{Fgfr1} mutant cells appear defective at traversing the primitive streak

Several explanations exist for the observed deficiencies in \textit{Fgfr1-/-} cell contribution to the anterior mesoderm and endoderm of chimeric embryos. Mutant cells may have initially colonized these lineages, but either failed to proliferate, or underwent cell death. Alternatively, mutant cells may have been precluded from contributing to these lineages at the time of their inception at gastrulation. To differentiate between these possibilities, chimeric embryos were examined at earlier stages of development.

Wholemount β-galactosidase staining of early streak-stage \textit{Fgfr1 +/-} \Leftrightarrow CD-1 chimeric embryos demonstrated a random distribution of ES-derived cells. β-galactosidase staining appeared more intense in posterior-lateral portions of the embryo because of the extra cell layer formed by the advancing mesodermal wings (Fig. 7A,B). In contrast, \textit{Fgfr1-/-} \Leftrightarrow CD-1 chimeras showed striking patterns in mutant cell distribution: \textit{Fgfr1-/-} cells accumulated along the primitive streak and were under-represented in the mesodermal wings, node and pre-chordal mesoderm (Fig. 7C,D). 20 E7.5 \textit{Fgfr1-/-} \Leftrightarrow CD-1 chimeric embryos that showed a large range in ES-derived cell contribution (from <5% to 95%) were chosen for histological analysis.

Serial sections confirmed that \textit{Fgfr1-/-} cells were accumulating within the ectoderm and posterior mesenchyme of the primitive streak (Fig. 8). These \textit{Fgfr1-/-} accumulations could be observed even in those chimeras which showed only minimal (<5%) ES-derived cell contribution (Fig. 8A-C). In very strong \textit{Fgfr1-/-} \Leftrightarrow CD-1 chimeras, patches of WT cells appeared to be selectively delaminating from an almost completely mutant epiblast layer (Fig. 8K,L). This suggests that \textit{Fgfr1-/-} cells were at a competitive disadvantage in traversing the primitive streak.
Figure 6. Analysis of Fgfr1-- cell contribution to the limb buds and nasal process of E10.5 Fgfr1-- ↔ CD-1 chimeric embryos.

(A) Whole-mount analysis of a β-galactosidase stained chimeric embryo at E10.5 showing the distribution of Fgfr1-- cells within the limb bud. Note the contribution of mutant cells to the apical ectodermal ridge (AER), and to the proximal mesenchyme of the limb, but the exclusion of Fgfr1-- cells from the more distal mesenchyme of the limb, within the progress zone (PZ). (B) Histological analysis of an E10.5 chimeric embryos demonstrating the exclusion of Fgfr1-- cells from the PZ. (C) Sagittal section through an E10.5 chimeric embryo showing the segregation of WT and Fgfr1-- cells in the mesenchyme of the nasal process (arrow). Fgfr1 mutant cells accumulate proximally and appear to be excluded from the outgrowing distal mesenchyme. Also note the distinct aggregations of Fgfr1-- cells within the neuroepithelium of the telencephalon (arrowhead).
Figure 6:
Figure 7. Whole-mount analysis of β-galactosidase stained chimeric embryos at E7.5 to E8.5.

(A) Early streak-stage and (B) mid streak-stage Fgfr1+/- ↔ CD-1 chimeras showing that Fgfr1+/- cells are randomly distributed throughout the embryo. Note the intense posterio-lateral β-galactosidase staining which corresponds to the advancing mesodermal wings (B); anterior is to the left. (C,D) E7.5 Fgfr1-/- ↔ CD-1 chimeras showing an accumulation of Fgfr1-/- cells along the posterior streak; anterior is to the left. Note the deficiency of Fgfr1-/- cell contribution to the node and pre-chordal mesoderm (arrow in D), and to the mesodermal wings. (E,F) Fgfr1-/- ↔ CD-1 chimeras at E8.0 (E), and at E8.5 (F) showing continued accumulation of Fgfr1-/- cells along the primitive streak, and within the allantois. Note the distinct accumulation of mutant cells within the primitive ectoderm of the posterior streak (arrow in F). Primitive streak (ps), node (n), allantois (al), head fold (hf). Scale bars: 50 μm (A-D); 100 μm (E-F).
Figure 7:
Figure 8. Histological analysis of whole-mount β-galactosidase stained Fgfr1-/− ↔ CD-1 chimeras at E7.5.

Primitive streak is to the right. Distal to more proximal transverse sections through 5 Fgfr1-/− ↔ CD-1 chimeras are shown; ES-derived cell contribution to the distal epiblast was approximately 5% (A-C), 30% (D-F), 80% (G-I) and 95% (J-L, and M-O). (A-C) Chimeric embryos with minimal ES-derived cell contribution show accumulations of Fgfr1-/− cells within the epiblast (arrow) and mesenchyme (arrowhead) of the posterior streak. (D-F) Stronger chimeras continue to show accumulations of Fgfr1-/− cells along the posterior midline (arrow); mutant cells are deficient at contributing to the mesodermal wings (arrowhead) when compared with ES-derived cell contribution to the epiblast layer. (G-I) The deficit of Fgfr1-/− mesodermal cells is quite pronounced in very strong chimeric embryos (arrowhead); mutant cells accumulate within the mesenchyme of the posterior primitive streak (arrow). (J-L) In chimeras that are almost completely Fgfr1-/− cell derived, WT cells appear to selectively delaminate from the primitive streak (arrows). Fgfr1-/− mesodermal cells accumulate beneath the primitive streak (arrowhead) while the leading edge of the mesodermal wings is composed largely of WT cells (chevron). (M-O) Another almost completely Fgfr1-/− cell-derived chimera showing WT cells at the leading edge of the mesodermal wings (arrows), and mutant cells accumulating within “pockets” formed from abnormal folding of the epiblast layer (arrowhead). Primitive streak (ps), epiblast (ep), mesodermal wings (w). Scale bars, 50μm.
Figure 8:
Although mutant cells contributed strongly to the epiblast of chimeric embryos, there was a striking deficit of Fgfr1-/- cells in the mesodermal wings (Fig. 8D-F, G-I). This defect was not absolute; as the Fgfr1-/- cell contribution increased, more mutant mesoderm cells were observed (Fig. 8G-I, J-L, M-O). Fgfr1-/- mesodermal cells tended to accumulate along the posterior streak (Fig. 8I,L), giving the impression of the thickened streak characteristic of homozygous Fgfr1 mutant embryos. This mutant mesodermal population also appeared less able to migrate laterally around the embryo, since the leading edges of the mesodermal wings were comprised largely of WT cells (Fig. 8K,O). Even in chimeras with over 95% Fgfr1-/- cell contribution, WT cells formed the leading edge of the mesodermal wings; mutant cells accumulated within "pockets" formed by abnormal buckling of the epiblast layer (Fig. 8O).

**Fgfr1 mutant cells are defective in epithelial to mesenchymal transition at the primitive streak**

Headfold to early somite-stage Fgfr1-/- ↔ CD-1 chimeric embryos showed accumulations of mutant cells within the primitive streak and allantois (Fig. 7E,F). A deficiency in mutant cell contribution to the paraxial and pre-somitic mesoderm was also observed; similar trends were not observed in the Fgfr1 +/- ↔ CD-1 chimeras (data not shown). 11 E8.5 Fgfr1-/- ↔ CD-1 chimeras with varying degrees of mutant cell contribution (<5 to 70%) were histologically analyzed, and all showed a deficit of Fgfr1 -/- cells in the cephalic mesenchyme, heart primordia and paraxial mesoderm populations (Fig. 9). Even chimeras that had very high Fgfr1-/- cell contribution to ectodermal lineages (60-70%) showed a near absence of mutant cells from anterior mesoderm (Fig. 9D). These chimeras appeared morphologically abnormal with kinked and misshapen neural folds.

Fgfr1-/- ↔ CD-1 chimeric embryos with minimal (<5%) ES-derived cell contribution showed accumulations of mutant cells within the epiblast layer at the posterior streak (Fig. 9A-C). Mutant cells accumulated as a distinct "block" of columnar epithelial cells, with WT cells forming mesenchyme on both sides of this accumulation (Fig. 9B). Also, in chimeras with high levels of ES-derived cell contribution, patches of WT cells could be seen delaminating from an almost entirely mutant epiblast layer (Fig.
Figure 9. Histological analysis of whole-mount β-galactosidase stained Fgfr1/-/- ↔ CD-1 chimeras at E8.5.

(A-C) Anterior to posterior, transverse sections through the primitive streak of a chimera with minimal Fgfr1/-/- contribution. Sections show the accumulation of Fgfr1/-/- cells within the posterior streak (arrowhead). Mutant cells collect in the epiblast layer while WT cells make an EMT on either side of this Fgfr1/-/- accumulation (arrows). (D) Transverse section through chimera that was approximately 70% Fgfr1/-/- cell-derived; note the deficiency of mutant cells in the cephalic mesoderm, and the misshapen neural folds (arrow). (E-H) Anterior to posterior sections through the caudal end of this chimera showing a lack of mutant cells in the paraxial mesoderm (arrowhead in F), and an accumulation of mutant cells within the posterior epiblast and lateral mesoderm (arrowhead in H). WT cells selectively delaminate from an almost entirely Fgfr1/-/- cell-derived epiblast layer (arrows). Primitive streak (ps), cephalic mesoderm (cm), neural fold (nf), paraxial mesoderm (pm), lateral mesoderm (lm). Scale bars, 50 μm.
This suggests that Fgfr1-/ cells are deficient in undergoing the epithelial to mesenchymal transition (EMT) which occurs at the streak. Despite apparent deficiencies in traversing the streak, Fgfr1-/ cells contributed strongly to the mesoderm of more posterior regions of the embryo and mutant cells were well represented in lateral mesoderm. All Fgfr1-/ \( \leftrightarrow \) CD-1 chimeras examined demonstrated strong ES-derived cell contribution to the allantois; the level of mutant cell contribution was highest at the base of the allantois and decreased distally (Fig. 7E,F). The remainder of the extra-embryonic mesoderm showed a striking deficiency in mutant cell colonization. Fgfr1-/ cells were occasionally observed in the blood islands in the mesodermal component of the yolk sac, but only in blood islands situated along the posterior midline (not shown).

**Fgfr1-/ \( \leftrightarrow \) CD-1 chimeras form entirely mutant secondary neural tubes**

At E9.5-10.5, Fgfr1-/ \( \leftrightarrow \) CD-1 chimeras showed caudal accumulations of mutant cells. Histological sections through the tails of such chimeras revealed that Fgfr1-/ cells appeared to form secondary neural tubes which were entirely mutant cell-derived (Figs. 5G, 10). 19 of the 22 Fgfr1-/ \( \leftrightarrow \) CD-1 chimeras examined had developed these secondary structures (Fig. 4); of the 3 chimeras which did not, 2 displayed only minimal ES-derived cell contribution.

To rule out the possibility of β-galactosidase stain seeping into adjacent WT cells, thus masking their contribution to secondary neural tubes, an aggregation was performed between ROSA26 embryos and the C15 ES cell line (Fgfr1-/; lacZ negative). In this case, secondary neural tubes did not show β-galactosidase staining, confirming that ectopic neural tubes were completely Fgfr1-/ cell derived (data not shown).

Secondary neural tubes ranged in size from small structures which appeared to "pinch off" from the primary neural tube (NT) in distal portions of the tail, to structures comparable in size to the primary NT, extending caudally from the forelimb bud level. The larger, secondary neural tubes originated from entirely Fgfr1-/ cell-derived expansions of the neural plate (Fig. 10), and extended laterally from the central neural groove. These ectopic neural plates were excluded from primary NT closure, and folded separately into secondary neural tubes (Fig. 10).
To verify the neural identity of these ectopic tubes, whole mount RNA in situ were performed using Sox-1, Sox-2 and Ncam probes. Sox-1 and Sox-2 are SRY related genes; Sox-1 expression is restricted to neuroectodermal lineages, and Sox-2 is initially expressed throughout the ectoderm and is later restricted to neuroepithelia (Robin Lovell-Badge, pers. comm.). Both Sox-1 (Fig. 11A) and Sox-2 (data not shown) were expressed in the primary and secondary neural tubes. NCAM, a neural cell adhesion molecule, was also expressed in both secondary and primary neural tubes, and showed normal expression in the surrounding somitic mesoderm (Fig. 11B). In situ were also performed using mox-1, twist, snail and Brachyury probes to ensure that the secondary neural tubes demonstrated no elements of mesodermal identity; mesoderm marker expression was not observed (not shown). Secondary neural tubes were often surrounded by ill-defined Fgfr1/-mesenchymal tissue (Fig. 10). The pattern of mox-1 expression in the tails of chimeric embryos suggests that this poorly organized mesenchymal tissue has paraxial mesoderm identity (Fig. 11E). However, owing to the nature of the in situ hybridization protocol, the genotype of mox-1 expressing cells could not be determined. Lack of Brachyury staining associated with the secondary NT verified histological analyses, which showed that secondary notochords were not associated with the ectopic neural tubes. Dorsal-ventral patterning of the secondary NT was also examined with Pax3 and Pax6, and shh probes. As expected, the secondary neural tubes, which lacked notochords, did not show floor plate expression of shh (data not shown). Pax3 is normally expressed in the dermamyotome and dorsal neural tube; Pax6 expression is restricted to lateral walls of the neural tube. Both Pax6 and Pax3 appeared to be expressed in their proper domains (Fig. 11C and D, respectively).
Figure 10. Progressive caudal to rostral transverse sections through the caudal end of a Fgfr1-/- CD-1 chimera at E9.5.

Secondary neural tubes begin as a lateral extension of the neural plate (arrowheads in A,B). The ectopic neural plate is excluded from the primary neural tube (C,D), and folds separately into a distinct secondary neural tube (E-I). Note the sharp distinction made between Fgfr1-/- and WT cells upon primary neural tube closure (arrows in B,C), and the absence of WT cells from the secondary neural tube. Neural tube (nt), secondary neural tube (snt). Scale bar, 100 μm.
Figure 10:
Figure 11. In situ hybridization analysis of secondary neural tubes from Fgfrl-/- ↔ CD-1 chimeras.

(A) Sox-1 staining is observed in the dorsal region of both primary and secondary neural tubes. (B) Ncam expression is present in both neural tubes in the tail of a Fgfrl-/- ↔ CD-1 chimera, as well as in the pre-somatic mesoderm. (C) Pax-6 expression is observed within both primary and secondary neural tubes. (D) Pax-3 expression is observed in its normal dorsal domain within the primary and secondary neural tubes, as well as in surrounding somitic mesoderm. (E) Mox-1 is expressed in the poorly organized mesenchyme surrounding primary and secondary neural tubes. Neural tube (nt), secondary neural tube (snt), paraxial mesoderm (m) Scale bars, 50 μm.
Figure 11:
**DISCUSSION**

Chimeric analysis has furthered our understanding of the developmental processes affected by the *Fgfr1* mutation. *Fgfr1-/-* ↔ tetraploid CD-1 aggregations demonstrated unequivocally that defects associated with the mutated *Fgfr1* allele are intrinsic to definitive embryonic germ layers and to the extra-embryonic mesoderm. In *Fgfr1-/-* ↔ diploid CD-1 chimeras, mutant cells contributed strongly to ectodermal lineages, and this argues against an early role for FGFR1 signalling in the development of the ectoderm and its derivatives. Furthermore, since the epiblasts of *Fgfr1+/-* and *Fgfr1-/-* ↔ CD-1 chimeras showed similar ranges in ES-derived cell contribution, this argues against a strong mitogenic role for FGFR1 in early development (although a specific role in mesoderm proliferation cannot be completely discounted).

**The primary defect associated with the *Fgfr1* mutation is a deficiency in traversing the primitive streak**

This study has also provided further insight into the role of FGFR1 in the morphogenesis and patterning of mesoderm at gastrulation. *Fgfr1-/-* ↔ CD-1 chimeras demonstrated strong deficiencies in the ability of mutant cells to populate not only the paraxial mesoderm (as already observed in the mutant analyses), but also the extra-embryonic, cephalic, heart, and axial mesoderm, plus the endodermal lineages of E9.5-10.5 chimeric embryos. Some mutant cells were found in all lineages, and this confirms previous studies into the differentiation capacity of FGFR1 deficient ES cell lines as teratomas, which suggested that FGFR1 is not required in a cell-autonomous manner for cell fate specification (Deng et al., 1994). Rather, the chimeric analysis suggests that FGFR1 is required for correct morphogenetic movement of mesodermal progenitor cells through the primitive streak at gastrulation. In *Fgfr1-/-* ↔ CD-1 chimeras, *Fgfr1-/-*-cells are deficient at traversing the primitive streak. *Fgfr1* mutant cells accumulate along the posterior midline and consequently fail to populate the mesodermal wings which have been identified as the source of presumptive extra-embryonic, heart and cephalic mesoderm (Parameswaran and Tam, 1995).
Axial and paraxial mesoderm are thought to be produced from stem-cell populations residing within the node and anterior portions of the primitive streak (Beddington, 1994; Tam and Beddington, 1987; Lawson et al., 1991; Nicolas et al., 1996). These progenitor populations form early in gastrulation, after extra-embryonic, cephalic and heart mesoderm progenitors have exited the streak (Lawson et al., 1991). Since Fgfr1-/- cells appear to be defective at delaminating from the primitive streak, and since chimeric embryos show deficiencies in mutant cell colonization of the node, pre-chordal and early paraxial mesoderm populations, we suggest that Fgfr1-/- cells were out-competed by WT cells in populating the axial and paraxial progenitor populations early in gastrulation. Interestingly, the resurgence of mutant cells within the posterior notochord may indicate later recruitment of Fgfr1-/- cells to the axial progenitor pool over the course of gastrulation.

Definitive endoderm is also derived from epiblast cells that have delaminated at or near the anterior end of the early primitive streak (Lawson et al., 1991). Since Fgfr1-/- cells are defective in traversing the streak, it is perhaps not surprising that there was a near absence of mutant cells in the gut of Fgfr1-/- ↔ CD-1 chimeric embryos. Slight deficiencies in ES-derived contribution to the endoderm were also observed in Fgfr1+/+ ↔ CD-1 chimeric embryos. This selection against Fgfr1+/+ cell contribution to the endodermal lineage may reflect an inherent bias in the distribution of 129 ES-progenitors within 129 ES cell ↔ CD-1 chimeras. The results of other chimeric analyses in our lab have shown that such biases against ES-derived cell contribution to endoderm exist independently of the Fgfr1 genotype (unpublished lab observations).

In contrast to the anterior mesoderm and endodermal populations, the limb bud, lateral mesoderm and allantois of chimeric embryos were well colonized by Fgfr1-/- cells. Thus, mutant cells were ultimately able to traverse the posterior streak. This skewed distribution of Fgfr1-/- mesoderm might indicate a differential requirement for FGFR1 at various A-P levels of the primitive streak. Fgf4 and Fgf5 are expressed predominantly within the anterior 2/3 of the egg cylinder and streak (Haub and Goldfarb, 1991; Niswander and Martin, 1992; Hebert et al., 1991) and could therefore be responsible for anterior streak-specific FGFR1 signalling. However, Fgfr1-/- cells were still observed accumulating within the epiblast and mesenchyme of the posterior streak. Thus, the fact that more mutant cells successfully traversed this portion of the streak may
simply reflect the "competitive" nature of the chimeric analysis: over the course of
gastrulation, WT cells preferentially exited and Fgfrl-/- cells progressively accumulated
within the streak. Eventually, Fgfrl mutant cells constituted the majority of the
mesodermal progenitor population, and were therefore forced to contribute to "posterior"
mesoderm lineages.

This competitive nature of the chimeric analysis might also explain differences
observed in the behaviour and patterning of Fgfrl-/- mesodermal progenitors between
homozygous mutant and chimeric embryos. Although cephalic, heart and axial
mesoderm were produced in homozygous Fgfrl mutant embryos, these lineages were
poorly colonized by mutant cells in Fgfrl-/- ↔ CD-1 chimeras where WT cells
demonstrated a competitive advantage in traversing the streak. Therefore, while the
homozygous mutant analyses indicate a specific role for FGFR1 in the formation of
paraxial mesoderm, we argue that the primary defect associated with the Fgfrl mutation
is a general deficiency in the ability of epiblast cells to traverse the primitive streak.
Although secondary roles for Fgfrl in cell survival or proliferation can not be discounted,
they alone do not seem sufficient to explain the absence of Fgfrl-/- cells from anterior
mesoderm and endodermal lineages at the time of their inception.

Defects in migration and cell adhesion are associated with the Fgfrl mutation

In addition to showing deficiencies in EMT at gastrulation, Fgfrl mutant cells
which managed to progress through the primitive streak tended to accumulate along the
posterior midline. This aberrant morphogenetic movement of Fgfrl mutant cells in
Fgfrl-/- ↔ CD-1 chimeras could be the result of defects in cell adhesion and/or cell
migration. Cell - extracellular matrix (ECM) interactions are necessary for cell
migration, and mouse embryos homozygous for mutations in fibronectin (a glycoprotein
component of the ECM) and in focal adhesion kinase (a non-receptor protein tyrosine
kinase thought to mediate integrin signalling) both show gastrulation phenotypes (George
et al., 1993; Furuta et al., 1995). Interestingly, chimeric analyses of T (Brachyury) gene
function have also shown accumulations of T/T cells at the posterior end of the embryo.
T/T cells were able to traverse the streak, but the nascent T/T mesodermal cells
accumulated beneath the primitive streak thus suggesting a role for T in regulating the
morphogenetic behaviour or adhesive properties of nascent mesoderm (Wilson et al., 1993; Wilson et al., 1995). Although the similarity between T and Fgfr1 mutant cell behaviour in chimeric embryos lends credence to the Brachyury-FGF regulatory pathway which has been established in Xenopus (Isaacs et al., 1994), homozygous Fgfr1 mutant embryos continue to express T (Yamaguchi et al., 1994; Deng et al., 1994).

A role for FGFR signalling in cell migration has been established by mutational analyses in other organisms (Reichman-Fried et al., 1994; DeVore et al., 1995). In particular, recent mutations of the Drosophila FGF-R2 gene, heartless (htl), have demonstrated phenotypes strikingly similar to those observed in this study: invaginated mesodermal cells remain aggregated along the ventral midline and fail to migrate in a dorsolateral direction (Gisselbrecht et al., 1996; Beiman et al., 1996). Because these muscle progenitors fail to acquire position-specific inductive cues, heartless embryos show a reduction in cardiac, visceral and dorsal somatic muscle fates. However, htl mesodermal precursors remain competent to receive inductive signals, and mesodermal fates can be rescued by ectopically expressed decapentaplegic protein (Gisselbrecht et al., 1996; Beiman et al., 1996). This is analogous to the results obtained in our Fgfr1 chimeric analysis which demonstrate that mutant Fgfr1 cells can still make all types of mesoderm, but fail to migrate properly through the primitive streak. Therefore, there may be a conserved role for FGFR signalling in the morphogenesis of mesoderm formation at gastrulation.

Defects in traversing the primitive streak could also involve altered adhesive properties of Fgfr1/- cells. Mutant cells which accumulated within the streak of Fgfr1/- <-> CD-1 chimeras maintained a columnar epithelial morphology, arguing that Fgfr1/- cells were failing to fully undergo an epithelial to mesenchymal transition (EMT). EMT events are thought to be regulated by a family of calcium dependent cell adhesion molecules (the cadherins). E-cadherin, for example, is expressed in all cells of the early egg cylinder but is down-regulated at gastrulation within the primitive streak and nascent mesodermal populations (Damjanov et al., 1986). Burdsal et al. (1993) have shown that function perturbing antibodies against E-cadherin can force an EMT in cultured epiblast tissue: epiblast cells lose cell-cell contacts, flatten and assume a mesenchymal morphology. Therefore, there appears to be a causal relationship between loss of cadherin function and EMT. FGFR1 may regulate the expression or function of these
adhesion molecules. Indeed, exogenous FGF has been shown to cause mesenchymal transformation of cultured epithelial cell lines (Boyer et al., 1992), and this FGF induced EMT has been associated with the cellular redistribution of E-cadherin. There is also some evidence that cell adhesion molecules may act upstream of FGF signalling by binding to and activating FGF receptors (reviewed by Green et al., 1996). Further study will be needed to determine the relationship between FGFR1 signalling and regulation of cell adhesion at gastrulation.

**Neuralization of Fgfr1-/- cells: morphogenetic movement and cell fate determination**

Secondary neural tubes, which are composed entirely of Fgfr1 mutant cells, form within the posterior trunk and tail of Fgfr1-/- × CD-1 chimeric embryos. They find their origins from expanded, Fgfr1-/- cell-derived neural plates which extend laterally from the neural groove and which presumably arise from the accumulation of mutant columnar epithelial cells observed earlier in the streak. Interestingly, these Fgfr1-/- cells adopt a neural over an epidermal fate. Studies in *Xenopus* have led to the proposal that neuralization of embryonic cells occurs when cells do not receive other inducing signals telling them to form epidermis, mesoderm, or endoderm (for review see Hemmati-Brivanlou and Melton, 1997). The formation of ectopic neural tissue by Fgfr1-/- cells may therefore demonstrate a similar neuronal default state for murine embryonic cells.

The formation of secondary neural tubes may be a common manifestation of disrupted morphogenesis at gastrulation: duplicated neural tubes have been observed in a number of spontaneously occurring mouse mutations which show defects in primitive streak formation and axis elongation. These mutations include *fused*, *vestigial tail* (*vt*), and *rib vertebrae* (as reviewed by Cogliatti, 1986; Gruneberg and Wickramaratne, 1974; Theiler and Varnum, 1985). Recently, *vt* has been shown to be a hypomorphic allele of *Wnt-3a* (Greco et al., 1996). *Wnt-3a* null mutant embryos lack caudal somites, have disrupted notochord and fail to form a tail bud (Takada et al., 1994). In addition, secondary neural tubes are formed, but, unlike Fgfr1 mutants, these tubes form from cells that have successfully traversed the streak (Yoshikawa et al., 1997). This has led Yoshikawa et. al. (1997) to propose that *Wnt-3a* regulates the choice of cell fate between neural and paraxial mesodermal cell lineages.
To date, Fgfr1 mutant and chimeric analyses have not demonstrated a role for FGFR1 signalling in cell fate specification; however, mutations affecting this pathway can result in similar neural anomalies. Hence, while FGFR1 and Wnt-3a may be playing different roles in the movement and specification of mesodermal progenitors at the primitive streak, disturbances in both systems result in similar phenotypic abnormalities. This study therefore underscores both the complexity of interactions that regulate mesoderm formation, and the importance of normal morphogenetic movements in the processes of mesodermal patterning and fate determination.
**EXPERIMENTAL PROCEDURES**

**ES cell isolation**

The *Fgfrl* mutant allele used in this study has been previously described (Yamaguchi et al., 1994). Briefly, exons 8-14 of the *Fgfrl* locus, which encode the transmembrane domain and most of the catalytic kinase domain, were replaced by homologous recombination with PGKneo; this should effectively eliminate catalytically active isoforms of the receptor. A second, independently derived mutation at the *Fgfrl* locus which truncated all major isoforms of the gene at the second extracellular immunoglobulin domain resulted in a similar mutant phenotype (Deng et al., 1994). Therefore, it is believed that both *Fgfrl* mutations are effectively null alleles.

To isolate ES cell lines homozygous for the *Fgfrl* mutation and marked with a ubiquitously expressed lacZ marker, *Fgfrl* heterozygotes (129 background) were first crossed with ROSA26 mice of a mixed background (Friedrich and Soriano, 1991). F1 progeny were genotyped for the *Fgfrl* locus by PCR (Yamaguchi et al., 1994). *Fgfrl+/-; ROSA26 lacZ +/-* mice were intercrossed, and the day that vaginal plugs were detected was designated embryonic day 0.5 (E0.5). Blastocysts were flushed, with M2 media, from the uterine horns of pregnant females at E3.5 (as described in Hogan et al., 1994), and zona pellucidae were removed by treatment with acid Tyrode's solution. Blastocysts were transferred to individual 10 mm wells containing a preformed feeder layer of mitotically inactivated embryonic fibroblasts and 1 ml of culture medium (Dulbecco's modified Eagle's medium [DMEM] supplemented with 100 μM non-essential amino acids, 1 mM sodium pyruvate, 1 μM 8-mercaptoethanol, 2 mM L-glutamine, 15% fetal calf serum and 1X leukemia inhibitory factor). Embryos were cultured at 37°C, 5% CO2 for 4-5 days to allow for inner cell mass (ICM) outgrowth. ICM outgrowths were then disaggregated into small, multi-cellular clumps by mouth pipetting with a series of finely drawn out Pasteur pipettes. Disaggregated outgrowths were cultured further until ES cell colonies developed. Colonies were picked and subcultured to establish cell lines. Cell lines were genotyped for the *Fgfrl* locus by Southern analysis (Yamaguchi et al., 1994) and the presence of the ROSA26 lacZ transgene was determined by β-galactosidase staining of ES cell colonies.
**Generation of chimeric embryos**

To assess the developmental potential of the ES cell lines generated, individual lines were aggregated with two tetraploid CD-1 embryos (Nagy et al., 1993). Previous studies have demonstrated that tetraploid cells do not contribute to fetal tissues of tetraploid embryo ↔ ES cell chimeras, therefore these chimeric embryos will be completely ES cell-derived (Nagy et al., 1993). Tetraploid CD-1 embryos were produced by the electrofusion of embryos at the two cell stage. Aggregates were transferred into the uteri of CD-1 foster mothers, chimeric embryos were dissected at early to mid-gestational stages, and developmental potential of each cell line assessed by gross phenotypic observation.

Diploid chimeric embryos were generated by aggregating an 8-10 cell clump of either Fgfr1+/- or Fgfr1-/- ES cells with CD-1 8-cell embryos, using the standard morulae aggregation technique (Nagy et al., 1993). Aggregates were transferred into the uteri of CD-1 foster mothers, chimeric embryos were dissected at early to mid-gestational stages, and the contribution of ES cells and CD-1 cells to the embryo was determined by whole-mount β-galactosidase staining.

**β-gal staining of chimeric embryos**

Embryos (or ES cells) were rinsed in 100 mM sodium phosphate pH 7.3, and then fixed in 0.2% gluteraldehyde, 2 mM MgCl2, 5 mM EGTA, 100 mM sodium phosphate pH 7.3 at room temperature for 15-30 minutes depending on size of embryo (or 5 minutes for ES cells). Embryos were then washed 3 times in wash buffer at room temperature (0.02% NP-40, 0.01% deoxycholate, 2 mM MgCl2, 100 mM sodium phosphate pH 7.3) for 15 minutes each (ES cells were washed 3X 5 minutes). Embryos or ES cells were stained in 1 mg/ml X-gal, 5 mM K3Fe(CN)6, 5 mM K4Fe(CN)6, 0.02% NP-40, 0.01% deoxycholate, 2 mM MgCl2, 100 mM sodium phosphate pH 7.3 at 37°C overnight. Samples were then rinsed with wash buffer, rinsed in PBS, and post-fixed overnight in 3.7% formaldehyde at 4°C.

After whole-mount β-galactosidase staining, chimeric embryos were photographed, dehydrated through an ethanol series and embedded in paraffin. Paraffin blocks were sectioned at 5 μm, sections were mounted onto glass slides, dewaxed, and counterstained with Nuclear Fast Red.
Whole mount RNA in situ hybridization

Whole-mount in situ hybridization was performed as described (Conlon and Rossant, 1992). For sectioning of whole-mount stained embryos, specimens were post-fixed in 3.7% formaldehyde overnight at 4°C. Embryos were embedded in paraffin, sectioned at 10 μm, mounted onto glass slides, dewaxed, and photographed using Nomarski optics.

The probes used for the whole-mount in situ hybridization studies were as follows: Brachyury (Herrmann, 1991), Shh (Echelard et al., 1993), Sox-1 and Sox-2 (unpublished, obtained from Robin Lovell-Badge), Pax3 (Goulding et al., 1991), Pax6 (Walther and Gruss, 1991), mox-1 (Candia et al., 1992), twist (Wolf et al., 1991), and snail. (Smith et al., 1992). The Ncam probe (obtained from Cynthia Faust) consisted of a 1 kb cDNA fragment spanning exons 3-9 (which contains nearly all IgG domains), cloned into the BamHI site of PBS+ (Stratagene) phagemid vector.
CHAPTER 3

FGF signalling regulates mesoderm cell fate specification and morphogenetic movement at the primitive streak

Portions of this chapter appear in the following publication:

SUMMARY

Although FGF signalling plays an integral role in the migration and patterning of mesoderm at gastrulation, the mechanism and downstream targets of FGF activity have remained elusive. Here, we demonstrate that FGFR1 orchestrates the epithelial to mesenchymal transition and morphogenesis of mesoderm at the primitive streak by controlling Snail and E-cadherin expression. Furthermore, we show that FGFR1 functions in mesoderm cell fate specification by positively regulating Brachyury and Tbx6 expression. Finally, we provide evidence that the attenuation of Wnt3a signalling observed in Fgfr1 -/- embryos can be rescued by lowering E-cadherin levels. We propose that modulation of cytoplasmic β-catenin levels, associated with FGF-induced downregulation of E-cadherin, provides a molecular link between FGF and Wnt signalling pathways at the streak.

INTRODUCTION

In early embryonic development, extensive cell proliferation, differentiation and the associated morphogenetic movements of gastrulation serve to generate the three definitive germ layers of the vertebrate body plan: endoderm, ectoderm and mesoderm. In the mouse, gastrulation begins around embryonic day 6.5 (E6.5) as embryonic ectoderm (epiblast) cells are recruited to the primitive streak, a transient structure which forms along the posterior midline of the embryo (reviewed in Hemavathy et al., 2000). At the primitive streak, epiblast cells undergo an epithelial to mesenchymal transition (EMT)
and then ingress between the epiblast and visceral endoderm to become incorporated into either the mesoderm or the definitive endodermal germ layers. Fate mapping studies demonstrate that the order and the site of progenitor-cell ingress through the primitive streak determine both the spatial distribution and the fate of mesodermal cells at gastrulation (Kinder et al., 1999). The morphogenesis, specification and patterning of mesoderm at the primitive streak are thought to be regulated by a network of intercellular inductive interactions (reviewed in Tam and Behringer, 1997).

Studies in Xenopus first demonstrated that fibroblast growth factors (FGFs) play important roles in specifying and patterning the mesodermal germ layer at gastrulation. FGFs have potent mesoderm-inducing activity, and can function as posteriorizing factors in the development and patterning of the anteroposterior axis. Experiments using dominant negative FGF receptor constructs have demonstrated a role for FGF signalling in the specification and maintenance of posterior mesodermal populations, and also in regulating the convergent-extension movements of gastrulating mesoderm (reviewed in Slack et al., 1996; Isaacs, 1997). An analogous role for FGF signalling at gastrulation has been established in the zebrafish (Griffin et al., 1995).

The first evidence that FGF signalling played a fundamental role during mouse gastrulation came from the targeted disruption of FGF receptor 1 (Fgfr1) (Yamaguchi et al., 1994; Deng et al., 1994). Fgfr1 is expressed throughout the epiblast prior to gastrulation and its expression becomes concentrated around the posterior streak as gastrulation proceeds (Orr-Urtreger et al., 1991; Yamaguchi et al., 1992). FGFR1 is one of four known FGF receptors, but it is the only receptor essential for development through early gastrulation (Colvin et al., 1996; Deng et al., 1996; Weinstein et al., 1998; Xu et al., 1998b). Embryos homozygous for null alleles of Fgfr1 (Fgfr1 -/-) die at gastrulation, and show accumulations of cells at the posterior streak, severe reductions in paraxial mesoderm formation and an apparent expansion of axial mesoderm (Yamaguchi et al., 1994; Deng et al., 1994). Chimeric analysis of Fgfr1 function revealed a defect in Fgfr1 -/- progenitor cell migration through the primitive streak; as a result, few Fgfr1 -/- cells contribute to mesoderm and endodermal lineages (Ciruna et al., 1997). Observed failures in EMT and the accumulation of Fgfr1 -/- progenitor cells within the streak indicated that FGFR1 might function to regulate cell adhesion and/or cell migration. In chimeric embryos, Fgfr1 -/- progenitor cells that accumulate at the primitive streak form
ectopic neural tubes (Ciruna et al., 1997; Deng et al., 1997). Therefore, in addition to its role in patterning axial and paraxial mesoderm populations, FGF signalling may negatively regulate the specification of neuroectoderm cell fate.

Mutational analyses of the known FGF genes have demonstrated that only Fgf4 and Fgf8 are required for early embryonic development. Fgf4 and Fgf8 are both expressed within the primitive streak at gastrulation (Niswander and Martin, 1992; Crossley and Martin, 1995). Targeted disruption of Fgf4 results in abortive post-implantation development, and therefore embryos die too early to assess a role for FGF4 in gastrulation (Feldman et al., 1995). However, embryos homozygous for null alleles of Fgf8 lose expression of Fgf4 in the primitive streak, and in the absence of both FGF8 and FGF4, gastrulation is severely disrupted (Sun et al., 1999). Embryonic mesoderm and endodermal tissues are not formed in Fgf8-/- embryos because progenitor cells fail to migrate away from the primitive streak (Sun et al., 1999).

It is clear therefore, that FGFs play an essential role in both the morphogenesis and patterning of mesoderm. However, functional analyses of mutations in the FGF pathway have discovered little, to date, of the downstream targets of FGF signalling at gastrulation. The goal of this study was to better understand the mechanisms by which FGFR1 signalling regulates the specification, EMT, migration, and patterning of progenitor cells at the primitive streak. Here we describe novel in vitro assays of mesoderm chemotaxis and cell migration that utilize primary cultures of explanted embryonic tissue, thus allowing physiologically relevant investigations into the morphogenetic movements of gastrulation. We also describe combined immunofluorescence and fluorescent RNA in situ hybridization analyses of chimeric embryos, which extend the utility of classical chimeric approaches in the mouse and have provided molecular insights into the downstream targets of FGFR1 activity. Although results indicate a possible chemotactic role for FGFR1 signalling, general cell migration defects were not observed in explanted Fgfr1-/- mesoderm cultures. Rather, we implicate abnormal intercellular adhesion in the failed EMT and aberrant morphogenesis observed at the primitive streak, and demonstrate that both Snail and E-cadherin are regulated by FGFR1 signalling. We also establish a role for FGF signalling in mesoderm cell fate specification and show that members of the T-box gene family, which have been implicated in posterior and paraxial mesoderm fate determination, are positively
regulated by FGFR1. Finally we demonstrate that FGFR1 indirectly regulates Wnt signalling activity at the primitive streak. We argue that ectopically expressed E-cadherin in Fgfr1 -/- progenitor cells sequesters free β-catenin from its intracellular signalling pool and thus attenuates Wnt signal transduction. Results provide a molecular link between FGF and Wnt signalling pathways at the primitive streak, and underscore the interdependent nature of morphogenesis and patterning at gastrulation.

RESULTS

Assessing Fgfr1 -/- cell migration in primary embryonic explant cultures

Cells from primitive streak mesoderm explants, when cultured on a fibronectin substratum, will scatter and migrate away from the center of the explant (Burdsal et al., 1993). This observation was used to devise an in vitro cell migration assay that would allow comparison of Fgfr1 mutant and wild-type (WT) mesodermal cells explanted from the primitive streak. Since the Fgfr1 -/- phenotype is characterized by retarded development, early lethality and aberrant mesoderm formation at gastrulation, Fgfr1 -/- ↔ WT chimeric embryos were used in this analysis as they allow for direct comparison of Fgfr1 -/- and WT cell behaviour in relatively normal embryonic environments. The primitive streaks from late-streak staged chimeric embryos (Figure 1A) were dissected and cultured for 3 days on fibronectin-coated glass slides in a chemically defined medium, after which time they were fixed and X-gal stained to distinguish Fgfr1 -/- or +/- cells (blue) from WT (pink). It was observed that Fgfr1 -/- mesodermal cells were capable of migrating from the periphery of primitive streak explants (n=21; Figure 1C). Since migration rate is a function of distance traveled over time, any deficiency in Fgfr1 -/- cell migration was expected to become manifest as a ring of “pink” cells at the periphery of the explant (formed by WT cells which would migrate further than their Fgfr1 -/- counterparts). However, Fgfr1 mutant and WT cell populations tended to segregate in culture (Figure 1C and D), making comparison of migration rates difficult. This segregation is characteristic of the behaviour of mutant cells along the primitive streak of Fgfr1 chimeric embryos (Ciruna et al., 1997), and was not observed in Fgfr1 +/- control explants (n=6; Figure 1B). bFGF was added to primitive streak cultures in an
attempt to accentuate potential deficiencies in Fgfrl +/- cell migration (n=19; Figure 1D). No striking differences between Fgfrl +/- and WT cell migration were observed.

In order to generate a mixed population of WT and Fgfrl +/- mesodermal cells for study, anterior epiblast explants were dissected from chimeric embryos (Figure 1E) and cultured with function perturbing anti- E-cadherin antibodies. WT and Fgfrl mutant cells within the anterior epiblast of primitive streak- staged chimeric embryos do not segregate from one another, but rather exist in a mixed, “salt and pepper” distribution (Ciruna et al., 1997). Furthermore, although cells of epiblast explants maintain an epithelial morphology in culture, it has been demonstrated that function-perturbing anti- E-cadherin antibodies will induce a non-reversible epithelial to mesenchymal transition (EMT) when added to epiblast cultures (Burdasal et al., 1993). WT and Fgfrl +/- mesodermal cells formed from chimeric epiblast explants did not segregate in culture (n=16; Figure 1G), and closely resembled Fgfrl +/- control explants (n=14; Figure 1F). In these mixed mesoderm outgrowths, Fgfrl +/- mesoderm migrated identically to WT cells (Figure 1G). Addition of bFGF to the explant culture system had no effect on relative migration rates (n=23; Figure 1H).

If Fgfrl mutant embryos had defects in extra-cellular matrix production, then deficiencies in Fgfrl +/- mesoderm migration might have been rescued in vitro by the presence of an exogenous fibronectin substratum. Fibronectin distribution in late streak-stage embryos was examined by immunofluorescence (Figure 1H). No difference was observed between WT (Figure 1I) and Fgfrl +/- (Figure 1J) embryos. Therefore, these results suggest that Fgfrl +/- mesodermal cells are not impaired in general cell migration. Mechanisms for the observed segregation of Fgfrl +/- and WT mesodermal populations as well as the consequences of anti- E-cadherin antibody treatment are discussed below.

**FGFR1 may function in chemo-atraction**

In *Drosophila* tracheal morphogenesis and *C.elegans* sex myoblast migration, chemotactic roles for FGFs are well documented (reviewed in Skaer, 1997; Chen and Stern, 1998). There is also evidence that during chick limb development, FGF signalling directs the migration of proximal mesenchymal cells towards the apical ectodermal ridge (AER; Li and Muneoka, 1999). To determine 1) whether primitive streak mesodermal
Figure 1. *in vitro* assays for mesodermal cell migration and chemotaxis.

(A-D) Primitive streak explants were dissected from late streak-staged chimeric embryos and cultured on a fibronectin substratum for 3 days to assess mesoderm cell migration. (B) No segregation of *Fgfr1* +/− and WT cells was observed in control *Fgfr1* +/− chimeric explants. (C) *Fgfr1* −/− cells (blue) are capable of migrating away from the periphery of chimeric explants. *Fgfr1* −/− and WT cell (pink) populations tended to segregate in culture. (D) Addition of bFGF to explant cultures had no effect on relative migration rates.

(E-H) Mixed populations of *Fgfr1* −/− and WT mesoderm were generated by culturing anterior epiblast explants of chimeric embryos with function perturbing anti-E-cadherin antibodies. After 3 days of culture on fibronectin, *Fgfr1* −/− chimeric explants (G) closely resembled controls (F), with no apparent differences in the rate of *Fgfr1* −/− and WT mesoderm migration. (H) Addition of bFGF to explant cultures had no effect on relative migration rates.

(I-J) Transverse sections through the primitive streaks of E7.5 WT (I) and *Fgfr1* −/− (J) embryos show normal fibronectin expression in *Fgfr1* mutants, as visualized by indirect immunofluorescence.

(K-L) FGF8-coated beads were implanted next to primitive streak explants to assay for chemotactic responses by WT (K) or *Fgfr1* −/− (L) mesodermal cells. Explants are shown after 48 hrs of culture; explant margins have been depicted as they were after 4hrs and 24hrs of culture. FGF8 attracted the outgrowth of WT, but not *Fgfr1* −/− mesodermal cells.
Figure 1:
cells exhibit a chemotactic response to FGFs, and 2) whether \textit{Fgfr1}^{-/-} mesoderm is deficient in such a response, the explant migration experiments were repeated and assayed for cell migration towards or away from a localized source of FGF.

Primitive streak explants were allowed to attach to a fibronectin substratum and were then embedded in collagen matrix in the presence of either BSA- or FGF8-coated heparin beads. The progress of mesoderm migration was followed over the course of two days. In approximately half of the WT explants (n=14/27; Figure 1K), an outgrowth of mesodermal cells was observed in the direction of the FGF-coated beads; no such localized outgrowth was observed when explants were cultured with BSA-beads (data not shown). Migration towards FGF-coated beads was never observed in \textit{Fgfr1}^{-/-} explants (n=16; Figure 1L). The outgrowth of WT cells towards FGF-coated beads (Figure 1K) could represent a localized mitogenic response or a directed chemotactic response. We favor a role for FGFR1 in chemotaxis, because mitogenic responses were not observed when FGF was added directly to the culture media of mesoderm explants (Figure 1D,H).

**Ectopic E-cadherin expression at the primitive streak of \textit{Fgfr1} mutants**

In \textit{Fgfr1} mutant analyses, observed failures in epithelial to mesenchymal transition at gastrulation and the accumulation and sorting of \textit{Fgfr1}^{-/-} cells at the base of the primitive streak (Yamaguchi et al., 1994; Deng et al., 1994; Ciruna et al., 1997), might also be explained by defects in cell adhesion. E-cadherin is normally expressed throughout the epiblast and endoderm of the early embryo but is down-regulated as progenitor cells undergo an EMT at the primitive streak; E-cadherin is not expressed in the nascent mesodermal germ layer (Figure 2A,G; Damjanov et al., 1986). Since down-regulation of E-cadherin expression has been directly implicated in the differentiation and migration of mesoderm at gastrulation (Burdsal et al., 1993), the expression of E-cadherin in the primitive streak of \textit{Fgfr1} mutant embryos was examined by immunofluorescence (Figure 2D,H,I).

Transverse sections through E7.5 mutant embryos revealed that \textit{Fgfr1}^{-/-} progenitor cells, accumulating within the swollen primitive streak, maintained high levels of E-cadherin at their cell membranes (Figure 2D). At E8.5, posterior transverse sections through \textit{Fgfr1}^{-/-} embryos revealed large masses of progenitor cells accumulating
beneath the primitive streak (Figure 2H,I). These cells expressed ectopically high levels of E-cadherin in a domain much larger than is normally found at the streak (compare Figure 2G), and at levels greater than the overlying ectoderm.

**E-cadherin expression is regulated by FGFR1 signalling**

To further evaluate the role for FGF signalling in cell adhesion, E-cadherin levels were examined in chimeric primitive streak cultures (Figure 3A-D). WT cell contribution was visualized by transgenic GFP expression (Figure 3B), and E-cadherin expression was determined by immunofluorescence (Figure 3C,D). It was observed that after 3 days of culture, patches of Fgfr1-/- mesodermal cells tended to maintain high levels of E-cadherin at their cell membranes (Figure 3D), whereas WT cells of the same explants had down-regulated E-cadherin expression.

To determine whether ectopic E-cadherin expression resulted from the cell-autonomous requirement for FGFR1 function at the streak, or from secondary effects due to disrupted gastrulation, E-cadherin levels were examined in WT and Fgfr1 -/- cells found side by side at the primitive streak of phenotypically normal chimeric embryos (Figure 3E-G). In this chimeric analysis, WT cells ubiquitously expressed the ROSA26 LacZ transgene (Friedrich and Soriano, 1991), and were visualized by anti- β-galactosidase staining (Figure 3F). No qualitative differences in E-cadherin expression could be discerned between WT epiblast cells and Fgfr1 -/- cells (asterisk) adjacent to the primitive streak. However, although WT mesoderm cells lost E-cadherin expression after traversing the streak, the few Fgfr1 -/- cells that entered the mesodermal germ layer maintained high levels of E-cadherin expression at their cell membranes (arrowhead). These studies provide strong evidence that FGFR1 signalling is required for normal down-regulation of E-cadherin expression at gastrulation.

**Snail expression is down-regulated in Fgfr1 mutants**

The zinc finger transcription factor Snail has been implicated in regulating the epithelial to mesenchymal transitions of gastrulation. In *Drosophila*, Snail functions to repress DE-cadherin expression and is required for mesoderm formation and invagination.
Figure 2. Localization of E-cadherin and β-catenin at the primitive streak.
(A-F) Confocal micrographs showing immuno-localization of E-cadherin (A,D) and β-catenin (B,E) in transverse sections through the primitive streak of E7.5 WT (A-C) or Fgfr1 -/- (D-F) embryos. (A) E-cadherin is normally expressed at the cell membrane of the primitive ectoderm (e), is down-regulated in progenitor cells as they traverse the primitive streak (ps), and is not expressed in nascent mesoderm (m). (B) WT β-catenin co-localizes with E-cadherin in the ectoderm, and accumulates in the cytosol of E-cadherin- negative mesodermal cells (arrowhead). (C) Overlay. (D) In Fgfr1 -/- embryos, progenitor cells accumulate within the primitive streak and maintain E-cadherin expression at their cell membranes. (E) β-catenin expression co-localizes with E-cadherin, and is not observed in the cytosol. (F) Overlay.
(G-I) De-convolved images of E-cadherin localization in the primitive streaks of WT (G) and Fgfr1 -/- (H and I) headfold-staged embryos. (H and I) In Fgfr1 mutants, E-cadherin is ectopically expressed in progenitor cells accumulating beneath the primitive streak.
Figure 2:
Figure 3. Down-regulation of E-cadherin requires FGFR1.

(A-D) *Fgfr1*^-/-* chimeric primitive streak explant after 3 days of culture on fibronectin. (A) DIC image of explant culture. (B) WT cells were distinguished by positive GFP expression; the border between WT and *Fgfr1*^-/-* cell populations has been indicated. (C) E-cadherin expression was visualized by immunofluorescence. Although E-cadherin expression was not observed above background levels in WT cells after 3 days of culture, patches of *Fgfr1*^-/-* cells tended to maintain E-cadherin expression at their cell membranes (arrows).

(E-G) Transverse section through the primitive streak of an E7.5 *Fgfr1*^-/-* chimera. (F) WT cells were distinguished by positive anti-β-galactosidase immunostaining. *Fgfr1*^-/-* cells are observed in the mesoderm (arrowhead) and in the epiblast layer (asterisk), accumulating adjacent to the primitive streak (ps). (E) Immunostaining for E-cadherin reveals that E-cadherin is not down-regulated by the few *Fgfr1*^-/-* cells that enter the mesoderm layer (arrowhead). (G) Overlay.
Figure 3:
at the ventral furrow; and in Xenopus and zebrafish. Snail homologues are expressed in the marginal zone of gastrulating embryos at the site of mesoderm involution (reviewed in Hemavathy et al., 2000). During mouse embryogenesis, mSnail is expressed in cell populations which will become migratory, including the primitive streak, nascent mesoderm, decondensing somites, neural crest, and mesenchymal cells of the limb bud (Figure 3A-D; Nieto et al., 1992; Smith et al., 1992). mSnail has been shown to directly repress E-cadherin expression and to induce a dramatic EMT when overexpressed in epithelial cell lines (Batlle et al., 2000; Cano et al., 2000). Furthermore, mouse embryos homozygous for a mutant mSnail allele die late in gastrulation with mesodermal cells retaining epithelial characteristics, including the expression of E-cadherin (T. Gridley, pers. comm.). Data suggest therefore, that mSnail represses E-cadherin expression at gastrulation, and plays an important role in the morphogenesis of the mesodermal germ layer.

mSnail expression was examined in WT and Fgfrl -/- embryos by whole-mount RNA in situ hybridization (Figure 4). As in WT embryos, mSnail was expressed in the very early primitive streak of E6.5 Fgfrl mutants (Figure 4E). However, this early signal diminished and only faint mSnail expression was observed in Fgfrl -/- embryos at mid-to late-streak stages (Figure 4F). At E8.5, only a small domain of mSnail expression was observed at the base of the allantois, at the most posterior end of the streak (Figure 4G).

Loss of mSnail expression in the primitive streak of Fgfrl mutants could therefore explain ectopic E-cadherin levels and the observed defects in EMT and cell migration. Although both the initial expression of mSnail at gastrulation (Figure 4E) and mSnail expression at the most posterior domain of the streak (Figure 4G) are independent of FGFR1 function, this is consistent with the Fgfrl -/- phenotype. Fate-mapping studies have demonstrated that these regions of the primitive streak will generate extra-embryonic mesoderm (Parameswaran and Tam, 1995; Kinder et al., 1999), a population that forms normally in Fgfrl -/- embryos. Our results demonstrate that FGFR1 is required for mSnail expression in the domain of the late-primitive streak fated to generate embryonic mesoderm. We propose therefore, that FGFR1 signalling maintains mSnail expression in the late primitive streak, thus promoting the down-regulation of E-cadherin (Figure 8).
Figure 4. *mSnai1* expression is lost in *Fgfr1* -/- embryos.
(A-D) *mSnai1* expression in WT embryos from E6.5 to E9.5. Abbreviations: primitive streak (ps); headfold (hf), allantois (al), somite (s); branchial arch (ba); limb bud (lb).
(E-G) In *Fgfr1* -/- embryos, *mSnai1* is expressed in the early primitive streak (E), but expression is lost as gastrulation proceeds (F). (G) At headfold stages, *mSnai1* expression is localized to a small domain at the base of the allantois (arrowhead).
Figure 4:
**FGFR1 regulates Tbx6 expression**

Although abnormal intercellular adhesion can account for defective morphogenetic movements at gastrulation, its contribution to the patterning defects observed in 
*Fgfrl* -/- embryos remains unclear. Fate mapping studies demonstrate that the order and the site of progenitor-cell ingress through the streak will determine the fate of mesodermal cells (Kinder et al., 1999). However, global abnormalities in morphogenesis through the streak do not adequately explain the very specific patterning defects observed in *Fgfrl* mutant analyses; in particular, severe reductions in paraxial mesoderm formation in *Fgfrl* -/- embryos (Yamaguchi et al., 1994; Deng et al., 1994) and the formation of ectopic neural tubes in *Fgfrl* -/- chimeric embryos (Ciruna et al., 1997; Deng et al., 1997). In an attempt to determine how FGFR1 regulates progenitor cell specification at the primitive streak, T-box gene expression was examined in *Fgfrl* mutant embryos. T-box transcription factors have been shown to play essential roles in early development, especially in the specification and patterning of the mesodermal germ layer (see reviews by Papaioannou and Silver, 1998; Smith, 1999). Furthermore, studies in zebrafish and *Xenopus* have established an intimate link between FGF signalling and the expression of T-box genes at gastrulation (Griffin et al., 1995; Griffin et al., 1998; and reviewed in Slack et al., 1996).

The T-box gene *Tbx6* plays a critical role in the specification and differentiation of paraxial mesoderm during gastrulation, and in the absence of *Tbx6*, cells destined to form somites differentiate into ectopic neural tubes (Chapman and Papaioannou, 1998). The expression of *Tbx6* was therefore examined in WT and *Fgfrl* mutant embryos by whole-mount RNA *in situ* hybridization (Figure 5A-D). *Tbx6* is expressed strongly throughout the late primitive streak and presomitic paraxial mesoderm (Figure 5A,C; Chapman et al., 1996). In *Fgfrl* -/- embryos, *Tbx6* expression was found to be much reduced. The onset of *Tbx6* expression was delayed until E8.0, and *Tbx6* was barely expressed above background levels, except for a small domain at the anterior streak (Figure 5B,D).

A chimeric analysis was performed to determine whether *Tbx6* is regulated by FGF signalling, or whether cells that would normally express *Tbx6* simply failed to form in *Fgfrl* -/- embryos due to retarded development and abnormal morphogenesis at the
streak. *Tbx6* expression was compared between *Fgfr1* -/- and WT progenitor cells found together in the epiblast layer of the primitive streak of phenotypically normal chimeric embryos (Figure 5E-G; n=8). *Tbx6* expression was visualized by fluorescent whole-mount RNA *in situ* hybridization (Figure 5F) and *Fgfr1* -/- cells, which expressed the ROSA26 LacZ transgene, were distinguished by anti- β-galactosidase immunofluorescence (Figure 5E). At E7.5, *Tbx6* is strongly expressed in the nascent mesoderm, and is expressed transiently in WT progenitor cells in the epiblast that are fated to traverse the primitive streak (Figure 5F). Adjacent *Fgfr1* -/- epiblast cells in the same region fail to express *Tbx6* (arrowhead). This suggests that *Tbx6* expression is positively regulated by FGFR1.

**FGFR1 is required for Brachyury expression in the posterior streak**

*Brachyury* (*T*) is the canonical member of the T-box gene family. Genetic and embryological studies across vertebrate species have revealed a conserved role for *T* in notochord maintenance, axis elongation, and the specification of posterior mesoderm populations (Smith, 1997). The domain of *T* expression is extended in *Fgfr1* -/- embryos in a manner which correlates well with the observed expansion of the axial mesoderm population (Deng et al., 1994; Yamaguchi et al., 1994). However, studies in *Xenopus* and zebrafish have demonstrated that FGF signalling is required for *Brachyury* expression at gastrulation (Griffin et al., 1995; and reviewed in Smith, 1997). A chimeric analysis was performed to re-examine the role for FGFR1 in regulating *T* expression at the primitive streak.

*T* expression was visualized by fluorescent whole-mount RNA *in situ* hybridization (Figure 6F,I), and *Fgfr1* -/- cells were distinguished by anti- β-galactosidase immunofluorescence (Figure 6E,H). Transverse sections of chimeric embryos at E7.5 show that *T* is expressed throughout WT primitive ectoderm and mesoderm of the streak; however, *T* expression is down-regulated in *Fgfr1* -/- progenitor cells within the primitive streak (Figure 6E-G, arrowhead). To determine whether *Fgfr1* is required for earlier expression of *Brachyury* at the streak, chimeric embryos were examined at E6.5 (Figure 6H-J). Sagittal sections reveal that *Fgfr1* -/- progenitor cells at the posterior streak (Figure 6H) have already down-regulated *T* expression (Figure 6I).
Figure 5. Tbx6 is down-regulated at the primitive streak of Fgfr1 -/- embryos.

(A-D) Whole-mount analysis of Tbx6 expression in WT (A,C) and Fgfr1 -/- embryos (B,C) showing that Tbx6 is down-regulated in the posterior streak of Fgfr1 mutants.

(E-G) Chimeric analysis of Tbx6 expression. (E) Fgfr1 -/- cell-contribution to chimeric embryos was distinguished by positive anti- β-galactosidase staining. (F) Tbx6 expression was visualized by fluorescent RNA in situ hybridization. At E7.5, Tbx6 is expressed in WT progenitor cells and in the nascent mesoderm (m) at the primitive streak (ps). Tbx6 expression is down-regulated in Fgfr1 -/- progenitor cells (arrowhead) at the streak. (G) Overlay.
Figure 5:
The expression of Brachyury in Fgfr1-/- embryos was re-examined at later stages of gastrulation, using standard whole-mount RNA in situ hybridization (Figure 6B). Fgfr1-/- embryos showed the characteristic expansion of T expression within the presumptive node and notochord. However, T expression was missing from the primitive streak, with the exception of a small patch of expression at the base of the allantois (Figure 6B). This suggests that FGFR1 is required for T expression within regions of the primitive streak fated to give rise to paraxial and lateral mesoderm populations (Parameswaran and Tam, 1995; Kinder et al., 1999).

Although previous analysis of Fgfr1-/- embryos demonstrated an expanded domain of T expression (Deng et al., 1994; Yamaguchi et al., 1994), we propose that early irregularities in T expression within the primitive streak were masked by patterning defects, particularly by the expansion of the node and axial mesoderm populations. The expression of a T-lacZ reporter transgene was examined in the Fgfr1 mutant background. The lacZ reporter is regulated by a 500bp proximal element of the Brachyury promoter that recapitulates endogenous Brachyury expression within the primitive streak only (Figure 7A; Clements et al., 1996). The T-lacZ reporter is not active in the axial mesoderm, node, and anterior portion of the early primitive streak (Figure 7A); the same T expression domains which are independent of FGFR1 and show expansion in Fgfr1 mutants. T-LacZ expression was not observed in Fgfr1 mutant embryos at E7.0 (n=4). At E8.0, some T-lacZ expression was observed in the allantois of mutant embryos, but no expression was observed within the primitive streak (n=5; Figure 7B). No T-lacZ activity was observed at E9.0 (n=3; data not shown). Chimeric analysis, gene expression profiles and T-lacZ reporter data suggest that FGFR1 is required for the initiation of T-lacZ expression within the streak, for maintained T expression in the medial region of the late-primitive streak, but not for T expression in the node, axial mesoderm or allantois.

Attenuated Wnt signalling in Fgfr1 mutants

Late in gastrulation, Brachyury is a direct target of the Wnt/ß-catenin signalling pathway. Two canonical TCF1 binding sites have been identified within the proximal element of the T promoter; mutation of these sites disrupts ß-catenin-dependent transactivation of reporter constructs in vitro, and abrogates primitive streak expression
Figure 6. FGFR1 regulates Brachyury expression in the primitive streak.

(A-B) In WT embryos (A), T is expressed throughout the primitive streak (ps), node and notochord (n). (B) In Fgfr1 -/- embryos, T expression is observed in the presumptive node and notochord, and at the base of the allantois (al). However, T expression is lost within the mid-segment of the streak.

(C-D) Wnt3a, which is also expressed throughout the WT primitive streak (C), is expressed normally along the streak of Fgfr1 -/- embryos (D).

(E-G) Transverse section through the primitive streak of an E7.5 chimeric embryo. (E) Fgfr1 -/- cell contribution to the chimera was distinguished by positive anti- β-galactosidase staining. (F) T expression was visualized by fluorescent RNA in situ hybridization. (G) Overlay, showing that Fgfr1 -/- cells, which accumulate at the primitive streak, down-regulate T expression (arrowhead).

(H-J) Sagittal section through the primitive streak of an E6.5 chimeric embryo. (H) Fgfr1 -/- cells were distinguished by positive anti- β-galactosidase staining. (I) T expression was visualized by fluorescent RNA in situ hybridization. (J) Overlay, showing that Fgfr1 -/- cells in the posterior primitive streak down-regulate T expression as early as E6.5 (arrow).
Figure 6:
of the reporter \textit{in vivo} (Yamaguchi et al., 1999; Arnold et al., 2000). Furthermore, in \textit{Wnt3a} \textsuperscript{-/-} embryos, \textit{T} expression is down regulated in a similar domain of the primitive streak to that observed to be affected in \textit{Fgfrl} \textsuperscript{-/-} embryos (Yamaguchi et al., 1999).

In addition to the loss of \textit{T} expression, the phenotype of \textit{Wnt3a} \textsuperscript{-/-} embryos has much in common with that of \textit{Fgfrl} mutant and chimeric embryos. \textit{Wnt3a} mutants show posterior truncations and lack paraxial mesoderm derivatives, they display abnormal morphogenesis at the primitive streak, and form ectopic neural tubes (Takada et al., 1994; Yoshikawa et al., 1997). Although \textit{Wnt3a} \textsuperscript{-/-} embryos develop further than \textit{Fgfrl} mutants, \textit{Wnt} and \textit{FGF} signalling appear to regulate similar morphogenetic and patterning events at gastrulation. Therefore, \textit{FGF} and \textit{Wnt} pathways may act in parallel, or alternatively, \textit{FGF} activity may regulate the \textit{Wnt} signalling pathway at the primitive streak.

The loss of both \textit{T} and \textit{T-LacZ} expression (direct targets of \textit{Wnt} signalling) in the late primitive streak of \textit{Fgfrl} \textsuperscript{-/-} embryos indicates that the \textit{Wnt3a} signalling pathway is being attenuated in the absence of \textit{FGFR1} activity. \textit{Wnt3a} expression was examined in \textit{WT} and \textit{Fgfrl} mutant embryos by whole-mount RNA in situ hybridization (Figure 6C,D). \textit{Wnt3a} expression is first detected at E7.5, and extends through much of the primitive streak (Figure 6C; Takada et al., 1994). In \textit{Fgfrl} \textsuperscript{-/-} embryos, \textit{Wnt3a} is expressed in its typical domain along the length of the streak (Figure 6D); this contrasts sharply with the observed loss of \textit{T} expression (Figure 6B). Thus, \textit{Wnt} signalling is being repressed in \textit{Fgfrl} \textsuperscript{-/-} embryos at a level downstream of ligand expression.

\textit{\beta-catenin} remains associated with ectopically expressed \textit{E-cadherin} in the streak of \textit{Fgfrl} \textsuperscript{-/-} embryos

The canonical \textit{Wnt} signalling pathway is regulated by the availability of 'stabilized' cytosolic \textit{\beta-catenin} (reviewed in Willert and Nusse, 1998). In the absence of \textit{Wnt} signal, cytosolic \textit{\beta-catenin} is believed to be phosphorylated by glycogen synthase kinase 3B (GSK3B), a serine/threonine kinase and negative regulator of the \textit{Wnt} pathway. Phosphorylation of \textit{\beta-catenin} destabilizes the protein, and targets it for destruction. However, in the presence of a \textit{Wnt} signal, GSK3B is inactivated. \textit{\beta-catenin} fails to be phosphorylated, it accumulates in the cytoplasm and then enters the nuclei where,
together with members of the LEF/TCF family of DNA binding proteins, it activates
transcription of Wnt responsive genes. β-catenin also functions at the cell membrane
where it is complexed with cadherin molecules and is required for intercellular adhesion
(see Willert and Nusse, 1998).

FGFR1 could regulate the Wnt signalling pathway at the streak by influencing
levels of cytosolic β-catenin. In Fgfr1 -/- embryos, E-cadherin is ectopically expressed at
the primitive streak (Figure 2D,H-I). It has been demonstrated that E-cadherin has a
potent ability to sequester free β-catenin to the cell membrane, and to prevent its
association with LEF/TCF proteins (Orsulic et al., 1999). Ectopic E-cadherin levels could
thereby attenuate a Wnt signalling response. The localization of β-catenin at the primitive
streaks of WT and Fgfr1 -/- embryos was therefore examined by confocal analysis
(Figure 2B,E). In E7.5 WT embryos, cytosolic levels of β-catenin rise after mesodermal
cells down-regulate E-cadherin and traverse the primitive streak (Figure 2B, arrowhead).
In Fgfr1 mutant embryos, however, β-catenin does not accumulate in the cytoplasm.
Rather β-catenin co-localizes with E-cadherin at the cell membrane of Fgfr1 -/-
progenitor cells accumulating within the primitive streak (Figure 2F). These results are
consistent with the possibility that high E-cadherin levels at the streak of Fgfr1 mutants
attenuate Wnt3a signalling by appropriating stabilized β-catenin from a cytosolic
“signalling-competent” pool.

**Disrupting E-cadherin in Fgfr1 -/- primitive streak explants restores Wnt activity**

If ectopic E-cadherin levels in Fgfr1 -/- embryos sequester free β-catenin and
repress Wnt signal transduction, then down-regulation of E-cadherin expression at the
primitive streak of Fgfr1 mutants should rescue Wnt signalling activity. To test this
hypothesis, primitive streak explants were cultured overnight (16-18 hours) with function
perturbing anti- E-cadherin antibodies: immunofluorescent analysis has demonstrated that
culture of explants in the presence of anti- E-cadherin antibodies causes the loss of E-
cadherin protein from the cell surface (Burdsal et al., 1993; and data not shown). T-lacZ
reporter activity was used to assay for a Wnt signalling response, as the T promoter
element has been shown to be a direct target of Wnt signalling (Yamaguchi et al., 1999;
Arnold et al., 2000).
WT primitive streak explants, cultured overnight on fibronectin, express T-LacZ in its normal domain along the primitive streak and nascent mesoderm (Figure 7C). WT primitive streak explants cultured overnight on Wnt3a-expressing 3T3 cells show a dramatic induction of T-LacZ activity (Figure 7E). This provides further evidence that Wnt signalling positively regulates T-LacZ reporter expression. When WT explants were cultured overnight on fibronectin in the presence of anti-E-cadherin antibodies, no changes from the control T-LacZ staining pattern were observed (Figure 7D). This indicates that anti-E-cadherin antibody treatment does not induce an ectopic or artifactual Wnt response in WT cells.

Explants encompassing the primitive streak of E8.5 Fgfr1 mutant embryos were then cultured overnight on a fibronectin substratum, in chemically defined media, with or without anti-E-cadherin antibodies (Figure 7F,G). Fgfr1 -/- control explants did not show T-lacZ activity within the primitive streak (n=9; Figure 7F). However, in the presence of anti-E-cadherin antibodies, T-lacZ reporter expression was induced (n=10; Figure 7G). Since explants were cultured in a “Wnt-free” chemically defined media, and since anti-E-cadherin treatment does not induce ectopic T-LacZ expression in WT embryos, results suggest that the disruption of ectopic E-cadherin in Fgfr1 -/- embryos can rescue endogenous Wnt signalling at the primitive streak.

Similar experiments were then performed examining endogenous T expression (Figure 7H-K). WT explants, cultured in chemically defined media alone, displayed the expected domains of T expression within the notochord, node and primitive streak (n=13; Figure 7H). No differences in Brachyury expression were observed after WT explants were cultured with anti-E-cadherin antibodies (n=15; Figure 7I). Fgfr1 -/- explants cultured without E-cadherin antibodies showed a strong medial domain of Brachyury expression at the anterior end of the explant, demarcating the characteristically expanded notochord of Fgfr1 mutant embryos (n=10; Figure 7J). Brachyury was not expressed in the primitive streak of Fgfr1 -/- explants, in accordance with in vivo analysis of T expression in Fgfr1 mutant embryos (Figure 6B). However, after overnight culture with anti-E-cadherin antibodies, Brachyury expression was observed in the primitive streak of Fgfr1 -/- explants (n=11; Figure 7K).
Figure 7. Down-regulating E-cadherin in Fgfr1-/- embryos restores Wnt signalling at the streak.

(A) In WT embryos, T-LacZ reporter activity recapitulates T expression in the primitive streak only; T-LacZ is not expressed in the notochord or node.

(B) In Fgfr1-/- embryos, T-LacZ is expressed in the allantois at E8.0; however, expression is never observed in the primitive streak (arrowheads).

(C-G) T-LacZ activity in primitive streak explants. (C) Explants from E7.5 WT embryos were dissected and cultured overnight (o/n) on fibronectin, and stained for T-LacZ activity. (D) WT explants cultured o/n with function perturbing anti-E-cadherin antibodies resembled controls. (E) WT explants cultured o/n with Wnt3a-expressing 3T3 cells showed a dramatic induction of T-LacZ activity. (F) E8.5 Fgfr1-/- explants cultured on fibronectin o/n did not express T-LacZ. (G) However, T-LacZ expression was rescued in the primitive streaks of Fgfr1-/- explants (asterisk) after o/n culture with anti-E-cadherin antibodies.

(H-I) Brachyury expression is observed throughout the primitive streak and notochord of E7.5 WT explant cultures. No differences in T expression were observed between explants cultured o/n with anti-E-cadherin antibodies (I) and untreated controls (H).

(J-K) Brachyury expression in E8.5 Fgfr1-/- explant cultures. (J) T expression is observed in the notochord at the anterior (a) end of Fgfr1-/- control explants; no staining is observed in the primitive streak. (K) After o/n anti-E-cadherin treatment, T expression is observed within the primitive streak (asterisk) at the posterior (p) end of Fgfr1-/- explants.
Figure 7:
Therefore, culture with function perturbing anti-E-cadherin antibodies, which result in down-regulated E-cadherin expression, can rescue both T-lacZ and endogenous Brachyury expression within the primitive streak of Fgfr1 mutant explants. This rescued T-lacZ and Brachyury expression was induced by endogenous Wnt3a signals, since explants were cultured in a Wnt-free chemically defined media. These results provide further evidence that the Wnt signalling pathway is intact and functional, but repressed in Fgfr1 -/- embryos. Presumably, anti-E-cadherin treatment relieves this repression by providing a cellular environment in which, after the reception of Wnt signals, stabilized β-catenin is free to accumulate and activate target genes without first being sequestered to the cell membrane by the overwhelming binding capacity of ectopically expressed E-cadherin.

DISCUSSION

Results from the Fgfr1 mutant expression analyses, chimeric studies, and in vitro explant experiments can be assembled into a minimal model for FGFR1 function at gastrulation (Figure 8). This study has defined a specific region of the primitive streak that requires FGFR1 signalling activity; this domain encompasses the paraxial and posterior embryonic mesoderm populations, but excludes the node, axial and extra-embryonic mesoderm. In the context of this domain, we propose that FGFR1 signalling orchestrates both the morphogenetic movement and cell fate specification events of gastrulation.

We have shown that FGFR1 regulates the morphogenesis and migration of mesodermal cells by differentially regulating intercellular adhesion properties of progenitor-populations in the primitive streak. More specifically, we demonstrate that FGFR1 signalling is required for the expression of mSnail - a key mediator of epithelial to mesenchymal transitions in development and disease (reviewed in Hemavathy et al., 2000). Furthermore, we propose that mSnail expression downstream of FGFR1 is required for the normal down-regulation of E-cadherin. Given the morphoregulatory roles for differential cell adhesion during embryogenesis (see Takeichi, 1995; Huber et al., 1996), ectopic E-cadherin expression at the primitive streak of Fgfr1 mutants provides a molecular explanation for the observed defects in EMT, progenitor cell migration, and
the sorting of Fgfrl -/- from WT cells during gastrulation. Although chemotaxis assays also indicate a possible role for FGF signalling in directing the migration of mesodermal cells from the primitive streak, the importance of this function in vivo remains unclear (Sun et al., 1999). However, other aspects of the Fgfrl mutant phenotype could be explained by chemotactic deficiencies. In particular, if FGFs direct the migration of limb mesenchymal cells towards the AER (Li and Muneoka, 1999), chemotactic defects could account for the accumulation of Fgfrl -/- cells in the proximal mesenchyme of chimeric limb buds, and their exclusion from the progress zone (Deng et al., 1997; Saxton et al., 2000).

Beyond its morphoregulatory role at gastrulation, FGFR1 also functions in the specification of mesoderm cell fate. Chimeric analyses demonstrate that FGFR1 is required for T and Tbx6 expression in the primitive streak. The down-regulation of T and Tbx6 expression in Fgfrl -/- mesoderm progenitor cells can account for both the reduction of paraxial and posterior mesoderm, and for the formation of ectopic neural tubes observed in Fgfrl mutant and chimeric analyses. As studies in zebrafish and Xenopus have also established the function of FGFs in T-box gene regulation and posterior mesoderm specification (Griffin et al., 1995; Griffin et al., 1998; and reviewed in Slack et al., 1996), these results further support an evolutionarily conserved pathway for FGF signalling at gastrulation.

Although the mechanisms by which FGFR1 signalling regulates both the morphogenesis and patterning of mesoderm at gastrulation have been discussed separately, the two pathways are intricately entwined. Fate mapping studies demonstrate that the order and site of progenitor-cell ingression through the primitive streak determines the spatial allocation and fate of mesodermal cells (Kinder et al., 1999; and reviewed in Tam and Behringer, 1997). Also, signals implicated in the specification and patterning of mesoderm play important morphoregulatory roles. Gene dosage and chimeric analyses of Brachyury function have demonstrated that the level of T expression in progenitor cell populations influences the timing and pattern of ingression through the primitive streak (Wilson et al., 1993; Wilson et al., 1995; Wilson and Beddington, 1997). T-box genes may also regulate cell adhesion and EMT at gastrulation. In zebrafish, the Brachyury homologue no tail, and the T-box gene spadetail have both been implicated as positive regulators of Snail expression (Thisse et al., 1993; Thisse et al., 1995). Although
regulation of mouse Snail by T has yet to be determined, it is intriguing that in late-gastrula staged Fgfr1-/- embryos, the only observed domain of mSnail expression (Figure 4G) overlaps with an Fgfr1- independent domain of T expression at the base of the allantois (Figure 6B). Therefore, T may positively regulate Snail expression at the primitive streak (Figure 8), providing another link between Brachyury expression, intercellular adhesion, and the morphogenesis of the mesodermal germ layer.

In addition, we propose that FGFR1 signalling indirectly regulates Wnt signal transduction at the primitive streak. In Fgfr1-/- embryos, although Wnt3a is expressed in the late primitive streak, direct targets of Wnt signalling (i.e. Brachyury and the T-LacZ reporter transgene) are not activated. We suggest that ectopic E-cadherin expression in Fgfr1 mutants attenuates Wnt3a signalling by sequestering free β-catenin from its intracellular signalling pool, and demonstrate that forced down-regulation of E-cadherin in Fgfr1-/- explants can rescue endogenous Wnt signalling at the primitive streak. Evidence that cadherins act as regulators of β-catenin signalling is well documented. E-cadherin and LEF-1 bind to partially overlapping sites in the central region of β-catenin (reviewed in Willert and Nusse, 1998); consequently, LEF-1 and E-cadherin form mutually exclusive complexes with β-catenin and compete for the same intracellular signalling pool (Orsulic et al., 1999). Furthermore, overexpression of cadherins during Drosophila and Xenopus embryogenesis has been shown to phenocopy Wnt/β-catenin signalling mutants (Sanson et al., 1996; Heasman et al., 1994; Fagotto et al., 1996).

It is well established that Wnt signalling stabilizes cytosolic levels of β-catenin by inhibiting its GSK3β-mediated phosphorylation and degradation (reviewed in Willert and Nusse, 1998). At gastrulation, loss of E-cadherin expression downstream of FGFR1 may facilitate a rapid intracellular transfer of membrane-bound β-catenin to the cytosolic “signalling” pool. Since down-regulation of E-cadherin alone is not sufficient to induce ectopic activation of T-LacZ and Brachyury expression in WT primitive streak cultures, signalling through the β-catenin pathway is still dependent on the activity of localized Wnt signals. However, FGF-mediated changes in cadherin levels and β-catenin localization could still regulate the threshold for and/or speed of Wnt signalling responses at gastrulation. We propose, therefore, that normal down-regulation of E-cadherin at the primitive streak not only regulates the EMT and migration of mesoderm progenitor cells at gastrulation, but also permits the rapid and uninhibited accumulation of cytosolic β-
catenin levels in response to localized Wnt signals. This competition for and opposing influences on the intracellular localization and function of β-catenin thus establishes a molecular link between the FGF and Wnt signalling pathways at gastrulation. Consequently, FGFR1 activity plays an indirect but permissive role in the propagation of Wnt signalling responses at the primitive streak. The fundamental inter-regulation of cell adhesion, morphogenesis and cell fate determination, as demonstrated in this analysis of FGFR1 function, serves to underscore the interdependent nature of morphogenesis and patterning at gastrulation, and the intricate network of inductive interactions which pattern and shape the developing embryo.
Figure 8. Model of FGFR1 signalling in the late-primitive streak.
A summary of the targets of FGF signalling at gastrulation, as based on genetic evidence presented in the text. Initiation of $T$ and $Tbx6$ expression in the posterior streak requires FGFR1 signalling. Later in gastrulation, maintenance of $T$ expression at the streak requires Wnt3a signalling (Yamaguchi et al., 1999), and maintenance of $Tbx6$ expression requires $T$ (Chapman et al., 1996). FGFR1 indirectly regulates $T$ expression at this stage by modulating Wnt3a signalling via regulation of the intracellular localization and hence accessibility of free $\beta$-catenin. Positive regulation of $Snail$ expression by Brachyury is only inferred.
Figure 8:
EXPERIMENTAL PROCEDURES

Mice

A colony of outbred mice heterozygous for a null allele of Fgfrl (Yamaguchi et al., 1994) was maintained for these studies. Male heterozygotes were crossed to ICR (Harland Sprague Dawley) random-outbred females to generate stock for timed matings. Noon of the day on which the vaginal plug was detected was considered as embryonic day 0.5 (E0.5). Fgfrl mutant embryos were identified by phenotypic characteristics after E8.5. At earlier stages, embryos were genotyped by standard polymerase chain reaction analysis using either extra-embryonic tissues or fragments of the embryo proper as a DNA source. The Fgfrl mutant allele was identified by a 0.9 kb DNA fragment amplified using a 5'-aagccacatcacc tgaggaa-3' and 5'-tgggattagataaetgcc tgc tc-3' primer pair, and the WT allele with a 5'-ttgaccggatctacacacacc-3' and 5'-gcacaccggggtatggggagc-3' primer pair. The annealing temperature was 58°C. Wild-type embryos for in situ hybridization, immunohistochemical analysis and explant studies were derived from timed matings between ICR males and females.

For detection of Brachyury expression in Fgfrl mutants, a T-lacZ reporter transgene was crossed into the Fgfrl mutant strain. The T-lacZ transgene was composed of a 0.5 kb fragment of the Brachyury promoter (Clements et al., 1996) driving expression of a β-galactosidase reporter cassette containing a nuclear localization signal. Males homozygous for the T-lacZ transgene were crossed to Fgfrl female heterozygotes. Male double heterozygotes were then crossed to female Fgfrl heterozygotes for timed matings. The presence of the T-lacZ reporter transgene was detected by PCR analysis using a 5'-gacaccagaccaactgtaatggtagcgac-3' and 5'-gcategcagctgggtatgggaat-3' primer pair, which are specific for the β-galactosidase gene; the annealing temperature was 58°C.

Generation of chimeric embryos

Diploid Fgfrl mutant chimeric embryos were generated by aggregating a 4-6 cell clump of homozygous Fgfrl mutant (Fgfrl -/-) ES cells with wild-type 8-cell embryos, using the standard morula aggregation technique (Nagy and Rossant, 2000). Depending on the experiment, WT embryos were derived from matings between ICR mice, between

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ICR females and ROSA26 LacZ transgenic males (Friedrich and Soriano, 1991), or between ICR females and EGFP transgenic males (which ubiquitously express a GFP transgene; Hadjantonakis et al., 1998). The Fgfr1 -/- ES cell lines used in this study have been previously described (Ciruna et al., 1997). Tetraploid Fgfr1 -/- chimeric embryos were generated by aggregating Fgfr1 -/- ES cells with two tetraploid ICR embryos (Nagy and Rossant, 2000). It has been demonstrated that the ICR tetraploid cells do not contribute to fetal tissues of tetraploid chimeric embryos (Nagy and Rossant, 2000) and that tetraploid Fgfr1 -/- chimeric embryos phenocopy natural Fgfr1 mutant embryos (Ciruna et al., 1997). Tetraploid Fgfr1 -/- chimeric embryos have therefore been used in some immunohistochemical and gene expression analyses of the Fgfr1 mutant phenotype.

Dissection and culture of explants

Anterior ectoderm and primitive streak explants were prepared from wild-type ICR, T-lacZ/+ , Fgfr1 mutant and Fgfr1 chimeric embryos, depending on the experiment. In all cases explants were dissected manually with glass needles in cold PBS, and were then transferred into a chemically defined culture medium (Dulbecco’s modified Eagle’s medium [DMEM] with streptomycin and penicillin, supplemented with 100 μM non-essential amino acids, 1 mM sodium pyruvate, 1 μM 8-mercaptoethanol, 2 mM L-glutamine, and 15% KNOCKOUT™ Serum Replacement [Gibco BRL]). In the case of anterior ectoderm explants, the dissected anterior halves of mid-streak staged chimeric embryos were first placed in a solution of 0.5% trypsin and 2.5% pancreatin in Ca2+- and Mg2+-free PBS for 15 minutes at 4°C, so as to separate tissue layers prior to being transferred into culture medium. The visceral endoderm and any underlying mesoderm were teased apart from the anterior epiblast and discarded. Primitive streak and anterior ectoderm explants were transferred to prepared wells in 8-chamber glass slides (Nunc) and cultured at 37°C, in 5% CO2 in air.

Explants were grown on fibronectin, on confluent layers of NIH 3T3 cells or on Wnt3a-expressing 3T3 cells (Kispert et al., 1998), depending on the experiment. Wnt3a-expressing 3T3 cells were provided by L. Reichardt. Fibronectin coated slides were prepared by incubating separate wells of the 8-chamber slides with a 20 μg/mL solution of fibronectin (from bovine plasma, Sigma) in PBS at 4°C overnight. Wells were then
washed 2-3 times with PBS before addition of culture medium. The culture medium was supplemented with bFGF (25 ng/mL; R&D Systems), heparin (1 μg/mL; Sigma), or with anti-E-cadherin antibodies (1:100 dilution of anti-uvomorulin antibodies; Sigma), depending on the culture applications and experiment.

For chemotaxis studies, primitive streak explants were prepared as above, and cultured overnight in fibronectin-coated wells of 8-chamber glass slides. Explants were then covered in collagen gel, FGF8 coated heparin beads or BSA control beads were placed adjacent to the explant and the culture slides were incubated at 37°C, undisturbed, until the collagen gel had set. Collagen was prepared by dissolving lyophilized rat tail collagen (Boehringer Mannheim) in 0.05% acetic acid, pH 3.0, overnight at 4°C to a final concentration of 3 mg/mL. The collagen gel solution was prepared on ice by mixing 8 volumes of the rat tail collagen solution with 1 volume of 0.2M Hepes pH7.3, and 1 volume of X10 concentrated DMEM high glucose with streptomycin and penicillin (Gibco BRL). The collagen solution was neutralized with 1N NaOH to pH 7.3, and heparin was added to a final concentration of 1 μg/mL. Heparin beads (H5263, Sigma) were soaked overnight either in PBS containing bovine serum albumin (BSA, 1mg/ml) to prepare the control BSA-beads, or in PBS-BSA with FGF8 (0.5 mg/ml) (b isoform; R&D Systems) to prepare FGF8-beads. After the collagen solution had set, culture medium supplemented with heparin was added to each of the culture wells and explants were incubated at 37°C, 5% CO₂ for 2 days. Individual explants were photographed 4hrs, 24 hrs and 48 hrs following the addition of heparin beads.

Fgfr1 mutant and WT cell contribution to chimeric explants was distinguished by β-galactosidase staining, as described by Ciruna et al. (1997). In the case of Fgfr1 mutant chimeras which had been aggregated with WT embryos expressing the EGFP transgene (Hadjantonakis et al., 1998), WT cells were distinguished by GFP expression using standard fluorescent microscopy.

Whole-mount RNA in situ hybridization and immunofluorescence

Standard alkaline phosphatase whole-mount RNA in situ hybridizations were performed as described by Conlon and Rossant (1992). Fluorescent whole-mount RNA in situ hybridizations were performed using a combination of the Conlon protocol (Conlon and Rossant, 1992) and the Tetramethyl-rhodamine Tyramide Signal Amplification
(TSA™) system for Fluorescence in situ Hybridization (NEN, Boston MA). Briefly, digoxigenin-labeled RNA probes were hybridized and washed as per Conlon and Rossant (1992). Embryos were then rinsed twice with TNT (0.1M Tris-HCl, pH 7.5, 0.15M NaCl, 0.1% Tween-20) and blocked for at least 1 hour at room temperature in TNB (TNT containing 1% NEN TSA-direct blocking reagent). All washes and incubations involved rocking unless otherwise specified. Peroxidase conjugated anti-digoxigenin antibodies (Boehringer Mannheim) were diluted to 750 mU/mL in cold TNB, and embryos were incubated with antibody overnight at 4°C. Embryos were rinsed 3X with TNT, and washed 6-8 times, 30 min. each, at room temperature in 5 mL volumes of TNT. Embryos were transferred to 2 mL buffer tubes and rinsed in NEN Amplification Diluent. Color reaction was initiated by adding to each tube 100 μL of Tyramide Working Solution (1:25 dilution of reconstituted Tetramethyl-rhodamine tyramide in NEN Amplification Diluent). The color reaction was allowed to develop at room temperature in the dark for 30 min. to 1 hour, without rocking. The reaction was stopped with 3 rinses of TNT, followed by 2X 30 min. washes in 5 mL volumes of TNT under low light conditions. Samples were routinely left to wash overnight at 4°C. Embryos were processed through SlowFade™ antifade reagent (Molecular Probes), and staining visualized by fluorescence microscopy using standard rhodamine filters. For deconvolution microscopy, embryos were first manually dissected into transverse sections using glass needles before being mounted onto glass coverslips. The probes used for the whole-mount in situ hybridization studies were as follows: Brachyury (Herrmann, 1991), Tbx6 (Chapman et al., 1996), and mSnail (Smith et al., 1992).

Embryos or primitive streak explants for whole-mount antibody staining were fixed with 4% paraformaldehyde in PBS overnight at 4°C. Embryos were then dehydrated in methanol, bleached with 5% H₂O₂ in methanol for 2-3 hours, and stored in methanol at -20°C. Samples were rehydrated at room temperature through a graded methanol/PBT (0.1% Triton X-100 in PBS) series and blocked for 2 hours in PBBT (1% BSA in PBT) +10% goat serum, followed by overnight incubation at 4°C with either a 1:500 dilution of anti-uvomorulin (Sigma), 1:1000 dilution of anti-β-catenin (Sigma) or 1:200 dilution of anti-human fibronectin (Sigma) in PBBT + 1% goat serum, depending on the experiment. Note, for anti-fibronectin staining, embryos and antibodies were blocked in PBBT only (no goat serum was added). Samples were washed 6-8 times in 5


mL volumes of PBT, for 30 min. each at room temperature, were blocked again for 1 hour in PBBT + 10% goat serum followed by overnight incubation at 4°C with 1:200 dilution of the appropriate species-specific Texas Red-X- or Oregon Green™ 488-conjugated 2° antibody (Molecular Probes) in PBBT + 1% goat serum. Samples were again washed 6-8X 30 min. in PBT and processed through SlowFade™ antifade (Molecular probes). Whole-mount stained embryos were manually dissected into transverse sections using glass needles, mounted onto glass coverslips, and staining was visualized by confocal or deconvolution microscopy. Explants were mounted with coverslips and immunostaining visualized by conventional fluorescence microscopy.

For combined fluorescent RNA in situ hybridization and β-galactosidase immunostaining, embryos were processed for fluorescent in situ hybridization as outlined above, except that embryos were incubated with both anti-digoxigenin and anti-β-galactosidase antibodies (1:200 dilution, Cappel). After developing the tyramide fluorescence reaction, embryos were washed 2X 30 min. with TNT, rinsed with PTW (0.1% Tween-20 in PBS), blocked for 1 hour in PTWB (1% BSA in PTW), and incubated overnight, at 4°C, with 1:200 dilution of anti-rabbit Oregon Green™ 488 antibody (Molecular Probes). Embryos were washed 6-8X 30 min. in PTW, and then manually sectioned, mounted and processed as described.
Expression of the T-box gene *Eomesodermin* during early mouse development

*Portions of this chapter appear in the following publication:*


**SUMMARY**

We report the expression pattern of a murine homologue of the *Xenopus laevis* T-box gene *Eomesodermin*. *mEomes* expression is first detected in the extra-embryonic ectoderm prior to gastrulation, and persists there until head-fold stages. In the embryo proper, *mEomes* is expressed throughout the early primitive streak, nascent mesoderm and in the anterior visceral endoderm. Although *mEomes* expression disappears from the embryo at late-streak stages, a second domain of *mEomes* expression is observed in the telencephalon beginning around E10.5.

**RESULTS AND DISCUSSION**

T-box genes constitute an ancient family of transcription factors related by a conserved DNA-binding domain (the T-box). The unique developmental expression patterns of T-box genes have implicated them in multiple cell fate specification events (as reviewed in Papaioannou and Silver, 1998). To identify new T-box genes that may play roles in mesoderm patterning and morphogenesis at gastrulation, we used degenerate PCR to amplify T-box motifs from a murine embryonic day 7.5 (E7.5) primitive streak cDNA library. Among the T-box clones isolated was a murine homologue of *Xenopus laevis Eomesodermin*, a pan-mesodermal marker expressed at midblastula transition (Ryan et al., 1996). Murine *Eomesodermin* (*mEomes*) has previously been recovered from an independent EST database screen (Wattler et al., 1998). To characterize the developmental expression pattern of *mEomes*, whole-mount RNA *in situ* hybridizations were performed.
mEomes expression is observed in the epiblast at the embryonic/extra-embryonic junction, just prior to gastrulation at E6.5 (Fig. 1B, arrowhead). As the primitive streak forms, and epiblast cells delaminate through the streak to establish the mesodermal germ layer, mEomes expression is observed throughout the primitive streak, the posterior third of the epiblast, and the nascent mesoderm (Fig. 1C-G). At the mid-streak stage, mEomes is also expressed in the distal and anterior visceral endoderm (Fig. 1D,G) in a domain which overlaps that of Cerberus-like and Goosecoid (Belo et al., 1997). Recent findings associate this domain with head organizer activity (reviewed in Bouwmeester and Leyns, 1997; Beddington and Robertson, 1998).

As gastrulation progresses and the streak elongates towards the distal tip of the egg cylinder, mEomes becomes restricted to the anterior primitive streak (Fig. 2A,C) and to the mesodermal cells most proximal to the streak (Fig. 2C; insert). mEomes expression disappears from the embryo at the late-streak stage, prior to morphological node formation (Fig. 2D).

In Xenopus, Eomesoderm in is expressed throughout the mesoderm in a ventral to dorsal gradient of increasing concentration, and it has been proposed that Eomes functions in initiating mesoderm differentiation and in progressively specifying more dorsal mesoderm fates (Ryan et al., 1996). The gradual confinement of mEomes expression to the anterior streak resembles the ventral to dorsal gradient of Eomes expression observed in Xenopus: fate mapping and lineage analysis of early mouse embryos have demonstrated that mesodermal cells which traverse the anterior primitive streak adopt "dorsal" cell fates (Lawson et al., 1991; Tam, 1989; Parameswaran and Tam, 1995). Therefore, it is conceivable that mEomes may also function in mesoderm fate determination.

At E5.0, mEomes is strongly expressed in the extra-embryonic ectoderm of the early egg cylinder (Fig. 1A). This represents a novel murine expression domain for Eomesoderm in since in early Xenopus embryos, Eomes shows only zygotic pan-mesodermal expression (Ryan et al., 1996). Over the course of gastrulation mEomes expression persists within the extra-embryonic ectoderm as it retracts towards the placenta to form the chorion (Figs. 1,2). Extra-embryonic ectoderm is initially derived from the polar trophectoderm lineage, soon after blastocyst implantation. Tanaka et. al. have recently reported that mEomes is expressed by trophoblast stem (TS) cells isolated
Figure 1. Early expression of mEomes.

(A-D) Whole-mount RNA in situ hybridizations showing extra-embryonic ectoderm expression of mEomes at egg cylinder stages (A); pre-streak epiblast expression of mEomes (B); and expression throughout the primitive streak and anterior visceral endoderm in early gastrula (C-D). (E-F) Transverse sections and, (G) sagittal section through mid-streak stage embryos. Abbreviations: (ee) extra-embryonic ectoderm; (ps) primitive streak; (ave) anterior visceral endoderm; (m) mesoderm. Scale bars: 100 μm.
Figure 1:
Figure 2. Late-streak expression of \textit{mEomes} and \textit{Fgfr2}.

(A,C,D) \textit{mEomes} expression in mid-late streak, late streak and early head-fold stage embryos, respectively. Note the restriction of \textit{mEomes} to the anterior streak (C) and its confinement within the primitive streak and adjacent mesodermal cells (C insert). (B,E) \textit{Fgfr2} expression in late-streak and early head-fold embryos, respectively. Note the similarities between \textit{mEomes} and \textit{Fgfr2} staining in extra-embryonic ectoderm and chorion. Abbreviations: (ee) extra-embryonic ectoderm; (ps) primitive streak; (ch) chorion; (m) mesoderm. Scale bars: 100 \textmu m.
Figure 2:
from extra-embryonic ectoderm at E6.5 (Tanaka et al., 1998). In the absence of FGF4, TS cells lose mEomes expression and differentiate. This and the similar extra-embryonic expression patterns of mEomes and of Fgfr2 (Arman et al., 1998; Fig. 2B,E) underscore the role for T-box genes as potential mediators of FGF signalling (Griffin et al., 1995; Griffin et al., 1998), and suggest a possible role for mEomes in the specification and patterning of trophoblast and extra-embryonic ectoderm lineages.

To investigate the possible regulation of mEomes expression by FGF signalling at the primitive streak, mEomes expression was examined in Fgfr1 -/- embryos. At late streak stages, mEomes expression was not confined to the anterior streak of Fgfr1 mutants; rather mEomes expression appeared to be expressed more broadly throughout the primitive streak of E8.5 Fgfr1 -/- embryos (Fig. 3A,B). This correlates well with the expansion of the anterior streak markers associated with the Fgfr1 mutant phenotype (Yamaguchi et al., 1994). At E9.5, mEomes was ectopically expressed in Fgfr1 -/- embryos, in two domains lateral to the posterior streak at the base of the allantois (Fig. 3C, asterisk). Thus, while FGFR1 signalling is not required for mEomes expression at gastrulation, patterning defects associated with the Fgfr1 mutant phenotype result in up-regulated and ectopic mEomes expression.

At E10.5, strong mEomes expression is seen in the forebrain of WT embryos. mEomes is expressed in the superficial layer of the telencephalic vesicle, but expression is excluded from the dorsal midline (Fig. 4A-D). This closely resembles the expression pattern of another mouse T-box gene, T-Brain-1 (Tbr-1; Fig. 4E) which Bulfone et al. (1995) have shown to be expressed in a single layer of post-mitotic cells in the mantle zone of the telencephalon at E10.5 (Bulfone et al., 1995). The expression domain of Tbr-1, however, extends more rostrally than that of mEomes, and the rostral margin of mEomes expression appears more defined than that of Tbr-1 (Fig. 4D-E). Xenopus Eomes is also expressed in the most anterior part of the brain, becoming strongly transcribed in the olfactory region of the telencephalon (Ryan et al., 1998). Therefore, XlEomes, mEomes and Tbr-1, which constitute a distinct subfamily of T-boxes (Wattler et al., 1998), may play a role in regionalizing the early forebrain.
Figure 3. Expression of *mEomes* in *Fgfr1* /-/- embryos.

(A,B) at E8.5, *mEomes* expression is not restricted to the anterior primitive streak of *Fgfr1* /-/- embryos; rather *mEomes* expression is unregulated throughout the length of the streak. (C) At E9.5, *mEomes* is ectopically expressed at the base of the allantois of *Fgfr1* mutants, on either side of the posterior primitive streak. Abbreviations: (ps) primitive streak; (al) allantois.
Figure 3:
Figure 4. T-box gene expression at E10.5.

(A-C) Wholemount and sections showing mEomes expression in the forebrain. mEomes is expressed in the superficial layer of the telencephalic vesicle (arrow in C). (D,E). Comparison of mEomes and Tbr-1 expression, respectively. Scale bars: 100 μm.
Figure 4:
EXPERIMENTAL PROCEDURES

Novel T-box motifs were amplified by PCR from an E7.5 primitive streak cDNA library (generous gift from Dr. Jonathan Pearce) using degenerate primers encoding amino acid sequences GTEMIITK and NPFAKGFR. Amplification products were analyzed by PAGE and amplicons in the expected size range (about 500 bp) were eluted from gel slices, subcloned and sequenced. NCBI-BLAST was used to identify sequences homologous to T-box genes. An isolated amplicon encoding the mEomes T-box was used as a probe for whole mount RNA in situ hybridizations, which were performed as described by Conlon and Rossant (Conlon and Rossant, 1992) and Henrique et al. (Henrique et al., 1995). A mEomes 3' RACE product, containing sequence downstream of the T-box including 3' UTR, was also used as an in situ probe and gave identical results. For sectioning of whole-mount stained embryos, specimens were postfixed in 3.7% formaldehyde overnight at 4°C, embryos were embedded in paraffin, sectioned at 10 μm, mounted onto glass slides, dewaxed, and some slides were counterstained with Nuclear Fast Red. Other in situ probes used were as follows: Fgfr2 probe consisted of a 2kb EcoRI extra-cellular and transmembrane fragment of bek cDNA (from Alka Mansukhani) cloned into Bluescript KS; the Tbr-1 probe consisted of an amino-terminal 1.3kb Tbr-1 EST obtained from Soares mouse p3NMF19.5 cDNA library (W09676).
CHAPTER 5

Discussion and Future Directions

The chimeric analyses performed in this study have provided further insight into cellular and molecular mechanisms that regulate the movement and specification of mesodermal progenitor cells downstream of FGF signalling at gastrulation. In chapter 2, analysis of the behaviour and distribution of Fgfrl -/- cells within chimeric embryos revealed a defect in mutant cell migration through the streak. As a result, Fgfrl -/- cells were deficient in populating not only the somitic mesoderm (as was previously observed in Fgfrl mutant analyses), but also the cephalic, heart, and axial mesoderm populations, as well as the endodermal lineages of E9.5-E10.5 chimeric embryos. Fgfrl -/- progenitor cells showed defects in the epithelial to mesenchymal transition and movement of mesodermal cells away from the primitive streak. Observed morphogenetic defects were consistent with abnormalities in Fgfrl -/- cell adhesion and/or cell migration.

In chapter 3, novel assays for mesoderm chemotaxis and cell migration were performed using primary cultures of explanted embryonic tissue. Although results indicated a potential chemotactic role for FGFR1 signalling at the primitive streak, general cell migration defects were not observed in explanted Fgfrl -/- mesoderm cultures. Rather, I demonstrated that FGFR1 signalling is required for the maintained expression of mSnail within the primitive streak, and for the down-regulation of E-cadherin expression at gastrulation. Snail has been shown to directly repress E-cadherin expression and to induce a dramatic EMT when overexpressed in epithelial cell lines (Battle et al., 2000; Cano et al., 2000), and the down-regulation of E-cadherin has been directly implicated in the differentiation and migration of mesoderm at gastrulation (Burdsal et al., 1993). Therefore, the loss of mSnail expression and ectopic E-cadherin levels in Fgfrl -/- embryos provide molecular explanations for the failed EMT and aberrant morphogenesis observed in Fgfrl mutant and chimeric embryos.

I also demonstrated that Fgfrl -/- cells, which had accumulated in the primitive streak of chimeric embryos, form ectopic neural tubes (chapter 2). This suggested that in addition to regulating paraxial and axial mesoderm formation (Yamaguchi et al., 1994; Deng et al., 1994), FGFR1 may play an unexpected role in negatively regulating neural
members of the T-box gene family, which have been implicated in the specification of posterior and paraxial mesoderm, are positively regulated by FGFR1. Tbx6, which functions in promoting paraxial mesoderm versus neuroectoderm fate (Chapman and Papaioannou, 1998), is down-regulated in the streak of Fgfr1 mutant and chimeric embryos. Similarly, Brachyury expression in the mid-primitive streak is also dependent upon FGFR1 signalling. I propose, therefore, that down-regulation of T and Tbx6 can explain both the absence of posterior and paraxial mesoderm in Fgfr1 mutants, and the formation of ectopic neural tubes in Fgfr1 -/- chimeras. Furthermore, analysis of T-box gene expression revealed that FGFR1 signalling is required in a very specific domain of the primitive streak, which includes those regions fated to generate paraxial and posterior mesoderm populations, but excludes the node, axial mesoderm and extraembryonic mesodermal lineages.

Finally, I showed that Wnt signalling is attenuated in the primitive streak of Fgfr1 mutant embryos, and suggest that ectopic E-cadherin levels – which have been implicated in abnormal mesoderm morphogenesis- act to sequester free β-catenin to the cell membrane, thus preventing its nuclear localization and association with TCF1/Leff proteins in response to localized Wnt signals. Furthermore, β-catenin remains co-localized with E-cadherin in the primitive streak of Fgfr1 -/- embryos, and the forced down-regulation of E-cadherin expression in Fgfr1 -/- embryonic explants can restore Wnt signalling at the streak. Results suggest a molecular link between FGF and Wnt signalling pathways at the primitive streak, and serve to underscore the interdependent nature of morphogenesis and patterning at gastrulation. Moreover, data suggests that modulations in cadherin levels can affect the intracellular distribution of β-catenin, and hence regulate β-catenin/Wnt signalling in vivo.

Is there physiological evidence that E-cadherin regulates morphogenesis and Wnt signalling at the primitive streak?

Genetic evidence that modulations in E-cadherin expression regulate mesoderm morphogenesis and Wnt activity at gastrulation is lacking. Embryos homozygous for mutations in E-cadherin die at implantation (Larue et al., 1994), and although mSnail -/- embryos die early in gastrulation with ectopic E-cadherin expression (T. Gridley, pers.
comm.), detailed analyses of mSnail-/− patterning and morphogenetic defects have not yet been reported. Fgfr1-/− and mSnail-/− phenotypes are not expected to be identical: mSnail is initially expressed in Fgfr1 mutant embryos and the Fgfr1-/− phenotype is associated with abnormal expression of T-box genes, which have also been implicated in regulating morphogenetic movement at the primitive streak. It would, however, be informative to perform a chimeric analysis of Snail function. If morphogenetic defects are caused by ectopic E-cadherin expression, one would expect that mSnail-/− cells would also accumulate in the primitive streak of mSnail-/− ↔ WT chimeric embryos and demonstrate failures in EMT and cell migration. Furthermore, one could examine T expression in the late primitive streak of mSnail-/− chimeras: if Wnt signalling is attenuated, T expression should be down-regulated in mSnail-/− cells. The effect of mSnail heterozygosity on the hypomorphic Fgfr1 phenotype could also be examined. Mice homozygous for hypomorphic Fgfr1 alleles gastrulate normally, but die at birth (Partanen et al., 1998). If mSnail is required to repress E-cadherin expression downstream of FGFR1, then heterozygosity for mSnail in an Fgfr1 hypomorphic background might disrupt normal gastrulation movements.

Alternatively, one could over-express E-cadherin in the primitive streak of WT embryos, using the proximal Brachyury promoter element, to determine whether ectopic E-cadherin expression can phenocopy Fgfr1-/− gastrulation defects. Furthermore, one could determine whether Wnt signalling is attenuated upon ectopic E-cadherin expression by analyzing Brachyury expression and T-LacZ reporter activity (Chapter 3) in these embryos. To demonstrate more conclusively that ectopic E-cadherin levels result in abnormal morphogenesis and attenuated Wnt signalling in Fgfr1 mutant embryos, E-cadherin expression could be reduced in Fgfr1-/− embryos through loss of a single copy of E-cadherin; embryos could then be examined for rescued mesoderm migration and Wnt activity at the streak.

Do FGFs play additional roles in regulating movement through streak?

Chimeric analysis of Shp2 function has revealed remarkable similarities with Fgfr1-/− chimeric embryos: Shp2 mutant cells accumulate in the posterior epiblast and primitive streak of chimeric embryos, are deficient at contributing to mesodermal
lineages, and also form ectopic neural tubes (Saxton and Pawson, 1999). Moreover, Shp2 mutant fibroblasts are unable to mount a positive chemotactic response to FGFs in vitro. Therefore, it is likely that Shp2 plays a key role downstream of FGFR1 signalling in regulating the morphogenetic movements of mesodermal cells at gastrulation (Saxton and Pawson, 1999).

Shp2 is believed to signal at focal contacts downstream of integrin engagement to promote both Src and focal adhesion kinase (FAK) activation. Shp2 therefore plays an important role in regulating the cell–extracellular matrix (ECM) interactions that control cell migration; indeed, fibroblasts mutant for Shp2 are impaired in their ability to spread and migrate on fibronectin (Yu et al., 1998, Oh et al., 1999). However, Fgfr1 -/- mesodermal cells do not show impaired migration on fibronectin (Chapter 3).

Furthermore, embryos which are mutant for fibronectin, or for FAK (which mediates all adhesion downstream of integrin signalling), do not show defects in mesoderm formation or in the migration of progenitor cells through the primitive streak (Georges-Labouesse et al., 1996; Furuta et al., 1995). Thus, defects in the morphogenesis of Fgfr1 -/- progenitors at the streak are unlikely to involve Shp2-dependent integrin signalling events.

Recently, studies in Xenopus have demonstrated that the convergence extension movements of gastrulation are dependent on the activation of Rho downstream of FGF and Shp2 signalling (O'Reilly et al., 2000). Interestingly, Rho has also been shown to play an essential role during Drosophila gastrulation, where it is required for presumptive mesodermal cells to undergo the cellular shape changes that are thought to drive ventral furrow formation (Barrett et al., 1997; Hacker and Perrimon, 1998). It is known that members of the Rho small GTPase family, which include Rho, Rac, and Cdc42, regulate actin cytoskeleton reorganization (reviewed in Hall, 1998). There is also growing evidence that Rho-like GTPases function in the maintenance of cadherin-mediated cell-cell adhesion. In epithelial cell lines, inactivation of Rac or Rho results in the dislocation of E-cadherin from adherens junctions and the loss of cell-cell adhesion; conversely, constitutively activated Rac mutants promote the accumulation of E-cadherin at sites of cell-cell contact, and enhanced intercellular adhesion (Evers et al., 2000). Interestingly, it has been reported that Rac1 deficient mouse embryos die at gastrulation, and show irregular foldings and thickenings within the epiblast layer, with no sign of mesoderm formation (Sugihara et al., 1998). Indeed, the Rac1 mutant phenotype resembles a failure...
in primitive streak formation, and suggests a role for Rac in promoting epithelial to mesenchymal transitions at gastrulation.

It is possible, therefore, that during mouse gastrulation, the activity of Rho-like GTPases may play a key role in regulating the morphogenesis of mesoderm progenitor cells downstream of FGFR1 and Shp2 signalling at the primitive streak, and that ectopic E-cadherin levels and failed EMT in Fgfr1-/- embryos are the result of abnormal Rac activity. In order to assess the roles for Rho, Rac and Cdc42 in regulating the morphogenetic movements of gastrulation, one could express dominant negative mutants of the Rho-like GTPases in WT embryos, under the control of the Brachyury promoter (Chapter 3). Transgenic embryos could then be analyzed for gastrulation phenotypes: dominant negative Rho constructs have been used successfully to inhibit gastrulation movements in both Drosophila and Xenopus (Barrett et al., 1997; Hacker and Perrimon, 1998; O'Reilly et al., 2000). Since Rac1-/- embryos appear to have defects in EMT (Sugihara et al., 1998), one might expect dnRac1 expression to phenocopy Fgfr1 mutant embryos. It would therefore be interesting to express an activated Rac1 construct in the primitive streak of Fgfr1-/- embryos: any rescue of the Fgfr1-/- phenotype might indicate that Rac1 functions downstream of FGFR1 signalling at gastrulation.

What is the role for FGFR1 signalling in axial mesoderm formation?

In Fgfr1 mutant embryos, there was an expansion of anterior primitive streak and notochord populations and a reduction in paraxial mesoderm formation. It was argued, therefore, that paraxial mesoderm progenitor cells were being re-directed towards an axial mesoderm fate (Yamaguchi et al., 1994). However, in chimeric embryos, Fgfr1-/- cells were deficient in contributing to the notochord, and thus did not show a tendency towards axial mesoderm formation (chapter 2).

The expansion of axial mesoderm in Fgfr1-/- embryos may in fact be secondary to the loss of T and Tbx6 expression within the paraxial mesoderm domain of the primitive streak. In zebrafish, the mutually antagonistic activities ofntl and spt within the developing axial and paraxial mesoderm populations, act to define the boundary between notochord and muscle populations (Amacher and Kimmel, 1998). It is possible that in the mouse, Tbx6 or T expression in the mid-primitive streak may also be required to repress
the formation of anterior midline structures, perhaps by inhibiting \textit{meomes} expression. Indeed, the expression of \textit{meomes} is expanded in \textit{Fgfr1} -/- embryos (chapter 4), and mutational analyses demonstrate a requirement for \textit{meomes} in the specification of the anterior streak (Russ et al., 2000). It would therefore be interesting to examine chimeric embryos for differences in \textit{meomes} expression between \textit{Fgfr1} -/- and WT cells found within the anterior- and mid-segments of the primitive streak.

The broadened domain of notochord cells found in \textit{Fgfr1} -/- embryos may also reflect abnormal morphogenesis of the axial mesoderm population. The morphogenetic movements undertaken by anterior midline tissues of late-primitive streak mouse embryo resemble the convergence extension movements observed in \textit{Xenopus} gastrula (Beddington, 1994; reviewed in Tam and Behringer, 1997). Since FGF signalling has been shown to regulate convergence extension in both zebrafish and \textit{Xenopus}, it is conceivable that anterior midline morphogenesis is also disrupted in \textit{Fgfr1} mutant embryos. \textit{T} and \textit{Shh}-positive cells could be counted in \textit{Fgfr1} -/- embryos to assess whether the axial mesoderm population is truly expanded, and Dil labeling of anterior streak or axial mesodermal cells of \textit{Fgfr1} -/- embryos should reveal whether anterior midline morphogenesis occurs normally (Beddington, 1994).

**Does FGFR1 signalling function in chemotaxis?**

\textit{Fgfr1} -/- cells from primitive streak explants were unable to mount a chemotactic response to a localized source of FGF (chapter 3). This indicates that during the morphogenesis of mesoderm at gastrulation, FGFs may act as chemo-attractants – a role for FGF signalling well documented in \textit{Drosophila} and \textit{C.elegans} (see Skaer, 1997 Chen and Stern, 1998). If chemo-attraction plays a role in mesoderm migration, it has been argued that FGFs produced by the visceral endoderm (VE), underlying the primitive streak, would act as the best source for such signals (Sun et al., 1999). Chimeric analyses have demonstrated that in the absence of FGF8 production by the VE, gastrulation proceeds normally - this argued against a physiological role for chemo-attraction at gastrulation (Sun et al., 1999). However, abundant FGF8 was still produced within the primitive streak, and may have compensated for the lack of FGF production by the VE. A more insightful experiment would involve the generation of tetraploid chimeras using
$Fgf8^{-/-}$ ES cells and WT embryos. In these chimeras, the visceral endoderm would provide the only source of FGF8, and one could then assess whether localized expression of FGF8 in the VE alone is sufficient to rescue migration through the streak.

It was argued in Chapter 3 that chemotactic deficiencies could explain other aspects of the $Fgfr1$ mutant phenotype. In particular, if FGFs direct the migration of limb mesenchymal cells towards the AER, as has been demonstrated in the chick (Li and Muneoka, 1999), chemotactic defects could explain the exclusion of $Fgfr1^{-/-}$ cells from the progress zone, and their accumulation in the proximal mesenchyme of chimeric limb buds (Chapter 2). In order to investigate the chemotactic potential of both WT and $Fgfr1^{-/-}$ limb bud mesenchymal cells, one could repeat the chemotaxis assays described in Chapter 3 using explants of proximal limb bud mesenchyme (instead of primitive streak explants) from E9.5-E10.5 $Fgfr1^{-/-}$ chimeric embryos.

It has also been demonstrated that mouse limb bud cells will extend cytoneme projections after brief culture in the presence of FGFs (Ramirez-Weber and Kornberg, 1999). It has been argued, therefore, that cytonemes are attributes of both vertebrate and invertebrate cells which may function in allowing cells to sense distant morphogenic cues. Since studies in Drosophila have demonstrated a requirement for FGF signalling in cytoneme formation (Ramirez-Weber and Kornberg, 1999), it would be interesting to determine whether $Fgfr1^{-/-}$ cells are capable of extending cytoneme processes, as this may provide insight into the role for FGF signalling in chemotactic migration. Furthermore, FGF induced chemotaxis is likely to involve Shp2 signalling: Shp2 mutant fibroblasts show an impaired chemotactic response to FGFs (Saxton and Pawson, 1999), and Shp2 $^{-/-}$ cells also accumulate in the proximal mesenchyme of the limb bud and fail to populate the progress zone of Shp2 $^{-/-}$ chimeric embryos (Saxton et al., 2000). Given the association between FGFR/Shp2 signalling and the activation of Rho-like GTPases (O'Reilly et al., 2000), and the fact that cytonemes are actin- and not microtubule-based (Ramirez-Weber and Kornberg, 1999), it is possible that cytoneme outgrowth may depend on Rac- or Cdc42- dependent actin cytoskeleton reorganization downstream of FGFR1 and Shp2 signalling.

It has been reported that, using high magnification fluorescent microscopy, cytonemes can be observed projecting from preparations of live cells (including limb bud cells) which express the green fluorescent protein (GFP) (Ramirez-Weber and Kornberg,
With the use of a two-photon confocal microscope, it should therefore be possible to investigate in situ cytoneme formation in fresh tissue samples explanted from chimeric mouse embryos. These chimeras could be generated from the aggregation of WT embryos with ES cells which ubiquitously express an enhanced GFP (EGFP) transgene (Hadjantonakis et al., 1998). In low-level chimeras, one would expect that only a small population of cells within the limb bud would express the EGFP transgene – this should facilitate the visualization of individual cytonemes. Similarly, one could introduce an EGFP transgene into Fgfr1 -/- ES cells, and then investigate cytoneme formation in low level Fgfr1 -/- chimeric limb buds. Using a similar approach, it should also be possible to investigate whether or not cytonemes are formed by WT and Fgfr1 -/- progenitor cells within the primitive streak.

Can other phenotypes of Fgfr1 -/- chimeras be explained by abnormal cell adhesion?

As discussed, I have implicated ectopic E-cadherin expression in the failed morphogenesis of Fgfr1 -/- mesoderm at gastrulation, and in the sorting of Fgfr -/- from WT cells at the primitive streak. Recently, an analysis of N-cadherin -/- chimeric embryos has provided further evidence that simple changes in cadherin expression are sufficient to cause cell-sorting in vivo (Kostetskii et al., 2001). N-cadherin -/- cells segregate from WT cells in the heart, somites, neural tube and neuroepithelium of chimeric embryos. Furthermore, these N-cadherin -/- cells form distinct aggregates that often form lumenal structures, and closely resemble the ectopic neural tubes and vesicles formed by Fgfr1 mutant cells within the neuroectoderm of Fgfr1 -/- chimeric embryos (Kostetskii et al., 2001). Therefore, intrinsic differences in cell adhesion properties may explain why ectopic neural tubes in Fgfr1 chimeras separate from the primary neural tube at boundaries with WT cell populations, and why they are composed entirely of Fgfr1 -/- cells. The expression of N-cadherin, and of other cadherin molecules, should therefore be examined in Fgfr1 -/- chimeric embryos at later stages of development (e.g. E9.5), in an attempt to identify differences in cell adhesion molecule expression.

Studies in the chick have demonstrated that limb mesenchymal cells will sort-out in culture according to their original proximodistal position within the limb bud (Ide et
al., 1994). This suggests that there are regional differences in cell adhesion along the proximodistal axis of the developing limb. In the mouse limb bud, several cadherin family members are expressed including N-cadherin, cadherin-6, cadherin-11 and PB-cadherin (Kimura et al., 1995; Inoue et al., 1997; Kitajima et al., 1999). In particular, cadherin-11 is strongly expressed in the mesenchymal cells of the progress zone, and is also expressed in branchial arch mesenchyme (Kimura et al., 1995). Since these are regions of the embryo where Fgfr1 is normally expressed, and from which Fgfr1 -/- cells are excluded in chimeric embryos (Chapter 2), it is possible that FGFR1 is required for the initiation of cadherin (cadherin-11?) expression in the limb and brachial arch, and that abnormal cell adhesion properties of Fgfr1 -/- cells results in their segregation from WT populations. Examination of cadherin expression in Fgfr1 -/- and WT cells of chimeric embryos should help identify potential targets of FGFR1 signalling.

What is the role for FGFR1 signalling after gastrulation?

Initially, it was hoped that the presence of WT cells in Fgfr1 -/- chimeric embryos would rescue the early lethality and gastrulation defects associated with the Fgfr1 mutant phenotype. This would have allowed investigations into the role for FGFR1 signalling in the development and patterning of mesodermal lineages which failed to form in Fgfr1 -/- embryos. Fgfr1 is expressed in the mesenchyme of most developing organs, including the kidney, heart and skin (Orr-Urtreger et al., 1991). Perhaps more striking, Fgfr1 is expressed in the presomitic mesoderm and in the rostral half of each newly formed somite - thus indicates a potential role for FGFR1 in the patterning or compartmentalization of the somitic mesoderm (Yamaguchi et al., 1992). However, due to the early requirement for FGFR1 signalling in the morphogenetic movement of progenitor cells through the primitive streak, very few Fgfr1 -/- cells contributed to the mesodermal lineages of phenotypically normal chimeric embryos.

In order to investigate the role for FGFR1 signalling in organogenesis and somitogenesis, FGFR1 function could be rescued at the primitive streak of mutant embryos by introducing an Fgfr1 cDNA transgene, under the transcriptional control of a primitive streak-specific promoter element, into the Fgfr1 -/- background. Presumably, expression of the Fgfr1 transgene would transiently rescue morphogenetic movement and
patterned defects; however, down-regulation of \( F_{gfr1} \) expression after gastrulation would still permit analysis of FGFR1 function in newly formed mesodermal lineages. The problem with "primitive streak-specific FGFR1 rescue" experiments lies in the identification of promoter constructs that will drive \( F_{gfr1} \) expression in the paraxial and posterior mesoderm domains of the primitive streak. The proximal Brachyury promoter element used for the \( T\)-\( LacZ \) transgene (described in Chapter 3) would be ideal; however, as demonstrated in Chapter 3, the \( T \) promoter element requires FGFR1 signalling and is not active in \( F_{gfr1}^{-/-} \) embryos. \( F_{gf8} \) and \( W_{nt3a} \) are both expressed normally within the primitive streak of \( F_{gfr1}^{-/-} \) embryos; unfortunately, primitive streak-specific promoter elements have not been reported for these genes. It would also be possible to target an \( F_{gfr1} \) cDNA transgene directly into the \( F_{gf8} \) or \( W_{nt3a} \) locus of \( F_{gfr1}^{-/-} \) ES cells. However, given the similarities between \( F_{gf8} \) and \( F_{gfr1} \) mutant phenotypes and the potential interactions between the FGF and Wnt signalling pathways at the primitive streak, heterozygosity at the \( F_{gf8} \) or \( W_{nt3a} \) loci in an \( F_{mgf}^{-/-} \) background may serve only to compound the \( F_{gfr1} \) mutant phenotype.

A second method of assessing FGFR1 function during somitogenesis and organogenesis would be to selectively eliminate FGFR1 function in specific mesodermal lineages of otherwise WT embryos, using Cre/loxP-mediated DNA recombination (Nagy, 2000). The loss of FGFR1 activity in specific tissues can be achieved by generating mice which carry a mutant (yet functional) \( F_{gfr1} \) allele, in which essential regions of \( F_{gfr1} \) are flanked by loxP sites (the recognition sequence for the site-specific DNA recombinase, Cre). Mice carrying a floxed \( F_{gfr1} \) allele are currently available in the lab (Juha Partanen, unbl. data). These mice could be mated to transgenic animals that express the \( cre \) gene under the control of a tissue specific promoter. Cre-mediated recombination of the "floxed" \( F_{gfr1} \) allele (to a null allele) would occur only in cells that express the transgene, resulting in a complete loss of FGFR1 function in those cells and their descendants.

Again, limitations to this strategy lie in generating transgenic lines that specifically express the \( cre \) gene in tissues or mesodermal lineages of interest. However, the repertoire of tissue specific \( cre \) transgenes is growing (Nagy and Mar, 2001), and it would currently be possible to examine FGFR1 function at the midbrain/hindbrain junction (\( Engrailed-2 \); Zinyk et al., 1998), in lateral plate and limb bud mesoderm.
(HoxB6: Lowe et al., 2000), heart and myotomal lineages (actin promoters; Miwa et al., 2000), embryonic endoderm and notochord populations (HNF3-alpha; Monaghan et al., 1993), and in paraxial and somitic lineages (HoxA1; Thompson et al., 1998). Indeed, it will soon be possible to study FGFR1 function in all developing organ systems, and to thus define the roles for FGF signalling in morphogenetic and patterning events throughout embryonic development.
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