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FGF Signaling Regulates Mesoderm Cell Fate Specification and Morphogenetic Movement at the Primitive Streak

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Summary

Although FGF signaling 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 signaling 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 signaling 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 Tam and Behringer, 1997). 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 ingression 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 signaling in the specification and maintenance of posterior mesoderm 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 signaling at gastrulation has been established in the zebrafish (Griffin et al., 1995).

The first evidence that FGF signaling 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). 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 signaling 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 postimplantation 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 signaling at gastrulation. The goal of this study was to better understand the mechanisms

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by which FGFR1 signaling regulates the specification, EMT, migration, and patterning of progenitor cells at the primitive streak. Here, we describe in vitro assays of mesoderm 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 we did not observe general cell migration defects in explanted *Fgfr1* −/− mesoderm cultures, we have implicated 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 signaling. We also establish a role for FGF signaling 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 signaling activity at the primitive streak. We argue that ectopically expressed E-cadherin in *Fgfr1* −/− progenitor cells sequesters free β-catenin from its intracellular signaling pool and thus attenuates Wnt signal transduction. Results provide a molecular link between FGF and Wnt signaling 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 the direct comparison of *Fgfr1* −/− and WT cell behavior 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 (Figures 1C and 1D), making comparison of migration rates difficult. This segregation is characteristic of the behavior of mutant cells along the primitive streak of *Fgfr1* chimeric embryos (Ciruna et al., 1997), and was not observed in *Fgfr1* +/− ↔ WT control explants (n = 6; Figure 1B). bFGF was added to primitive streak cultures in an attempt to accentuate potential deficiencies in *Fgfr1* −/− cell migration (n = 19; Figure 1D). No striking differences between *Fgfr1* −/− and WT cell migration were observed.

In order to generate a mixed population of WT and *Fgfr1* −/− 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 *Fgfr1* 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 nonreversible epithelial to mesenchymal transition (EMT) when added to epiblast cultures (Burdsal et al., 1993). WT and *Fgfr1* −/− mesodermal cells formed from chimeric epiblast explants did not segregate in culture (n = 16; Figure 1G), and closely resembled *Fgfr1* +/− ↔ WT control explants (n = 14; Figure 1F). In these mixed mesoderm outgrowths, *Fgfr1* −/− mesoderm migrated identically to WT cells (Figure 1G). The addition of bFGF to the explant culture system had no effect on relative migration rates (n = 23; Figure 1H).

If *Fgfr1* mutant embryos had defects in extracellular matrix production, then deficiencies in *Fgfr1* −/− 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 (Figures 1I and 1J). No difference was observed between WT (Figure 1I) and *Fgfr1* −/− (Figure 1J) embryos. Therefore, these results suggest that *Fgfr1* −/− mesodermal cells are not impaired in general cell migration.

Ectopic E-Cadherin Expression at the Primitive Streak of *Fgfr1* Mutants

In *Fgfr1* mutant analyses, observed failures in EMT and the accumulation and sorting of *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 downregulated as progenitor cells undergo an EMT at the primitive streak; E-cadherin is not expressed in the nascent mesodermal germ layer (Figures 2A and 2G; Damjanov et al., 1986). Since downregulation 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 *Fgfr1* mutant embryos was examined by immunofluorescence (Figures 2D, 2H, and 2I).

Transverse sections through E7.5 mutant embryos revealed that *Fgfr1* −/− progenitor cells, accumulating

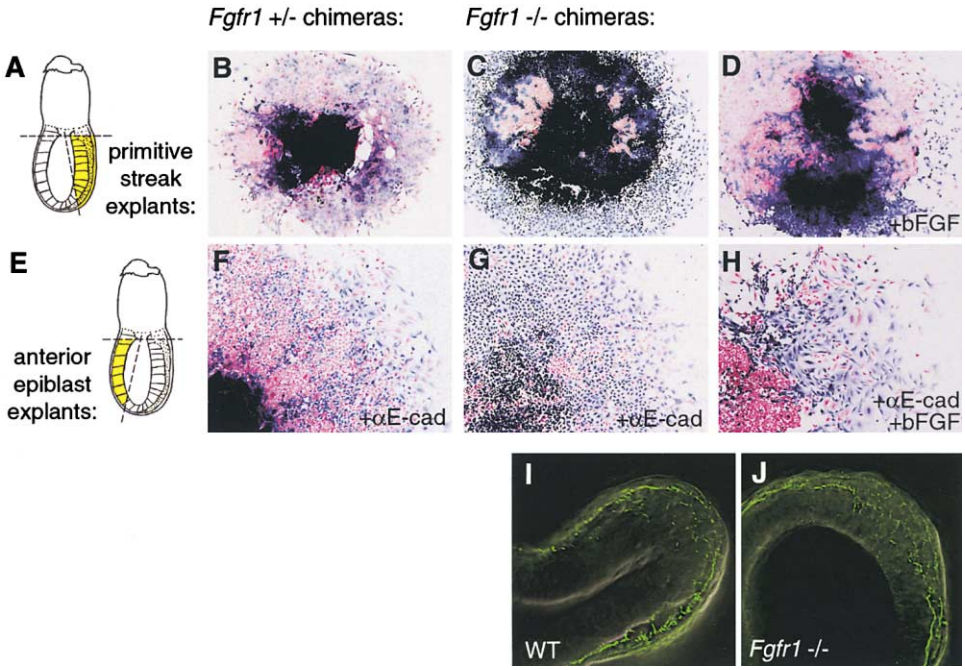

Figure 1. In Vitro Assays of *Fgfr1* −/− Mesoderm Cell Migration

(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) Control *Fgfr1* +/− ↔WT chimeric explant.

(C–D) *Fgfr1* −/− ↔ WT chimeric explants. Explants were X-gal stained to distinguish *Fgfr1* +/− and *Fgfr1* −/− cells (blue) from WT (pink).

(D) bFGF was added to cultures to assess its effect on relative migration rates.

(E–H) Mixed populations of *Fgfr1* mutant and WT mesoderm were also generated by culturing anterior epiblast explants of chimeric embryos with function-perturbing anti-E-cadherin antibodies. Explants were cultured for 3 days on fibronectin.

(F) Control *Fgfr1* +/− ↔WT chimeric explant.

(G–H) *Fgfr1* −/− ↔ WT chimeric explants.

(H) Addition of bFGF had no effect on relative migration rates.

(I–J) Transverse sections through the primitive streaks of E7.5 WT (I) and *Fgfr1* −/− (J) embryos showing immunofluorescent detection of fibronectin expression.

within the swollen primitive streak, maintained high levels of E-cadherin at their cell membranes (Figure 2D). At E8.5, posterior transverse sections through *Fgfr1* −/− embryos revealed large masses of progenitor cells accumulating beneath the primitive streak (Figures 2H and 2I). 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.

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 (Figures 2J–2L). In these chimeras, WT cells ubiquitously expressed the ROSA26 *lacZ* transgene (Friedrich and Soriano, 1991), and were visualized by anti-β-galactosidase staining (Figure 2K). 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 (Figure 2L, arrowhead). These studies provide strong evidence that FGFR1 signaling is required for normal downregulation of E-cadherin expression at gastrulation.

Snail Expression Is Downregulated 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 at the ventral furrow; and in *Xenopus* and zebrafish, *Snail* homologs 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 (Figures 3A–3D; 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

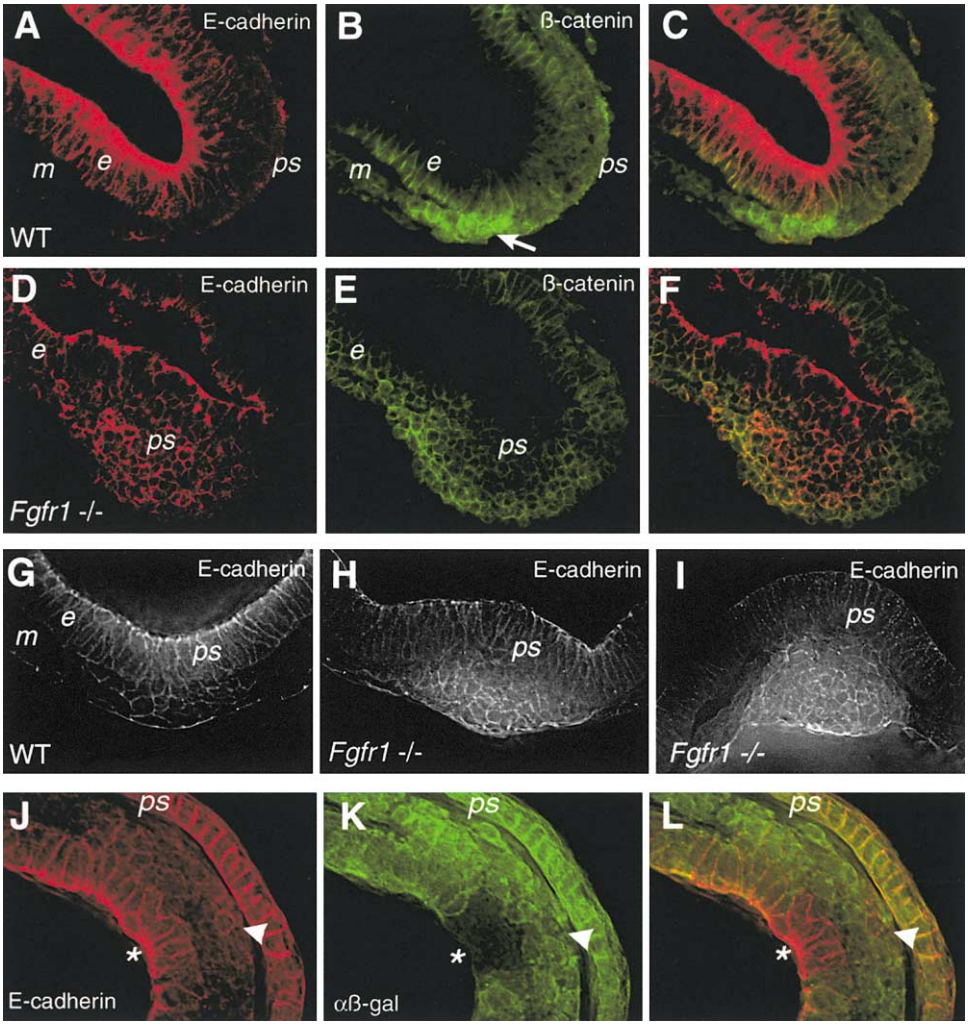

Figure 2. Localization of E-Cadherin and β-Catenin at the Primitive Streak

(A–F) Confocal micrographs showing immunolocalization of E-cadherin (A and D) and β-catenin (B and E) in transverse sections through the primitive streaks of WT (A–C) or *Fgfr1* −/− (D–F) embryos at E7.5.

(C and F) Overlay of E-cadherin and β-catenin staining in WT and *Fgfr1* −/− primitive streaks, respectively.

(G–I) Deconvolved images of E-cadherin localization in transverse sections through the primitive streaks of WT (G) and *Fgfr1* −/− (H and I) headfold-staged embryos.

(J–L) E-cadherin expression in a transverse section through the primitive streak of an E7.5 *Fgfr1* −/− ↔ WT chimera.

(J) E-Cadherin was detected by immunofluorescence.

(K) WT cells were distinguished by positive anti-β-galactosidase immunostaining. The arrowhead indicates an *Fgfr1* −/− mesodermal cell.

(L) Overlay. ps, primitive streak; m, mesoderm; e, epiblast.

characteristics, including the expression of E-cadherin (T. Gridley, personal communication). 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 *Fgfr1* −/− embryos by whole-mount RNA in situ hybridization (Figure 3). As in WT embryos, *mSnail* was expressed in the very early primitive streak of E6.5 *Fgfr1* mutants (Figure 3E). However, this early signal diminished, and only faint *mSnail* expression was observed in *Fgfr1* −/− embryos at mid to late streak stages (Figure 3F). At E9.0, only a small domain of *mSnail* expression was observed at the base of the allantois, at the most posterior end of the streak (Figure 3G, arrowhead).

Loss of *mSnail* expression in the primitive streak of *Fgfr1* 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 3E) and *mSnail* expression at the most posterior domain of the streak (Figure 3G) are independent of FGFR1 function, this is consistent with the *Fgfr1* −/− phenotype. Fate-mapping studies have demonstrated that these regions of the primitive streak will generate extraembryonic mesoderm (Parameswaran and Tam, 1995; Kinder et al., 1999), a population that forms normally in *Fgfr1* −/− 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

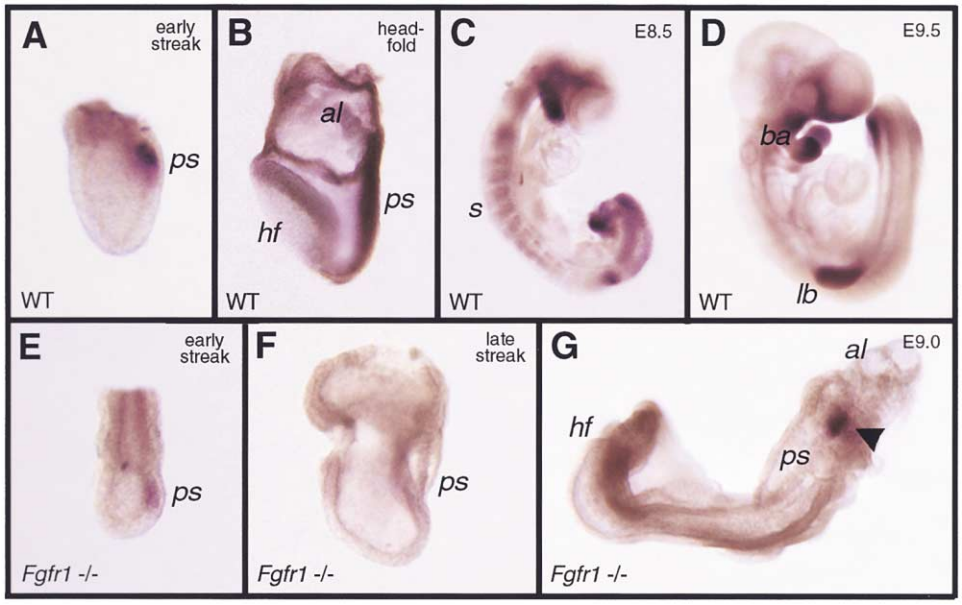

Figure 3. *mSnail* Expression Is Lost in *Fgfr1* −/− Embryos

(A–D) *mSnail* expression in WT embryos from E6.5 to E9.5.

(E–G) *mSnail* expression in *Fgfr1* −/− embryos from E7.5 to E9.0. ps, primitive streak; hf, headfold; al, allantois; s, somite; ba, branchial arch; lb, limb bud.

FGFR1 signaling maintains *mSnail* expression in the late primitive streak, thus promoting the downregulation of E-cadherin (Figure 7).

FGFR1 Regulates *Tbx6* Expression

Although abnormal intercellular adhesion can account for defective morphogenetic movements at gastrulation, its contribution to the patterning defects observed in *Fgfr1* −/− embryos remains unclear. Fate-mapping studies demonstrate that the order and the site of progenitor cell ingression 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 *Fgfr1* mutant analyses, in particular severe reductions in paraxial mesoderm formation in *Fgfr1* −/− embryos (Yamaguchi et al., 1994; Deng et al., 1994) and the formation of ectopic neural tubes in *Fgfr1* −/− 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 *Fgfr1* 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 signaling and the expression of T box genes at gastrulation (Griffin et al., 1995, 1998; 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 *Fgfr1* mutant embryos by whole-mount RNA in situ hybridization (Figures 4A–4D). *Tbx6* is strongly expressed throughout the late primitive streak and presomitic paraxial mesoderm (Figures 4A and 4C; Chapman et al., 1996). In *Fgfr1* −/− 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 (Figures 4B and 4D).

A chimeric analysis was performed to determine whether *Tbx6* is regulated by FGF signaling, or whether cells that would normally express *Tbx6* simply failed to form in *Fgfr1* −/− 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 (Figures 4E–4G; n = 8). *Tbx6* expression was visualized by fluorescent whole-mount RNA in situ hybridization (Figure 4F), and *Fgfr1* −/− cells, which expressed the ROSA26 *lacZ* transgene, were distinguished by anti-β-galactosidase immunofluorescence (Figure 4E). 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 4G). 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

Genetic and embryological studies across vertebrate species have revealed a conserved role for *Brachyury*

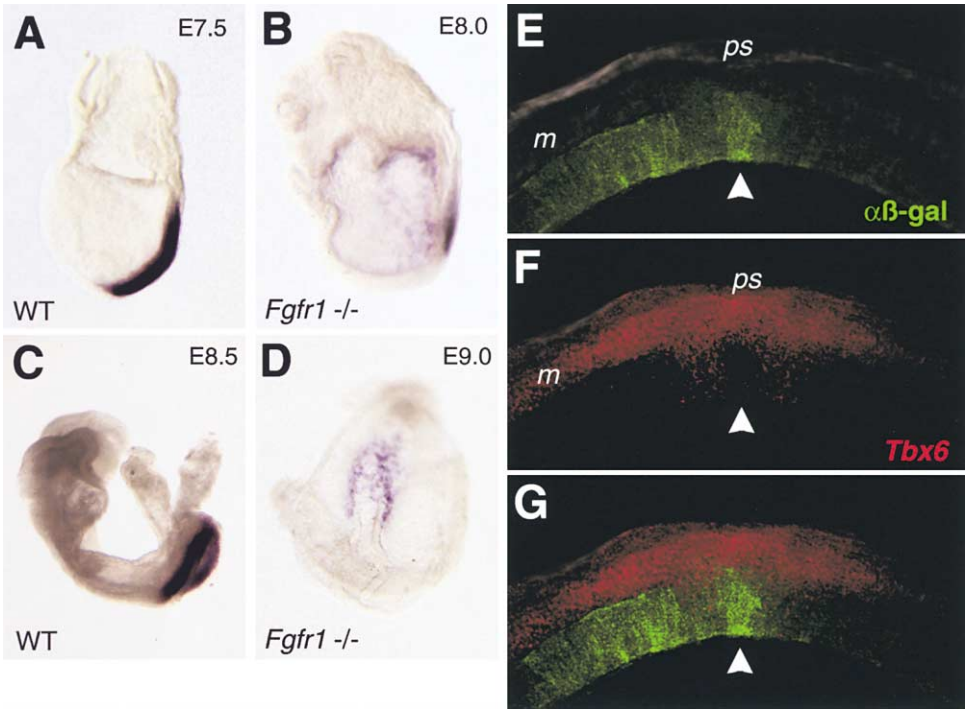

Figure 4. *Tbx6* Is Downregulated at the Primitive Streak of *Fgfr1* −/− Embryos

(A–D) Whole-mount analysis of *Tbx6* expression in WT (A and C) and *Fgfr1* −/− embryos (B and C).

(E–G) *Tbx6* expression in a transverse section through the primitive streak of an E7.5 *Fgfr1* −/− ↔ WT chimeric embryo.

(E) *Fgfr1* −/− cells were distinguished by positive anti-β-galactosidase staining.

(F) *Tbx6* expression was visualized by fluorescent RNA in situ hybridization. Arrowhead indicates *Fgfr1* −/− progenitor cells within the primitive streak.

(G) Overlay. ps, primitive streak; m, mesoderm.

(*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 signaling is required for *Brachyury* expression at gastrulation (Griffin et al., 1995; reviewed in Smith, 1997). A chimeric analysis was performed to reexamine the role for FGFR1 in regulating *T* expression at the primitive streak.

T expression was visualized by fluorescent whole-mount RNA in situ hybridization (Figures 5F and 5I), and *Fgfr1* −/− cells were distinguished by anti-β-galactosidase immunofluorescence (Figures 5E and 5H). 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 downregulated in *Fgfr1* −/− progenitor cells within the primitive streak (Figures 5E–5G, arrowhead). To determine whether *Fgfr1* is required for earlier expression of *Brachyury* at the streak, chimeric embryos were examined at E6.5 (Figures 5H–5J). Sagittal sections reveal that *Fgfr1* −/− progenitor cells at the posterior streak (arrow) have already downregulated *T* expression (Figure 5J).

The expression of *Brachyury* in *Fgfr1* −/− embryos was reexamined at later stages of gastrulation, using standard whole-mount RNA in situ hybridization (Figure 5B). *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 5B). 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 500 bp proximal element of the *Brachyury* promoter that recapitulates endogenous *Brachyury* expression within the primitive streak only (Figure 6A; 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 6A), 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

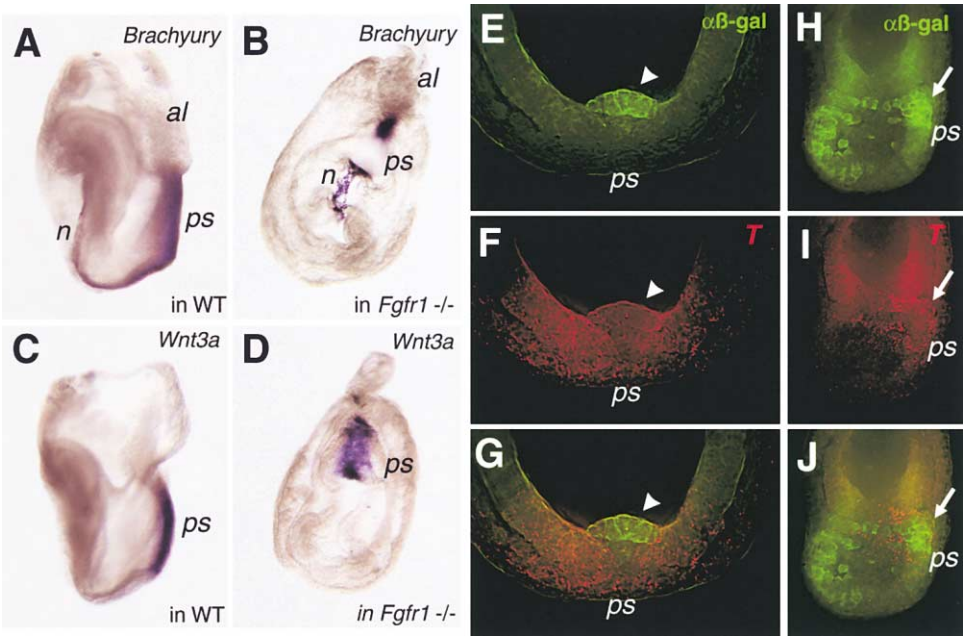

Figure 5. FGFR1 Regulates *Brachyury* Expression in the Primitive Streak

(A–B) Whole-mount analysis of *Brachyury* (*T*) expression in WT (A) and *Fgfr1* −/− embryos (B).
(C–D) Whole-mount analysis of *Wnt3a* expression in WT (C) and *Fgfr1* −/− embryos (D).
(E–G) Transverse section showing *T* expression in the primitive streak of an E7.5 *Fgfr1* −/− ↔ WT 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. Arrowhead indicates *Fgfr1* −/− cells accumulating at the streak.
(G) Overlay.
(H–J) Sagittal section showing *T* expression in 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. Arrow indicates *Fgfr1* −/− cells in the posterior primitive streak.
(J) Overlay. ps, primitive streak; n, notochord; al, allantois.

expression was observed within the primitive streak (n = 5; Figure 6B). 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 and 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 Signaling in *Fgfr1* Mutants

Late in gastrulation, *Brachyury* is a direct target of the Wnt/β-catenin signaling pathway. Two canonical TCF1 binding sites have been identified within the proximal element of the *T* promoter; mutation of these sites disrupts β-catenin-dependent transactivation of reporter constructs in vitro, and abrogates primitive streak expression of the reporter in vivo (Yamaguchi et al., 1999; Arnold et al., 2000). Furthermore, in *Wnt3a* −/− embryos, *T* expression is downregulated in a similar domain of the primitive streak to that observed to be affected in *Fgfr1* −/− embryos (Yamaguchi et al., 1999).

In addition to the loss of *T* expression, the phenotype of *Wnt3a* −/− embryos has much in common with that of *Fgfr1* mutant and chimeric embryos. *Wnt3a* mutants show posterior truncations and lack paraxial mesoderm derivatives, display abnormal morphogenesis at the primitive streak, and form ectopic neural tubes (Takada et al., 1994; Yoshikawa et al., 1997). Although *Wnt3a* −/− embryos develop further than *Fgfr1* mutants, Wnt and FGF signaling appear to regulate similar morphogenetic and patterning events at gastrulation. Therefore, FGF and Wnt pathways may act in parallel, or alternatively, FGF activity may regulate the Wnt signaling pathway at the primitive streak.

The loss of both *T* and *T-lacZ* expression (direct targets of Wnt signaling) in the late primitive streak of *Fgfr1* −/− embryos indicates that the Wnt3a signaling pathway is being attenuated in the absence of FGFR1 activity. *Wnt3a* expression was examined in WT and *Fgfr1* mutant embryos by whole-mount RNA in situ hybridization (Figures 5C and 5D). *Wnt3a* expression is first detected at E7.5, and extends through much of the primitive streak (Figure 5C; Takada et al., 1994). In *Fgfr1* −/− embryos, *Wnt3a* is expressed in its typical domain along the length of the streak (Figure 5D); this contrasts sharply with the observed loss of *T* expression (Figure 5B). Thus, Wnt signaling is being repressed in *Fgfr1* −/− embryos at a level downstream of ligand expression.

β-Catenin Remains Associated with Ectopically Expressed E-Cadherin in the Streak of *Fgfr1* −/− Embryos

The canonical Wnt signaling pathway is regulated by the availability of "stabilized" cytosolic β-catenin (reviewed in Willert and Nusse, 1998). In the absence of

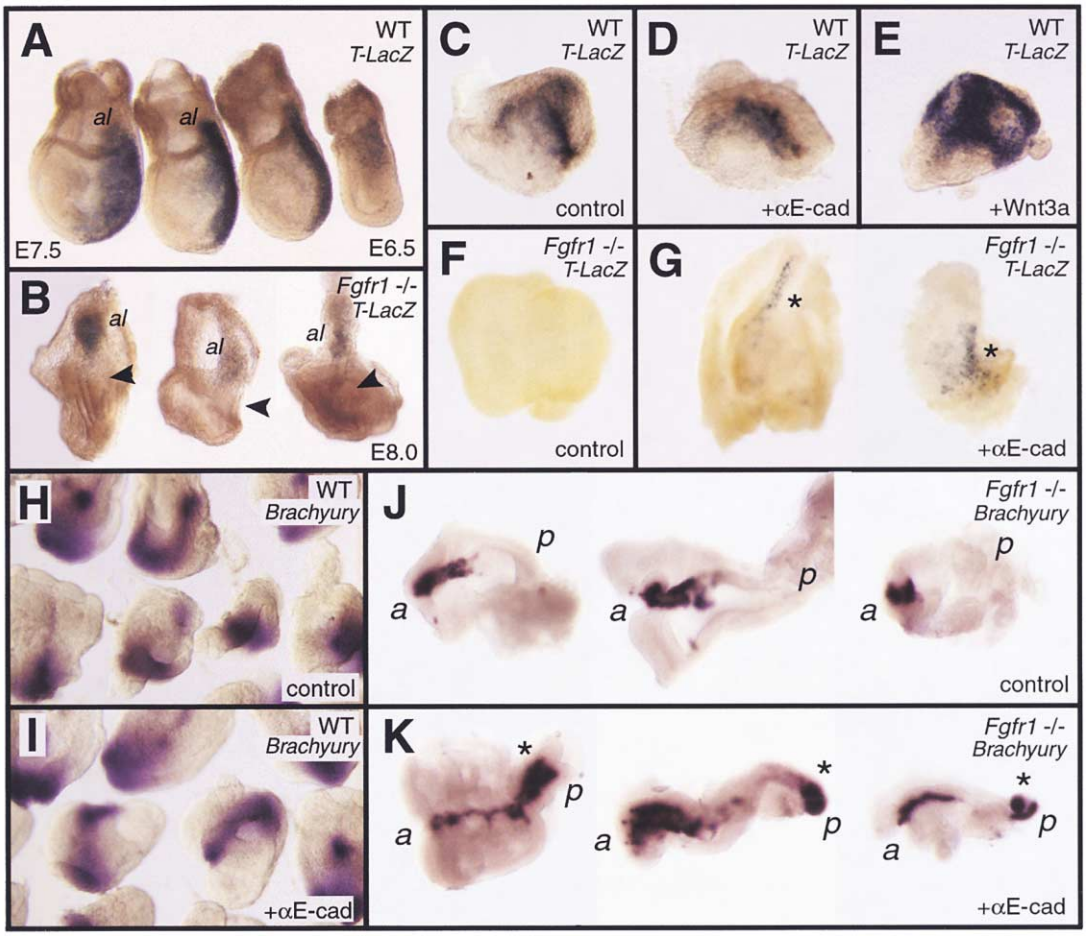

Figure 6. Downregulating E-Cadherin in *Fgfr1* −/− Embryos Restores Wnt Signaling at the Streak

(A–B) X-gal staining reveals *T-lacZ* reporter activity in WT (A) and *Fgfr1* −/− embryos (B). Arrowheads indicate the location of the primitive streak.

(C–E) *T-lacZ* activity in primitive streak explants from E7.5 WT embryos after overnight culture on a fibronectin substrate (C); on fibronectin, in the presence of function-perturbing anti-E-cadherin antibodies (D); and on Wnt3a-expressing 3T3 cells (E).

(F–G) *T-lacZ* activity in primitive streak explants from E8.5 *Fgfr1* −/− embryos after overnight culture on a fibronectin substrate (F); and on fibronectin, in the presence of function-perturbing anti-E-cadherin antibodies (G).

(H–I) *Brachyury* expression in E7.5 WT explants after overnight culture on fibronectin, in the absence (H) or presence (I) of anti-E-cadherin antibodies.

(J–K) *Brachyury* expression in E8.5 *Fgfr1* −/− explants after overnight culture on fibronectin, in the absence (J) or presence (K) of anti-E-cadherin antibodies. al, allantois; a, anterior; p, posterior.

Wnt signal, cytosolic β-catenin is believed to be phosphorylated by glycogen synthase kinase 3β (GSK3β), a serine/threonine kinase and negative regulator of the Wnt pathway. Phosphorylation of β-catenin destabilizes the protein, and targets it for destruction. However, in the presence of a Wnt signal, GSK3β is inactivated. β-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 signaling pathway at the streak by influencing levels of cytosolic β-catenin. In *Fgfr1* −/− embryos, E-cadherin is ectopically expressed at the primitive streak (Figures 2D and 2H–2I). 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 signaling response. The localization of β-catenin at the primitive streaks of WT and *Fgfr1* −/− embryos was therefore examined by confocal analysis (Figures 2B and 2E). In E7.5 WT embryos, cytosolic levels of β-catenin rise after mesodermal cells downregulate E-cadherin and traverse the primitive streak (Figure 2B, arrow). In *Fgfr1* mutant embryos, however, β-catenin does not accumulate in the cytoplasm. Rather, β-catenin colocalizes 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 signaling by appropriating stabilized β-catenin from a cytosolic "signaling-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 the downregulation of E-cadherin expression at the primitive streak of *Fgfr1* mutants should rescue Wnt signaling activity. To test this hypothesis, primitive streak explants were cultured overnight (16–18 hr) with function-perturbing anti-E-cadherin antibodies; immunofluorescent analysis has demonstrated that the 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 signaling response, as the *T* promoter element has been shown to be a direct target of Wnt signaling (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 6C). WT primitive streak explants cultured overnight on Wnt3a-expressing 3T3 cells show a dramatic induction of *T-lacZ* activity (Figure 6E). This provides further evidence that Wnt signaling 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 6D). 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 (Figures 6F and 6G). *Fgfr1* −/− control explants did not show *T-lacZ* activity within the primitive streak (n = 9; Figure 6F). However, in the presence of anti-E-cadherin antibodies, *T-lacZ* reporter expression was induced (n = 10; Figure 6G, asterisk). 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 signaling at the primitive streak.

Similar experiments were then performed examining endogenous *T* expression (Figures 6H–6K). 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 6H). No differences in *Brachyury* expression were observed after WT explants were cultured with anti-E-cadherin antibodies (n = 15; Figure 6I). *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 6J). *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 5B). However, after an overnight culture with anti-E-cadherin antibodies, *Brachyury* expression was observed in the primitive streak of *Fgfr1* −/− explants (n = 11; Figure 6K, asterisks).

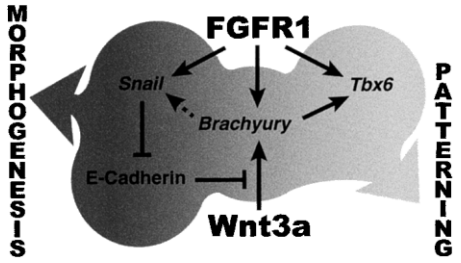

Figure 7. Model of FGFR1 Signaling in the Late Primitive Streak

A summary of the targets of FGF signaling at gastrulation, as based on genetic evidence presented in the text. Initiation of *T* and *Tbx6* expression in the posterior streak requires FGFR1 signaling. Later in gastrulation, maintenance of *T* expression at the streak requires Wnt3a signaling (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 signaling via regulation of the intracellular localization and hence accessibility of free β-catenin. Positive regulation of *Snail* expression by Brachyury is only inferred.

Therefore, culture with function-perturbing anti-E-cadherin antibodies, which results in downregulated 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 signaling 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 7). This study has defined a specific region of the primitive streak that requires FGFR1 signaling activity; this domain encompasses the paraxial and posterior embryonic mesoderm populations, but excludes the node, axial, and extraembryonic mesoderm. In the context of this domain, we propose that FGFR1 signaling 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 signaling 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 downregulation 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 *Fgfr1* −/− from WT cells during gastrulation.

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 downregulation of *T* and *Tbx6* expression in *Fgfr1* −/− mesoderm progenitor cells can account for both the reduction of paraxial and posterior mesoderm, and for the formation of ectopic neural tubes observed in *Fgfr1* 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, 1998; reviewed in Slack et al., 1996), these results further support an evolutionarily conserved pathway for FGF signaling at gastrulation.

Although the mechanisms by which FGFR1 signaling regulates both the morphogenesis and patterning of mesoderm at gastrulation have been discussed separately, the two pathways are intricately entwined. 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, 1995; Wilson and Beddington, 1997). Furthermore, T box genes may also regulate cell adhesion and EMT at gastrulation. In zebrafish, the *Brachyury* homolog *no tail*, and the T box gene *spadetail* have both been implicated as positive regulators of *Snail* expression (Thisse et al., 1993, 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 3G) overlaps with an *Fgfr1*-independent domain of *T* expression at the base of the allantois (Figure 5B). Therefore, *T* may positively regulate *Snail* expression at the primitive streak (Figure 7), providing another link between *Brachyury* expression, intercellular adhesion, and the morphogenesis of the mesodermal germ layer.

In addition, we propose that FGFR1 signaling 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 signaling (i.e., *Brachyury* and the *T-lacZ* reporter transgene) are not activated. We suggest that ectopic E-cadherin expression in *Fgfr1* mutants attenuates Wnt3a signaling by sequestering free β-catenin from its intracellular signaling pool, and demonstrate that forced downregulation of E-cadherin in *Fgfr1* −/− explants can rescue endogenous Wnt signaling at the primitive streak. Evidence that cadherins act as regulators of β-catenin signaling 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 signaling pool (Orsulic et al., 1999). Furthermore, overexpression of cadherins during *Drosophila* and *Xenopus* embryogenesis has been shown to phenocopy *Wnt*/β-*catenin* signaling mutants (Sanson et al., 1996; Heasman et al., 1994; Fagotto et al., 1996).

It is well established that Wnt signaling 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 also facilitate a rapid intracellular transfer of membrane-bound β-catenin to the cytosolic "signaling" pool. Since downregulation of E-cadherin alone is not sufficient to induce ectopic activation of *T-lacZ* and *Brachyury* expression in WT primitive streak cultures, signaling 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 signaling responses at gastrulation. We propose, therefore, that normal downregulation 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 signaling pathways at gastrulation. Consequently, FGFR1 activity plays an indirect but permissive role in the propagation of Wnt signaling responses at the primitive streak. The fundamental interregulation 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.

Experimental Procedures

Mice

A colony of outbred mice heterozygous for a null allele of *Fgfr1* (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). *Fgfr1* mutant embryos were identified by phenotypic characteristics after E8.5. At earlier stages, embryos were genotyped by standard polymerase chain reaction analysis using either extraembryonic tissues or fragments of the embryo proper as a DNA source. The *Fgfr1* mutant allele was identified by a 0.9 kb DNA fragment amplified using a 5′-aagccaccatcacctgag gaa-3′ and 5′-tgggattagataaatgcctgctc-3′ primer pair, and the WT allele with a 5′-ttgaccggatctacacacacc-3′ and 5′-gcacaccggggtatg gggagc-3′ primer pair. The annealing temperature was 58°C. Wild-type embryos for in situ hybridization, immunostaining, and explant studies were derived from timed matings between ICR males and females.

For detection of *Brachyury* expression in *Fgfr1* mutants, a *T-lacZ*

reporter transgene was crossed into the *Fgfr1* 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 *Fgfr1* female heterozygotes. Male double heterozygotes were then crossed to female *Fgfr1* heterozygotes for timed matings. The presence of the *T-lacZ* reporter transgene was detected by PCR analysis using a 5′-gacaccagaccaactggtaatggtagcgac-3′ and 5′-gc atcgagctgggtaataagcgttggcaat-3′ primer pair, which are specific for the β-galactosidase gene; the annealing temperature was 58°C.

Generation of Chimeric Embryos

Diploid *Fgfr1* mutant chimeric embryos were generated by aggregating a 4- to 6-cell clump of homozygous *Fgfr1* mutant (*Fgfr1*−/−) 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 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 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 immunofluorescent 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 nonessential amino acids, 1 mM sodium pyruvate, 1 μM β-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 Ca^{2+}- and Mg^{2+}-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% CO_2 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 the 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.

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 (1992) and the tetramethyl-rhodamine tyramide signal amplification (TSA™) system for fluorescence in situ hybridization (NEN). Briefly, digoxigenin-labeled RNA probes were hybridized and washed as per Conlon and Rossant (1992). Embryos were then rinsed twice with TNT (0.1 M Tris-HCl [pH 7.5], 0.15 M NaCl, and 0.1% Tween-20) and blocked for at least 1 hr 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 three times 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 hr, without rocking. The reaction was stopped with three rinses of TNT, followed by two 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 was 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% H_2O_2 in methanol for 2–3 hr, 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 hr in PBBT (1% BSA in PBT) plus 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 plus 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, and were blocked again for 1 hr in PBBT plus 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 plus 1% goat serum. Samples were again washed 6–8 times for 30 min each 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; Cappell). After developing the tyramide fluorescence reaction, embryos were washed twice for 30 min each with TNT, rinsed with PTW (0.1% Tween-20 in PBS), blocked for 1 hr 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 6–8 times for 30 min each in PTW, and then manually sectioned, mounted, and processed as described.

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