Fgf8 is required for anterior heart field development
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In the mouse embryo, the splanchnic mesodermal cells of the anterior heart field (AHF) migrate from the pharynx to contribute to the early myocardium of the outflow tract (OT) and right ventricle (RV). Recent studies have attempted to distinguish the AHF from other precardiac populations, and to determine the genetic and molecular mechanisms that regulate its development. Here, we have used an Fgf8flox allele to demonstrate that Fgf8 is expressed within the developing AHF. In addition, we use both a hypomorphic Fgf8 allele (Fgf8neo) and Cre-mediated gene ablation to show that Fgf8 is essential for the survival and proliferation of the AHF. Nkx2.5Cre is expressed in the AHF, primary heart tube and pharyngeal endoderm, while TnT-Cre is expressed only within the specified heart tube myocardium. Deletion of Fgf8 by Nkx2.5Cre results in a significant loss of the Nkx2.5Cre lineage and severe OT and RV truncations by E9.5, while the remaining heart chambers (left ventricle and atria) are grossly normal. These defects result from significant decreases in cell proliferation and aberrant cell death in both the pharyngeal endoderm and splanchnic mesoderm. By contrast, ablation of Fgf8 in the TnT-Cre domain does not result in OT or RV defects, providing strong evidence that Fgf8 expression is crucial in the pharyngeal endoderm and/or overlying splanchnic mesoderm of the AHF at a stage prior to heart tube elongation. Analysis of downstream signaling components, such as phosphorylated-Erk and Pea3, identifies the AHF splanchnic mesoderm itself as a target for Fgf8 signaling.

KEY WORDS: Fgf8, Anterior heart field, Cardiogenesis, Cell survival, Proliferation, Pea3, Bmp4, Erk, Mouse

INTRODUCTION
Myocardial progenitors are derived from early migratory mesoderm that forms bilateral heart fields. These populations organize and coalesce generating the heart field crescent immediately rostral to the forming anterior intestinal portal in the mouse. Through a combination of proliferation and reorganization of the lateral fields, a subset of these myocardial progenitors form the primary heart tube (reviewed by Abu-Issa et al., 2004; Harvey, 2002). The primary heart tube gives rise to the cardiac inflow tract (atria) and the left ventricle. The remaining chambers of the heart, specifically the cardiac outflow tract (OT) and the right ventricle (RV) are derived from the pharyngeal mesodermal contiguous with the primary heart tube. Recent studies in chick and mouse demonstrate that cells within the developing splanchnic mesoderm (SM), as well as possibly the pharyngeal arch core mesoderm (CM), migrate from the pharynx and contribute to the myocardial wall of the OT and RV during early stages of development (Kelly et al., 2001; Mjaatvedt et al., 2001; Waldo et al., 2001). These myocardial precursors have been defined as the anterior heart field (AHF), which we use to describe specifically the SM and CM that contribute to the myocardium of the OT and RV.

In more recent studies, several transcription factors (Isl1, Tbx1, Nkx2.5, Foxh1, Mef2c) and extracellular signaling molecules (SHH, FGF10, BMP4) that are expressed within or adjacent to the SM and CM have been functionally implicated in AHF development (Baldini, 2004; Cai et al., 2003; Dodou et al., 2004; Lyons et al., 1995; Hu et al., 2004; Jiao et al., 2003; Kelly et al., 2001; Liu et al., 2004; von Both et al., 2004; Xu et al., 2004) (Washington Smoak et al., 2005). Moreover, extensive genetic analyses have shed some light on the regulatory network that guides its development. For example, Shh has been implicated as a regulator of Tbx1 expression through the Foxa1, Foxc1 and Foxc2 transcription factors (Yamagishi et al., 2003), and Tbx1 in turn may regulate Fgf8 expression (Hu et al., 2004). Interestingly, heterozygosity for Fgf8 decreases the incidence of cardiac defects of Tbx1Cre embryos (Hu et al., 2004; Vitelli et al., 2002) and Fgf8 hypomorphic mutants have OT defects, such as double outlet right ventricle and persistent truncus arteriosus (Abu-Issa et al., 2002; Frank et al., 2002).

Fgf8 is a member of a large gene family encoding extracellular ligands that have a wide range of cellular effects depending on the developmental context. Several studies have indicated that there are at least four non-mutually exclusive roles for Fgf8 in the development of OT defects. First, there is a primary requirement for Fgf8 during gastrulation, and loss of Fgf8 results in failure to form a crescent of heart precursor cells (Sun et al., 1999). Second, Fgf8 is required for establishing the left-right axis and cardiac looping. Abnormalities in this process may affect OT development (Albertson and Yelick, 2005; Meyers and Martin, 1999). Third, Fgf8 is required for migratory cardiac neural crest cell survival, and therefore OT seption defects could arise from neural crest cell deficiencies (Abu-Issa et al., 2002; Macatee et al., 2003). Finally, Fgf8 is expressed in the precardiac SM as early as E7.75 (Crossley and Martin, 1995; Kelly et al., 2001) and could have a direct role in AHF maintenance and/or specification. Reduction of the AHF population, and subsequent shortening of the OT, could result in OT seption defects. In zebrafish, it has been demonstrated that the loss of fgf8 results in the severe truncation of the ventricular myocardial segment (Reifers et al., 2000).

In this study, we have used genetic manipulations to distinguish between these possibilities to better understand how Fgf8 regulates OT development. We find that Fgf8 is required for AHF proliferation and survival; furthermore, we define the responsive tissues as well as the expression domains of Fgf8 that are crucial for AHF development.
MATERIALS AND METHODS

Mouse lines

All mouse lines used in this study were maintained on mixed genetic backgrounds. Floxed, null and hypomorphic alleles of Fgf8 (respectively named Fgf8\textsuperscript{lox,lo}x, Fgf8\textsuperscript{lo} and Fgf8\textsuperscript{hypo}) have been previously described (Meyers et al., 1998). The Fgf8\textsuperscript{lox,lo}x ‘knock in’ line was used to follow Fgf8 gene expression and was used interchangeably as a null allele (D.B. and R. Martin, unpublished). Nkx2.5\textsuperscript{Cre} (Moses et al., 2001) and TnT-Cre (Jiao et al., 2003) were crossed to the Cre-reporter line Rosa26R (R26R) (Soriano, 1999) to determine the domains of DNA recombination as shown by β-galactosidase (β-gal) activity in the progeny. Bmp4\textsuperscript{floox} and Ptc1\textsuperscript{lacZ} lines have been described previously (Goodrich et al., 1996; Lawson et al., 1999).

Generation of Cre-mediated Fgf8\textsuperscript{null} mutants

To generate Nkx2.5\textsuperscript{Cre}, Fgf8\textsuperscript{floox} embryos, male mice heterozygous for Nkx2.5\textsuperscript{cre} and Fgf8 were mated to females homozygous for the Fgf8 floxed allele (Fgf8\textsuperscript{floox}). The embryos were genotyped for Cre and Fgf8 alleles as previously described (Meyers et al., 1998; Sun et al., 2002). Similar protocols were used to generate Nkx2.5\textsuperscript{Cre}, Fgf8\textsuperscript{floox,lo}x and TnT-Cre; Fgf8\textsuperscript{loox,lo}x mutant embryos. For Cre lineage trace analysis in the conditional mutant background, Nkx2.5\textsuperscript{Cre}, Fgf8\textsuperscript{loox,lo}x males were crossed to females homozygous for both Fgf8\textsuperscript{loox} and R26R. Fgf8\textsuperscript{loox,lo}x, Ptc1\textsuperscript{lacZ} and Bmp4\textsuperscript{floox} embryos were genotyped for lacZ by staining for β-gal activity as described below.

lacZ staining

For embryos containing the R26R reporter allele or Bmp4\textsuperscript{floox}, tissues were fixed for 20 minutes at room temperature in 2% formaldehyde/0.2% glutaraldehyde in PBS with 0.02% NP40, then stained overnight at 37°C in standard X-gal stain. For Fgf8\textsuperscript{loox,lo}x and Ptc1\textsuperscript{lacZ} embryos, tissues were fixed for 2-3 minutes at room temperature, followed by standard X-gal stain for 48 to 72 hours at 37°C.

In situ hybridization

Digoxigenin antisense riboprobes were synthesized using template plasmids described in the following: Fgf8 (Crossley and Martin, 1995), Wnt11 (Majumdar et al., 2003), Atof, Mhc, Mlc2v (Christoffels et al., 2000) and Pea3 (Finkberg and Neubuser, 2002). Whole-mount mRNA in situ hybridization was performed essentially as previously described (Neubuser et al., 1997). All results represent at least three mutants and three control embryos.

Immunohistochemistry

All antibodies were diluted in blocking solution containing 3% milk, 0.1% Triton X in PBS. Antibodies were diluted as indicated: phospho-Histone H3 (1:500; Upstate Biotechnology), phospho-Erk (1:200; Cell Signaling Technologies) and AP2α (1:100; Developmental Studies Hybridoma Bank). Alexa Fluor 594 anti-rabbit IgG (Molecular Probes) and Cy2 anti-mouse IgG (Jackson Immuno) secondary antibodies were diluted 1:500. pErk staining was amplified with 1:200 biotinylated anti-rabbit IgG (Vector Laboratories), followed by 1:50 HRP-streptavidin (Jackson Immuno). For peroxidase colorimetric detection, embryos were bleached in 3% H2O2 in PBS, and incubated for 10 minutes at room temperature in 0.67 mg/ml 3,3-diaminobenzidine (DAB, Sigma) in PBS containing 0.1% Tween 20.

Cell death

LysoTracker Red (Molecular Probes) staining has been previously described as a marker for cells death (Zucker et al., 1999). We have performed LysoTracker Red staining as described by Abu-Issa et al. (Abu-Issa et al., 2002), with the exception that DMEM was substituted for Lactated Ringer’s Solution.

RESULTS

Fgf8 hypomorphic embryos exhibit cardiovascular and craniofacial defects

Previous analyses of Fgf8 hypomorphic mutants with variable levels of Fgf8 expression have revealed a range of cardiovascular phenotypes (Abu-Issa et al., 2002; Frank et al., 2002). Eighty-five percent of Fgf8\textsuperscript{hypo} hypomorphic mutants survive to term with various cardiac defects. Approximately 50% of these embryos have abnormal left-right axis establishment (data not shown), based on earlier ratios of laterality markers as well as organ laterality at term (Meyers and Martin, 1999). The remaining survivors exhibit severe craniofacial and cardiovascular defects, such as double outlet right ventricle (data not shown). These defects are reminiscent of DiGeorge/Velocardiofacial Syndrome and appear statistically and phenotypically independent of left-right defects. Instead, these defects are consistent with significant losses of neural crest cells (Abu-Issa et al., 2002; Macatee et al., 2003) and possibly deficiencies in AHF development.

In order to determine whether defects in the AHF contribute to these observed phenotypes, we examined Fgf8\textsuperscript{hypo} and Fgf8\textsuperscript{floox,lo}x embryos for OT or RV abnormalities at embryonic day (E) 9.5, prior to any substantial contribution of neural crest cell (NCC)-derived populations. Most of the mutant embryos that survive early gastrulation defects exhibit moderate (Fig. 1C) or severe (Fig. 1D) truncations of the OT and RV when compared with stage-matched controls (Fig. 1A). Most of these moderate and severe mutants presumably die by E10.5 owing to circulation failure, as evidenced by pericardial edema (data not shown). The remaining hypomorphic mutants demonstrate mild truncation of the OT/RV (Fig. 1B). These mild Fgf8\textsuperscript{hypo} mutants presumably progress to term with a variety of OT defects such as double outlet right ventricle, which may be the result of the OT/RV hypoplasia. No defects in the atria or left ventricle were evident in our analysis at this stage.

Surprisingly, we noted strong β-gal activity in the developing OT and RV of Fgf8\textsuperscript{hypo} and Fgf8\textsuperscript{floox,lo}x embryos at E9.5–E10.5, where Fgf8 mRNA has not previously been detected. This expression pattern is consistent with a transgenic Fgf8\textsuperscript{lacZ} line which has detectable β-gal activity in the OT (Hu et al., 2004). Given the abnormal OT/RV phenotype and this unexpected expression, we compared the expression of both Fgf8 mRNA and Fgf8\textsuperscript{hypo} relative to AHF development. Fgf8\textsuperscript{hypo} faithfully recapitulates Fgf8 mRNA expression pattern in all areas examined between E7.5 and E10.5 with some notable differences described below.

Analysis of Fgf8 expression relative to heart development

At E7.5, both Fgf8 mRNA and Fgf8\textsuperscript{hypo} expression are present in bilateral fields immediately caudal to the primary heart field (Crossley and Martin, 1995). These cells ingress inward with the foregut pocket, moving both dorsally and rostrally in relation to the primary heart field. Consistent with this model, both Fgf8 mRNA and Fgf8\textsuperscript{hypo} are expressed in the SM contiguous with the developing OT between E8.0 and E8.5 (Fig. 2C,C’ and 2D,D’, respectively).

Fig. 1. Fgf8 hypomorphs have variable length outflow tracts. (A-D) Fgf8\textsuperscript{floox,lo}x embryos that undergo relatively normal gastrulation demonstrate OT of variable lengths from mild (B) to moderate and severe (C,D) when compared with control at E9.5 (A). OT, outflow tract; V, ventricle; 1, first pharyngeal arch.
Later, between E9.0 and E10.5, Fgf8 mRNA is expressed in the pharyngeal endoderm and ectoderm in close proximity to the AHF, but not detected in the SM or OT (Fig. 2E,E’,G,I). By contrast, β-gal activity is still detected within the SM and extensively in the OT and RV (Fig. 2F,F’,H). To better define which cells are expressing lacZ, we sectioned whole-mount stained embryos at multiple stages. Transverse sections at E8.0 demonstrate expression within the SM extending from the OT myocardium caudally to where the foregut has not closed (Fig. 2K1-K3). In addition, we detect expression at E9.5-E10.0 in the central area of the developing first and second pharyngeal arch, consistent with non-neural crest CM (Fig. 2F,L,L’,J). These Fgf8lacZ-positive CM cells are contiguous with the developing OT wall.

To determine the nature of the discrepancy between Fgf8 mRNA expression and Fgf8lacZ β-gal activity, we analyzed the OT of the heart at E9.5 for lacZ mRNA. Although, lacZ mRNA was detectable by in situ hybridization in domains of Fgf8lacZ β-gal activity (Fig. 3A,B), the mRNA levels appear much lower in proportion to protein activity. Additionally, very low levels (<1% of limb expression) of Fgf8 message are detected in the OT by RT-PCR (Fig. 3C), which again are out of proportion with β-gal activity. This finding suggests that most of the activity in OT and RV myocardium is the result of residual β-gal protein carried within cells that originated from the AHF external to the developing heart tube, consistent with AHF development models.

Given the expression of Fgf8lacZ within the SM and CM, and the cardiac defects associated with Fgf8 hypomorphic mutants, we hypothesized that Fgf8 expression is required within the AHF for the development of the OT and RV. To test this hypothesis, we performed genetic ablation of Fgf8 using the tissue-specific Cre lines Nkx2.5Cre and TnT-Cre.

**Nkx2.5Cre, but not TnT-Cre, mediates ablation of Fgf8 in the AHF**

The Nkx2.5 locus targets most cardiogenic populations when expressing Cre (Moses et al., 2001; Stanley et al., 2002); however, early cardiac crescent expression of the Nkx2.5-Cre allele used in this study is somewhat mosaic (data not shown). Nevertheless, complete recombination in the cardiac fields is observed at E8.0-E8.5 prior to heart tube elongation (Fig. 4B,B’). Although mesodermal expression of Fgf8 precedes robust Nkx2.5Cre expression, we can use the Nkx2.5Cre allele to ablate Fgf8 in the AHF prior to the elongation process. Nkx2.5Cre also targets cells in the heart tube, the ventral pharyngeal endoderm and the anterior ectoderm of the first pharyngeal arch (Fig. 4B,B’,E,E’,E’). As revealed by the Fgf8lacZ line, Fgf8 is expressed in the pharyngeal endoderm, ectoderm, the AHF and the lineage of the AHF (Fig. 4A,A’,D,D’,D’). Therefore, Fgf8 will functionally be ablated from the AHF, endoderm, first pharyngeal arch ectoderm and subsequent derivatives of these tissues.

By contrast, TnT-Cre drives DNA recombination in the primary heart field cardiac crescent as early as E7.5, but not in the AHF precursors (Jiao et al., 2003). Between E8.5 and E11.5, the expression domain is exclusive to the entire myocardium including OT, ventricles and atria. Importantly, TnT-Cre is expressed in AHF derivatives only after these cells have migrated into the heart tube proper and differentiated to form the OT and RV myocardium.
Although slightly dilated, while the presumptive OT and RV are almost completely absent (Fig. 5A,B). Only a small segment of myocardium joins the left ventricle to the aortic sac. To better define the regions of heart tube that are missing, we performed in situ hybridization analysis on the Nkx2.5Cre+; Fgf8crex/mutants and littermate controls with a number of regionally specific markers (Fig. 5C-3). Wnt11, previously described as a marker specific for the truncus of the OT of the heart (Cai et al., 2003), is completely absent, indicating that mutants are deficient in this region of the OT (Fig. 5C,D). We used the chamber-specific markers atrial natriuretic factor (Anf) and myosin light chain 2v (Mlc2v) to further characterize the remnant anterior heart segment. Anf specifically marks the left ventricle and both atria at E9.5 (Fig. 5E) (Christoffels et al., 2000), while Mlc2v is specifically expressed by ventricular myocardium (Fig. 5G) (Cai et al., 2003). We observe in our Nkx2.5Cre+, Fgf8crex/-mutants that the small anterior heart segment does not express Anf (Fig. 5F), but does express Mlc2v (Fig. 5H). This analysis suggests that the remnant region is primarily residual right ventricular myocardium, with very little contribution by OT myocardium.

As a final means to characterize the mutant heart tubes, we bred the Bmp4lacZ “knock in” allele onto wild-type and mutant backgrounds, and stained the embryos for β-gal activity in whole mount in situ hybridization at E9.5, but very low levels are seen within the OT and RV (Fig. 5I). Fgf8 transcripts are present in the OT at very low levels compared with limb bud controls and HPRT positive controls (Fig. 4C) shown by semi-quantitative RT-PCR analysis at E9.5. These data suggest that Fgf8 expression accurately reflects Fgf8 mRNA expression and that β-gal activity is more prominent in OT/RV than either lacZ or Fgf8 mRNA. OT, outflow tract; LB, limb bud; NEG, negative control tissue.

We defined the regions of functional Fgf8 deletion by in situ hybridization for the deleted exons of the Fgf8lox allele (Fig. 4G-J). We confirmed that Fgf8 expression is greatly reduced in the AHF of Nkx2.5Cre+; Fgf8crex/-embryos as early as E8.0 (compare Fig. 4G with 4H) and virtually absent by E9.5 (compare Fig. 4J with 4I), while maintained in other areas of expression such as brain, surface ectoderm and tail bud (where Nkx2.5Cre is not expressed). Conversely, TnT-Cre recombinase resulted in no discernable change in Fgf8 mRNA expression in the SM or endoderm (data not shown). Phenotypic comparison between TnT-Cre; Fgf8crex/-embryos and Nkx2.5Cre+/; Fgf8crex/-mutants identify and define the domains of Fgf8 expression that are necessary for OT and RV development.

The Nkx2.5Cre lineage is reduced in the Nkx2.5Cre+; Fgf8crex/-mutants
As Nkx2.5Cre is expressed in the AHF, we can use the Cre reporter line R26R (Soriano, 1999) to trace the fate of these cells. Using this strategy, we analyzed the Nkx2.5Cre lineage in the Fgf8 conditional mutant background. At stages prior to heart tube elongation (E8.0), we do not observe any change in the number of R26R lacZ-expressing cells of Nkx2.5Cre+; Fgf8crex/-; R26R mutants. However, differences become noticeable by E8.5-E9.0 as the AHF cells move from the SM and into the heart tube (Fig. 6A,B). By E9.0-E9.5, we observe a significant reduction in β-gal-positive cells in the SM (Fig. 6C-H) and the pharyngeal endoderm (Fig. 6E,F), evidenced by the severe thinning of these cell layers (Fig. 6E,F). β-gal-positive cells of the CM are also significantly decreased in mutants
The overall reduction in the Nkx2.5Cre lineage strongly suggests that loss of these cells results in the severe heart tube truncation phenotype that is observed in Fgf8 conditional mutants. Fgf8 lacZ cells are reduced, but not completely absent in mutants.

As Fgf8lacZ also marks AHF cells, we used the Fgf8lacZ allele as a null allele in our ablation scheme to determine the fate of Fgf8-expressing cells in Nkx2.5Cre+; Fgf8lacZ/lox mutant embryos. Phenotypically, Nkx2.5Cre+; Fgf8lacZ/lox embryos are indistinguishable from Nkx2.5Cre++; Fgf8lox/lox embryos. As seen in whole-mount and section, the overall number of Fgf8lacZ-positive cells in the conditional mutants is mildly reduced (Fig. 6I), and, as expected, ectodermal expression remains intact. Again, as seen with the Nkx2.5Cre lineage, we observe a loss of AHF cells as shown by the thinning of the SM cell layer (Fig. 6I'). Interestingly, most of the AHF cells that remain in the SM and CM are Fgf8lacZ β-gal-positive (Fig. 6J').

We also observe that the OT/RV cells in Nkx2.5Cre++; Fgf8lacZ/lox mutants are Fgf8lacZ positive. Although consistent with our data indicating that this segment is remnant RV, this finding is surprising in that we expected a lineage autonomous function of Fgf8, i.e. that deletion of Fgf8 would result in a loss of all the Fgf8lacZ-expressing cells of the heart tube. There are several explanations for the persistence of Fgf8lacZ-expressing cells in Nkx2.5Cre/+; Fgf8lacZ/lox embryos. First, this subset of AHF cells may develop independently of Fgf8. Second, deletion of Fgf8 by Nkx2.5Cre may not be early enough to affect a small population of AHF as Fgf8 expression precedes Nkx2.5 expression. Alternatively, residual RNA or protein after genetic deletion can result in some functional rescue of the earliest AHF cells, which form the RV. The latter two explanations are more likely given that at early stages, R26R data clearly demonstrates a relatively normal number of Nkx2.5-positive cells of the AHF, and residual Fgf8 mRNA is observed in mutants (Fig. 4H, Fig. 6B) despite complete recombination between E8.0 and 9.5 (Fig. 4C',E',E' and data not shown).
Erk phosphorylation is reduced in Nkx2.5Cre/++; Fgf8flox/– mutants

FGF8 can function through the receptor tyrosine kinase pathway, leading to the phosphorylation of the MAP kinase Erk (Corson et al., 2003; Katz and McCormick, 1997; Rommel and Hafen, 1998; Sato and Nakamura, 2004). Distribution of phospho-Erk (pErk) can therefore identify tissue targets undergoing active signaling as a result of Fg8 activity in anterior heart development. We first observe pErk staining in the SM at E8.5 (data not shown). pErk persists until E9.0, where it is observed in the SM as well as the emerging OT, but not significantly in the pharyngeal endoderm (Fig. 7A,A’). After E9.5, when AHF cells have been added and looping is complete, there is an absence in pErk staining in the SM and OT, indicating absence of active signaling through this pathway (data not shown). This decrease in Erk phosphorylation temporally coincides with the observed downregulation of Fgf8 mRNA, suggesting that Fg8 signals through Erk in the AHF. If this hypothesis is correct, then loss of Fgf8 will result in the loss of Erk phosphorylation in the SM. Therefore, we compared the pErk levels in wild-type and Nkx2.5Cre/++; Fgf8flox/– mutant embryos.

In early cardiac crescent stages, there is no detectable difference in pErk levels between wild-type and mutant embryos (data not shown). This finding is consistent with the fact that at these early stages, Nkx2.5Cre expression appears low and incomplete, as revealed by the R26R reporter gene. Between E8.5 and E9.5 as the heart tube undergoes elongation, Nkx2.5Cre/++; Fgf8flox/– embryos do not stain positively for pErk in the SM, indicating a loss of signal specifically within these AHF cells (Fig. 7B,B’). It therefore appears that Fgf8 signaling through Erk phosphorylation within the SM is necessary for AHF addition to the myocardium.

Genetic targets Pea3 and Bmp4 are reduced in Nkx2.5Cre/++; Fgf8flox/– mutants

To further test our hypothesis that FGF8 signals in an autocrine fashion to the AHF, we analyzed downstream genetic components of the Fgf8 pathway. Members of the ETS family of transcription factors, Pea3, Erm and Er81, are transcriptional targets of FGF8 signaling (Corson et al., 2003; Munchberg and Steinbeisser, 1999; Raible and Brand, 2001; Roehl and Nusslein-Volhard, 2001). Pea3 is expressed in both tissues in wild-type embryos. Pea3 expressed in both tissues in wild-type embryos, and is absent in both tissues after Fgf8 ablation. These data suggest that FGF8 may signal directly to the SM to activate Erk, and that a secondary signal (independent of Erk) may be transduced both in an autocrine fashion to the SM and by a paracrine mechanism to the endoderm to initiate Pea3 expression. Alternatively, FGF8 could regulate Pea3 expression independently of Erk activation.

In consideration of the potential crosstalk between the AHF and the pharyngeal endoderm, we determined if Fgf8 ablation altered other extracellular signaling pathways (Shh and Bmp4) known to be involved in OT development (Liu et al., 2004) (Washington Smoak et al., 2005). We do not observe any alterations of Shh expression in the endoderm of Nkx2.5Cre/++; Fgf8flox/– mutants (Fig. 7G,H). Pch1lacZ expression, which is upregulated in response to activation of the hedgehog pathway (Goodrich et al., 1996), is likewise unaltered in the endoderm and SM (Fig. 7E,E’,F,F’). However, examination of Bmp4lacZ expression in the SM of Nkx2.5Cre/++; Fgf8flox/– mutants (Fig. 7I,J and Fig. 5I) indicates a reduction in gene expression. These data reveal that Fgf8 does not regulate the hedgehog pathway in the SM, but may regulate Bmp4. In addition, they suggest that the loss of pErk and Pea3 expression are indeed the result of Fgf8 ablation and not the result of dysplastic SM and endoderm, as these tissues are still capable of expressing Pch1 and Shh, respectively.

Fgf8 is required for proliferation and survival of the precardiac mesoderm and pharyngeal endoderm

Lineage tracing of the Nkx2.5Cre domain has revealed a reduction in both the endoderm and SM populations. Therefore, we analyzed cell proliferation in the pharyngeal regions from E8.5 to
E9.5 using antibodies for phosphorylated histone H3 (pHH3), which marks mitotic cells. In $Nkx2.5^{Cre/+}, Fgf8^{flox/-}$ mutants, we have observed a significant decrease in cell proliferation in both the SM and pharyngeal endoderm (Fig. 8A,B). A comparison of wild-type and mutant embryos reveals that mitotic indices are reduced by ~50% in the SM and by 60% in the pharyngeal endoderm, although no significant differences in mitotic indices were noted in the outflow region, the left ventricle, atria (Fig. 8G) or the pharyngeal arches (data not shown).

LysoTracker Red analysis from E8.5 to E9.5 reveals that the SM and surrounding tissue undergo excess cell death. Specifically at E9.0, there is an increase in cell death in the SM immediately distal to the emerging OT (Fig. 8C,D), as well as the endoderm adjacent to the SM. By E9.5, once heart tube elongation has been completed, no differences are detected in the SM. However, excess cell death has expanded into the ventral pharyngeal endoderm (data not shown). These data, along with the proliferation studies, reveal that $Fgf8$ is necessary for the expansion and maintenance of the precardiac mesoderm, as well as pharyngeal endoderm, and suggest that the truncation of the OT and RV is a result of the reduction in these cell populations.

**Neural crest cells undergo excess cell death in $Nkx2.5^{Cre/+}, Fgf8^{flox/-}$ mutants**

We and others have previously described abnormal cell death of neural crest cells (NCCs) in $Fgf8$ hypomorphs (Abu-Issa et al., 2002; Frank et al., 2002). Interestingly, tissue-specific ablation of $Fgf8$ in the $Nkx2.5^{Cre}$ domain also results in excess cell death of NCCs in the pharyngeal arch mesenchyme at E9.5 (Fig. 8E,F). The excess cell death in the pharyngeal arch mesenchyme encompasses much of the migratory streams of NCCs up to the dorsal neural tube. These migratory NCCs are specified and migrate in relatively normal streams from the dorsal neural tube as seen by the markers $Ap2$ and $Crabp1$ (data not shown). In addition, we see increased cell death of presumptive cardiac NCCs in close proximity to the OT (Fig. 8D).

**DISCUSSION**

**FGF8 signaling to the AHF is required for OT and RV development**

In this study, we identify $Fgf8$ expression domains that are required for the maintenance and specification of the AHF. Elimination of the $Fgf8$ gene specifically from the $Nkx2.5^{Cre}$ domain results in aberrant AHF development and ultimately OT/RV truncation. By
contrast, our studies using TnT-Cre demonstrate that Fgf8 expression in the entire primary and AHF-derived myocardium is not crucial. No obvious defects are noted in primary heart field development with either Nkx2.5 or TnT-Cre ablations, suggesting that Fgf8 expression is not required in these tissues within the time frame of the Cre expression. Therefore, Fgf8 expression must be required in the AHF and/or the pharyngeal endoderm for OT/RV outgrowth and elongation. Interestingly, Fgf8 ablation in all mesoderm, but not endoderm, can phenocopy Nkx2.5<sup>Cre/+; Fgf8<sup>flox/–</sup> embryos (Park et al., 2006). Although these data suggest that Fgf8 expression in the SM alone is required for heart tube elongation, this phenotype (driven by Mesp1<sup>Cre</sup>) could represent an earlier defect in mesodermal specification and/or maintenance. Erk phosphorylation is restricted to the SM, and not endoderm, demonstrating that FGF8 signaling to the AHF is crucial for OT/RV outgrowth and development.

Other studies have also used tissue-specific gene deletion to determine the specific functions of the various Fgf8 expression domains in the pharynx. Tbx1-Cre, like Nkx2.5<sup>Cre</sup>, is expressed in both pharyngeal endoderm and mesoderm. Tbx1-Cre ablation of Fgf8 results in DiGeorge-like defects reminiscent of late term Fgf8 hypomorphs (Brown et al., 2004), but the majority of these mutants do not exhibit the early heart tube truncation phenotype seen in Nkx2.5<sup>Cre/+; Fgf8<sup>flox/–</sup></sup> embryos. As Nkx2.5<sup>Cre</sup> is a null allele, and Nkx2.5 may lie genetically downstream of Fgf8 (Alsan and Schulthesis, 2002), Nkx2.5 heterozygosity may contribute to early OT/RV truncation. However, the Nkx2.5<sup>Cre/+; Fgf8<sup>flox/–</sup></sup> mutants can be phenocopied through the use of other Cre lines, such as Isl1<sup>Cre</sup> (Park et al., 2006). Furthermore, we observe the heart tube truncation phenotype in Fgf8 hypomorphs where Nkx2.5 gene dose is unaltered.

Phenotypic differences between Nkx2.5<sup>Cre/+; Fgf8<sup>flox/–</sup></sup> and Tbx1-Cre; Fgf8<sup>flox/–</sup> embryos may be the result of slightly different Cre expression domains. Brown et al. have shown that DNA recombination in the endoderm is complete at E10.5, but the extent of endodermal Tbx1-Cre expression at earlier stages may not be complete (C. Brown, personal communication). Incomplete deletion of Fgf8 in the endoderm earlier in development may explain the discrepancy between Tbx1-Cre; Fgf8<sup>flox/–</sup> and Nkx2.5<sup>Cre/+; Fgf8<sup>flox/–</sup></sup> mutants. This explanation suggests a crucial role for early endodermal Fgf8 expression and AHF expression in the development and outgrowth of the OT/RV. In support of this hypothesis, Fgf8 ablation specifically in the AHF does not phenocopy the early heart tube truncation phenotype (Park et al., 2006). These data, therefore, suggest a requirement for both domains of Fgf8 expression for heart tube elongation. Although it remains unclear whether or not endodermal and mesodermal sources of FGF8 have separate and distinct functions, we have nevertheless shown that Fgf8 has a role in determining the length of the OT/RV through proliferation and survival of the AHF.

**Fig. 7. Cell signaling analysis in Nkx2.5<sup>Cre/+; Fgf8<sup>flox/–</sup></sup> mutants.** Immunohistochemistry analysis demonstrates pErk is localized in the SM, but not the PE (A,A'). Nkx2.5<sup>Cre</sup> elimination of Fgf8 results in loss of pErk from the SM (B,B'). RNA in situ hybridization demonstrates that Pea3 is expressed in the developing arches as well as SM and PE (C,C'). Loss of Fgf8 results in continued arch ectoderm expression (D, black arrowhead) but loss of SM and PE expression (D'). Pitch1<sup>lacZ</sup> β-gal activity (E-F) and Shh expression (G,H) demonstrate that the SM and PE are still capable of Pitch1 expression, and that Fgf8 does not regulate this pathway in these tissues. Bmp4 is downregulated within the SM (I,J), suggesting a requirement for FGF8 signaling. OT, outflow tract; SM, splanchnic mesoderm; PE, pharyngeal endoderm; Nt, neural tube/notochord.
It is interesting to speculate that the OT septation defects seen in near term Fgf8 hypomorph embryos, such as double outlet right ventricle, may reflect alignment defects owing to variable lengthening of the OT. However, it is also possible that alterations in the number or specification of cardiac NCCs entering the OT could also account for such defects. NCCs also have a role in OT elongation as NCC laser ablation results in shortened OT in chick (Farrell et al., 2001; Yelbuz et al., 2002). Distinguishing between such possibilities will be difficult because of potential interactions between the two fields. We are currently investigating the requirement for the Fgf receptor genes Fgfr1 and Fgfr2 in the AHF to determine if Fgf signaling has a direct influence on AHF development. Preliminary data indicate that loss of these receptors from the Nkx2.5Cre domain results in OT septation defects, but not OT/RV truncation (G. Smyth and E.N.M., unpublished).

Neural crest cells and the anterior heart field
Expansion of the pharyngeal arches is the result of NCCs migrating into the pharyngeal region, forming much of the mesenchyme. Loss of Fgf8 from the AHF and endoderm results in marked cell death of migratory NCCs (Fig. 8F), similar to that seen in Fgf8flox– embryos (Abu-Issa et al., 2002; Macatee et al., 2003). This expansive cell death can account for the pharyngeal arch hypoplasia observed in Fgf8 mutants. It was surprising to observe that NCCs located as far as the dorsal neural tube undergo excess cell death in Nkx2.5Cre+; Fgf8flox– embryos. This observation leads to two hypotheses. First, given the close apposition and function of the AHF and NCCs in OT development, it is possible that the AHF and NCCs are interdependent. That is, the development of the NCCs depends on signals from the AHF (perhaps through Bmp4). Defective AHF, owing to loss of Fgf8, negatively impacts NCC development indirectly. It follows then that pharyngeal arch hypoplasia would result from the loss of NCCs.

The second possibility is that FGF8 protein from the AHF is able to diffuse long distances to directly influence NCC development. However, NCC-specific deletion of both Fgfr1 and Fgfr2 does not affect cardiac NCC development, suggesting an indirect effect of FGF8 on these cells in the lower arches (G. Smyth and E. Meyers, unpublished). Alternatively, FGF8 may signal directly to the NCCs through other Fgfr receptors.

Role of the pharyngeal arch core mesoderm
Interestingly at E9.5, Fgf8flox–/– β-gal activity persists within the CM of the pharyngeal arches, but neither Fgf8 nor lacZ mRNA transcripts are detected (data not shown). Previous work has shown that this population of cells is derived from the paraxial mesoderm (Trainor et al., 1994), and Fgf8 appears to be expressed in this region as early as E8.0 (Crossley and Martin, 1995). The persistence of β-gal activity again could represent a lineage trace of this mesoderm into the core of the pharyngeal arches rather than nascent gene expression.

Gross analyses of the AHF markers Fgfl0lacZ, Tbx1lacZ, and Fgf8lacZ, as well as the Nkx2.5Cre lineage trace, reveal that the CM cells are contiguous with the OT (Brown et al., 2004; Kelly et al., 2001). Dil-labeling studies suggest that these CM cells are part of the AHF, and contribute cardiomyocytes to the OT at early stages (Kelly et al., 2001). We have found that like in the SM, there is a marked reduction of the Nkx2.5Cre-positive cells in the CM of conditional Fgf8 mutants. The reduction of CM may not only contribute to OT/RV truncation, but also to pharyngeal arch hypoplasia. Additionally, Fgf8 expression may be required in the CM for the survival of the NCCs that populate the pharyngeal arches.
At E9.5, when heart tube elongation has been completed, we observe a persistence of Fgf8lacZ and Nkx2.5Cre-positive cells in the CM. Although these cells remain contiguous with the OT myocardium, it is unlikely that at this stage they continue to contribute to the OT. Instead, these cells are known to give rise to the muscles of the jaw (Trainor et al., 1994). It is intriguing to speculate that OT/RV myocardium and the muscles of the jaw arise from a common lineage represented by the Fgf8lacZ allele and Nkx2.5Cre line. At the least, the two populations share common genetic regulators required for myogenesis and/or for proper development, such as Tbx1, Isl1 and Nkx2.5 (Cai et al., 2003; Kelly et al., 2004; Lyons et al., 1995).

**Cellular functions of Fgf8 in the anterior heart field**

Aside from cell proliferation and survival, Fgf8 signaling has a very specific role in gene regulation. Fgf8 signaling has been shown to modulate the expression of Wnt (Grieshammer et al., 2005) and Bmp (Lewandoski et al., 2000) genes to pattern the embryo. Fgf8 has also been shown in vivo to be necessary for expression of various transcription factors such as Pea3, Erm and Tbx2 (Firmberg and Neubuser, 2002; Munchberg and Steinbeisser, 1999; Raible and Brand, 2001; Roehl and Nusslein-Volhard, 2001). Hence, we have found that Fgf8 is necessary for Pea3 expression within the AHF itself, and may also modulate Bmp4 expression levels.

Many ETS and Fgf proteins have roles in epithelial-to-mesenchymal transition and cell migration (reviewed by Hsu et al., 2004; Thiery, 2002). Fgf1 and Fgf2 have been shown in vitro to induce the internalization and nuclear localization of the cell-adhesion molecule E-cadherin (Bryant et al., 2005). In addition, Fgf signals can activate cell motility through the small GTPase Rho, as seen in Drosophila (Schumacher et al., 2004), and through activated Mapk to promote myosin light chain kinase phosphorylation (Klemke et al., 1997). Developmentally, the loss of the Fgf ligand heartless in Drosophila demonstrates defects in mesodermal migration (Beiman et al., 1996). In mouse, Fgf8-null (Sun et al., 1999) and Fgfr1-null embryos (Ciruna and Rossant, 2001) have altered cadherin expression, and exhibit migrational defects during gastrulation. We speculate that Fgf8 signaling in the AHF increases cell motility at both the transcriptional and cellular levels, allowing for the migration of cells into the heart tube. Loss of this motility signal may disrupt the AHF program, resulting in increased cell death and decreased proliferation. Studies are currently under way to address this possibility.

**References**


Fgf8 and AHF development
