FGF10/FGFR2b signaling is essential for cardiac fibroblast development and growth of the myocardium

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SUMMARY
The epicardium serves as a source of growth factors that regulate myocardial proliferation and as a source of epicardial-derived cells (EPDC), which give rise to interstitial cardiac fibroblasts and perivascular cells. These progenitors populate the compact myocardium to become part of the mature coronary vasculature and fibrous skeleton of the heart. Little is known about the mechanisms that regulate EPDC migration into the myocardium or the functions carried out by these cells once they enter the myocardium. However, it has been proposed that cardiac fibroblasts are important for growth of the heart during late gestation and are a source of homeostatic factors in the adult. Here, we identify a myocardial to epicardial fibroblast growth factor (FGF) signal, mediated by FGF10 and FGFR2b, that is essential for movement of cardiac fibroblasts into the compact myocardium. Inactivation of this signaling pathway results in fewer epicardial derived cells within the compact myocardium, decreased myocardial proliferation and a resulting smaller thin-walled heart.

KEY WORDS: Epicardium, Cardiac fibroblast, Cardiomyocyte, FGF10, FGFR2b, EPDCs

INTRODUCTION
The epicardium comprises the outer layer of the heart and provides a source of cardiac fibroblasts, vascular smooth muscle cells and pericytes during heart development (Mikawa and Fischman, 1992; Mikawa and Gourdie, 1996; Dettman et al., 1998; Merki et al., 2005; Cai et al., 2008; Snider et al., 2009). The formation of the epicardial layer begins after heart looping at embryonic day (E) 9.5 in the mouse (Kalman et al., 1995). Epicardial cells arise from the pro-epicardial organ, which is a transient structure located close to the sinus venosus on the ventral body wall (Hiruma and Hirakow, 1989; Vincent and Buckingham, 2010). Cells from the pro-epicardium migrate to the atrioventricular groove and then from the base of the heart to the apex, covering the heart as a single cell layer. Concurrently, a capillary plexus grows from the dorsal atrioventricular groove and expands towards the apex and ventrally to envelop the entire heart (Kattan et al., 2004; Lavine et al., 2006; Red-Horse et al., 2010). As epicardial cells migrate to cover the heart, a subset of epicardial cells undergo epithelial-to-mesenchymal transitions (EMT) and delaminate from the epicardium. These cells are termed epicardial-derived cells (EPDCs). Once EPDCs acquire mesenchymal phenotype, they migrate further into the compact myocardium where they differentiate into smooth muscle cells and cardiac fibroblasts. These differentiated smooth muscle cells and cardiac fibroblasts become part of the mature coronary vasculature and interstitial mesenchyme of the heart. PDFGRβ and Alk5 are factors that regulate epicardial-derived vascular smooth muscle cells (Mellgren et al., 2008; Sridurongrit et al., 2008). By contrast, factors that regulate migration of cardiac fibroblasts have not been identified.

The FGF family comprises 18 signaling ligands and four receptors (FGFRs) (Ornitz and Itoh, 2001; Itoh and Ornitz, 2008; Turner and Grose, 2010). Fgfr1, Fgfr2 and Fgfr3 undergo alternative splicing that results in b and c splice variants (Dell and Williams, 1992; Werner et al., 1992). The b splice variants are preferentially expressed in epithelial and epithelial-like tissues, such as the epicardium (Marguerie et al., 2006). By contrast, c splice variants are preferentially expressed in mesenchymal tissues. FGF ligands are classified in subfamilies based on phylogenetic similarities. Members of each subfamily of FGFs share similar biochemical properties, such as affinity for specific FGFRs and FGF splice variants (Itoh and Ornitz, 2004). FGFR2c is efficiently activated by members of the FGF9 subfamily (FGF9, FGF16 and FGF20) (Ornitz et al., 1996; Zhang et al., 2006). By contrast, FGF3, FGF7, FGF10 and FGF22 are ligands that activate FGFR2b. Downstream FGF signal transduction can proceed via three main pathways: Ras/MAPK pathway, phospholipase Cγ (PLCγ)/Ca2+ pathway and the PI3 kinase/Akt pathway (Eswarakumar et al., 2005; Lemmon and Schlessinger, 2010).

The expression of several FGFs and FGFRs in cardiac and vascular mesoderm, mesothelium and endoderm suggests an important role for these molecules in development of the heart. In zebrafish and avian models, FGF signaling has been implicated as important for epicardial cells to undergo EMT, to enter the myocardium, and potentially to differentiate into coronary smooth muscle cells, interstitial cardiac fibroblasts, coronary endothelial cells and cardiomyocytes (Mikawa and Gourdie, 1996; Morabito et al., 2001; Perez-Pomares et al., 2002; Lepilina et al., 2006). The FGF ligands FGF1, FGF2 and FGF7 were shown to stimulate EMT in cultured epicardial cells (Morabito et al., 2001) and pharmacological inhibition of FGF signaling impaired epicardial EMT (Pennisì and Mikawa, 2009); however, retroviral expression of a dominant-negative FGFRI in epicardial and endothelial precursors in the pro-epicardial organ did not affect epicardial EMT but did prevent the progeny of pro-epicardial-derived cells from invading the myocardium (Pennisì and Mikawa, 2009). These studies suggest that FGF signaling is necessary for epicardial and...
endothelial development, but do not define the precise FGF signaling pathways that regulate each of these lineages or determine whether signaling is direct or indirect.

In previous studies, we identified an epicardial-to-myocardial FGF signaling pathway in which FGF9, expressed in the epicardium, signals to FGFR1c and FGFR2c in the myocardium to control myocardial proliferation and, indirectly, vascular formation (Lavine et al., 2005). Other members of the FGF9 subfamily have also been shown to function during heart development. For example, FGF16 is expressed in the midgestation heart (Lavine et al., 2005) and functions to regulate myocardial proliferation (Hotta et al., 2008). Several studies have identified expression of Fgf7 and Fgf10 in the developing myocardium, and one study showed that mice lacking the b splice variant of Fgr2 (Fgr2b+) developed a thin-walled heart (Morabito et al., 2001; Marguerie et al., 2006). These observations suggest that FGF signals emanating from the myocardium might directly regulate epicardial development or function. In this study, we show that FGF10 signals to the epicardium through FGFR1 and FGFR2b. In turn, these receptors control movement of EPDCs into the compact myocardium. Inactivation of this pathway results in fewer EPDCs within the compact myocardium and results in reduced cardiomyocyte proliferation and a smaller heart.

**MATERIALS AND METHODS**

**Mice**

Mouse lines used were Fgr2b—/- (Revest et al., 2001), Fgf7—/- (Guo et al., 1996), Fgf10—/- (Min et al., 1998), Wt1-Cre (Zhou et al., 2008), Fgr2b+/Fgfr2f/f (Shaffer et al., 2008), Fgfr1–/– (Yu et al., 2003), Rosa26-rtTA (Belteki et al., 2005), TetO-Fgf10 (Clark et al., 2001) and McI1vnlacZ-24 transgenic mouse (Kelly et al., 2001).

**Histology**

Paraffin sections (5 μm) were stained with Hematoxylin and Eosin (H&E) for general visualization. Myocardial area was calculated with the contouring tool using Canvas X software. Cross-sectional area of the heart was defined as the measure of total muscle, including both chambers in one mid-frontal section. Atrial area was not included. Fgr2b–/–/Fgfr2f/f, Fgfr2b–/–/Mlc1vnlacZ-24 positive nuclei relative to the total number of nuclei was counted from two small areas from each embryo. Statistical significance was determined using Student’s t-test, with n representing number of embryonic hearts examined.

**Immunohistochemistry and immunofluorescence**

For immunohistochemistry, paraffin sections (5 μm) were dewaxed, rehydrated, incubated in methanol/hydrogen peroxide, antigen unmasked and blocked in 10% goat serum. Antigen unmasking was performed by incubating sections in 1% trypsin for 5 minutes at room temperature or by pressure cooking in citrate buffer for 15 minutes. Primary antibodies used were FGRFR2 (rabbit IgG, Abcam, ab10648), BrdU (mouse IgG, Becton and Dickinson, 1:100), activated caspase 3 (BD Pharmingen, 557035) and Snaill1 (a gift from A. Garcia de Herreros Madueno, Universitat Pompeu Fabra, Barcelona, Spain). Expression was visualized using the Histostain SP broad spectrum (DAB) kit from Invitrogen (95-9643). Immunofluorescence was performed on primary embryos without blocking endogenous peroxidase activity. Primary antibodies used were Wt1 (mouse IgG, Dakocytomation, M561), vimentin (mouse IgM, Abcam, ab20346), pERK (mouse IgG, Santa Cruz, sc-7383), desmin (mouse IgG, Research Diagnostics, RDI-PRO10519), Pecam (rabbit IgG, Abcam, ab28364), smooth muscle cell actin (mouse IgG, Sigma, C-6198), E-cadherin (mouse IgG2a, BDTransduction, #61038), β-catenin (mouse IgG1, BDTransduction, #610181), troponin (mouse IgG2a, Developmental Studies Hybridoma Bank, CT3-s) and actinin2 (rabbit IgG, gift from Jean Nearbonne laboratory, Washington University in St Louis, MO, USA). Secondary antibodies were incubated for 1 hour and visualized with a Zeiss confocal microscope or Zeiss apotome microscope.

Staining for β-galactosidase was performed as described previously (Soriano, 1999).

**In situ hybridization**

Tissues were fixed in 4% formaldehyde, embedded in paraffin and sectioned (5 μm). In situ hybridization was performed as previously described (Wilkinson, 1992). The Fgf10 in situ probe was provided by B. Hogan (Bellusci et al., 1997).

**Proliferation analysis**

For embryos, pregnant females at E15.5 and E17.5 were injected IP with BrdU (50 μg/g body weight), 30 minutes prior to sacrifice. BrdU immunohistochemistry was performed as described above. Sections were counterstained with Hematoxylin. For statistical analysis, two areas from three different specimens were analyzed per stage. The number of BrdU-positive nuclei relative to the total number of nuclei was counted from two 63 × fields per section. Data are shown as mean±s.e.m.

**Heart explant culture**

Hearts were dissected under aseptic conditions at E17.5, then labeled with 50 μM CFSE [5-(and-6)-carboxy-2′,7′-dichlorofluorescein diacetate succinimidyl ester, Invitrogen, C1165] for 1 hour and placed in glass scintillation vials containing 1 ml of media (DMEM, 2 μg/ml heparin, antibiotic and antimycotic). Vehicle (0.25 μl/ml DMSO), FGF10 (10 nM, Peprotech), FGF9 (10 nM, Peprotech) or PD173074 (25 nM, Pfizer) was added to the vials. Vials were incubated for 48 hours on a rocker at 37°C in 5% CO2 with loose caps. Hearts were harvested, fixed in 10% formalin or 4% formaldehyde and embedded in paraffin prior to sectioning.

**Epidermal live imaging**

Hearts were dissected under aseptic conditions at E15.5 and set in 1% collagen-coated delta T dishes (Fisher) overnight in 350 μl of media (DMEM, 5% horse serum, 2 μg/ml heparin and antibiotic and antimycotic). Hearts were then removed from the dish, leaving foci of epicardial cells attached to the dish. Adherent cells were washed and 2 ml of media was added to the cultures. FGF inhibitor (PD173074, 22 nM) was added as indicated. Cultures were placed in a live imaging chamber on a Leica DMI 6000B microscope and maintained at 37°C in 5% CO2. Images were taken every 10 minutes at 20× magnification for a period of 24 hours using a Retiga Exi camera. Images were prepared and exported using the CIMAT software (C. Little, UMKC, Kansas City, USA). Images were analyzed using the Manual Tracking plug-in for Image J software. X and Y coordinates and scaling were used to calculate the distance, displacement, speed and velocity of cells in culture.

**RESULTS**

**Fgf10 signaling to the epicardium regulates heart size**

The phenotype of Fgr2b+/− mice and the presence of appropriate ligand expression in the heart suggested that FGF signaling might regulate epicardial function and, indirectly, myocardial development. Such a signal, from cardiomyocytes, fibroblasts or vascular cells in the compact myocardium may feedback to the epicardium to control the function of epicardial or EPDCs, which could indirectly regulate heart size during development. To test the hypothesis that a myocardial to epicardial signal could regulate development of the heart, we measured the cross-sectional area of the whole heart and the thickness of the compact myocardium in Fgr2b+/−, Fgf7+/− and Fgf10+/− embryos at several developmental time points. At earlier stages (E13.5 to E15.5), Fgr2b+/− hearts appeared normal in external morphology (data not shown), but at E17.5, Fgr2b+/− embryos and Fgf10+/− embryos both appeared smaller (Fig. 1A-E). The width of the compact myocardium
**Fig. 1.** Fgfr2b and Fgf10 regulate heart size. (A-D’) Hematoxylin and Eosin staining of hearts at E17.5. Control (A,A’), Fgfr2b+/− (B,B’), Fgf7+/− (C,C’) and Fgf10+/− (D,D’). Broken rectangles in A-D indicate magnified areas in A’-D’. Red lines indicate wall thickness. Scale bars: 500 μm in A-D; 100 μm in A’-D’. (E) Quantification of the relative area of the heart: control, n=8; Fgfr2b+/−, n=8, **P<0.001; Fgf10+/−, n=8, *P<0.02; Fgf7+/−, n=5. (F) Quantification of left ventricular wall thickness (red line in A’-D’). Fgfr2b+/−, n=6, *P<0.02; Fgf10+/−, n=9, * P<0.003; Fgf7+/−, n=5. (G,H) Short axis in utero echocardiogram at E17.5 of control (G) and Fgfr2b+/− (H) hearts. Area measured (broken white lines) and wall thickness measured (yellow lines) are placed at end diastole according to Movie 1 in the supplementary material. LV, left ventricle; S, septum; RV, right ventricle. (I-K) Quantification of left ventricular posterior wall diameter at end diastole (LVPWd), n=7, **P=0.0003; right ventricular posterior wall diameter at end diastole (RVPWd), n=7, *P<0.02; interventricular septum diameter at end diastole, n=7, **P<0.001. Control hearts are a mix of wild-type, Fgfr2b+/− and Fgf7+/−, Fgf10+/− genotypes. Asterisks indicate statistically significant difference compared with controls. Data are mean±s.e.m.

Fgfr2b−/− embryos (as shown by Marguerie et al., 2006) and Fgf10−/− embryos were significantly (P<0.02, P<0.003, respectively) thinner than age-matched control embryos (Fig. 1A’-D’). Fgf7−/− embryos did not show a significant difference in thickness of the compact myocardium. We were able to generate two Fgf7−/−;Fgf10−/− embryos at E17.5, and the hearts of both appeared smaller in size compared with Fgf10−/− hearts, suggesting possible redundancy with FGF7. In utero echocardiography also showed a decrease in diastolic wall thickness in E17.5 Fgfr2b−/− hearts when compared with littermate controls (Fig. 1G-J; see Movie 1 in the supplementary material). Consistently, the interventricular septum of Fgfr2b−/− hearts was also thinned (Fig. 1K). We also examined the formation of coronary vessels in Fgfr2b−/− and Fgf10−/− hearts. Endothelial vessels formed normally compared with controls (see Fig. S1 in the supplementary material). Taken together, these data suggest that FGF10 signals to FGFR2b in the epicardium or in EPDCs to control heart size.

Based on these phenotypes, we hypothesized that FGFR2b should be expressed in epicardial cells and FGF10 should be expressed in cardiac myocytes or other cell types within the compact myocardium. In situ hybridization localized Fgj10 mRNA expression within the myocardium of wild-type hearts at E17.5 (Fig. 2A,B). No expression was observed in Fgf10−/− hearts or with a sense probe. In addition, the expression of FGF10 in the heart was investigated by using a lacZ gene trap reporter allele (Mailleux et al., 2005). Expression of FGF10-lacZ was localized to the compact myocardium (see Fig. S1 in the supplementary material), consistent with the in situ hybridization expression pattern. FGFR2 protein expression was examined using an antibody that detects all splice forms (Fig. 2C,D). In wild-type hearts, FGFR2 expression was observed in both the epicardial layer and the myocardial layer. By contrast, in Fgfr2b−/− hearts, the expression of FGF2 was reduced in the epicardial layer but present throughout the myocardium. This expression pattern supports a model in which myocardial-derived FGF10 signals to FGFR2b in the epicardium or in EPDCs to control heart size.

Fgfr2b−/− is a germline knockout with multiple developmental defects. Therefore, to determine whether FGFR2 signaling in epicardial cells and EPDCs could be responsible for the observed cardiac phenotypes in Fgfr2b−/− embryos, we used Wt1-Cre to inactivate a floxed allele of Fgfr2 in the epicardium and in EPDCs. Because FGF10 can also signal to FGFR1b, and FGFR1 and FGFR2 often show functional redundancy, we simultaneously inactivated conditional alleles of both Fgfr1 and Fgfr2. Mice with the genotype Wt1-Cre, Fgfr1fl/fl, Fgfr2fl/fl (referred to as Fgfr1/2f/f(Wt1-Cre)) showed reduced expression of FGFR2 in the epicardium (Fig. 2E,F). These mice also showed a thin-walled compact myocardium similar to that seen in Fgfr2b−/− and Fgf10−/− embryos (Fig. 3E). However, mice
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Regulation of epicardial development by FGF signaling

To determine whether loss of epicardial FGFR1 and FGFR2 in Fgf10−/− mice affects epicardial development, we examined the rate of proliferation of epicardial cells and the number of epicardial-derived cells localized within the compact myocardium in Fgf10−/− hearts. At E15.5 and E17.5, there was no change in proliferation of epicardial cells between Fgf10−/− mice and littermate controls (Fig. 4F).

To determine whether epicardial EMT, delamination from the epicardium or EPDC migration was defective in Fgf10−/− hearts, we examined the expression of Wt1, a protein expressed in epicardial cells and EPDCs (Fig. 5A-I). At E13.5, fewer Wt1-positive cells had initiated invasion of the myocardium in Fgf2b−/− hearts (Fig. 5A-E). Consistent with this observation, more Wt1 positive cells were found within the epicardium and subepicardium of Fgf2b−/− hearts (Fig. 5A-E). This indicates that FGFR2 signaling directly regulates myocardial growth.

Fig. 2. Expression of Fgf10 and Fgfr2 in the left ventricle of the heart at E17.5. (A,B) Fgf10 in situ hybridization showing Fgf10 mRNA in cells within the compact myocardium in wild-type hearts (A). No expression of Fgf10 was observed in Fgf10−/− hearts (B). (C,D) Immunohistochemistry showing expression of FGFR2 throughout the heart in wild-type heart (C). In Fgfr2b−/− heart (D), FGFR2 levels are reduced in the epicardial layer. Broken lines indicate the border between the myocardial and epicardial layer. Insets show higher magnifications of the boxed areas. (E,F) FGFR2 expression in control heart (E) and Fgfr1/2Wt1-Cre heart (F), showing reduced staining in the epicardium in the conditional knockout. Scale bars: 20 µm.

Fig. 3. Decreased heart size after epicardial conditional inactivation of Fgfr1 and Fgfr2 with Wt1-Cre. (A-D') Hematoxylin and Eosin staining of E17.5 control (A,A'), Fgfr1Wt1-Cre (B,B'), Fgfr2Wt1-Cre (C,C') and Fgfr1/2Wt1-Cre (D,D') hearts. Fgfr1/2Wt1-Cre hearts are smaller compared with controls and display a thinner compact myocardium. Broken rectangles in A-D indicate magnified areas in A'-D'. (E) Quantification of the left ventricle wall thickness (red line in A'-D'). control, n=14; Fgfr1Wt1-Cre, n=5; Fgfr2Wt1-Cre, n=10; Fgfr1/2Wt1-Cre, n=9, **P<0.001. Scale bars: 500 µm in A-D; 100 µm in A'-D'. (F) Quantification of heart cross-sectional area normalized to body weight: Fgfr1/2Wt1-Cre, n=10, **P<0.003; Fgfr1Wt1-Cre, n=5; Fgfr2Wt1-Cre, n=9. Control hearts contain Wt1-Cre and are a combination of wild-type and floxed alleles of Fgfr1 and Fgfr2. Asterisks indicate statistically significant difference compared with controls. Data are mean±s.e.m.
FGF-mediated regulation of epicardial development

To further characterize the EPDCs within the myocardium, hearts were stained with an antibody to vimentin, a marker of cardiac fibroblasts (Fig. 5M-P). Consistent with the decreased number of Wt1-positive cells within the myocardium of Fgf10–/– hearts, the number of vimentin-positive cells was also decreased in Fgf10–/– hearts compared with controls. These loss-of-function and gain-of-function studies support a model in which FGF signaling regulates migration of a subset of EPDCs (that will become cardiac fibroblasts) into the compact myocardium.

**FGF signaling regulates EPDC migration**

To determine whether FGF10 signaling regulates migration of EPDCs into the myocardium, hearts were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) to label epicardial cells (Morabito et al., 2001), allowing their location to be imaged following explant culture. CFSE is permeable to cells, but once inside a cell, esterases cleave the molecule, which traps it in the cytosol. To determine whether epicardial cells could be specifically labeled, dissected E17.5 wild-type hearts were treated with CFSE for 1 hour and then fixed, sectioned and immunostained for Wt1. CFSE and Wt1 were co-localized in the epicardial cell layer, and Wt1 was also present in EPDCs that had already migrated into the myocardium prior to labeling with CFSE (Fig. 6A-C, arrows highlight Wt1+ cells that have already migrated into the myocardium). To determine whether FGF10 activated FGF signaling in CFSE-labeled cells, CFSE-labeled and FGF10-treated explants were stained for p-Erk, a downstream target of activated FGFRs. Exposure to FGF10 for 48 hours resulted in an increase in p-Erk labeling of CFSE+ cells in the epicardium and myocardium, but not of CFSE- cells within the compact myocardium (Fig. 6D-F). This increase in p-Erk labeling in response to FGF10 was blocked by treatment with the FGFR inhibitor PD173074.

To determine whether EPDC migration into the myocardium responded to FGF10, E17.5 heart explants were treated with CFSE for 1 hour, washed, and then cultured for 48 hours with or without FGF10 and PD173074. In addition, to determine the specificity of FGF signaling, explants were also treated with FGF9, a ligand that is expressed in the epicardium that signals to cardiomyocytes (Fig. 6G-K). In response to treatment with FGF10, explants showed a significant \( P<0.003 \) increase in CFSE-labeled cells within the sub-epicardial space and compact myocardium. Addition of the FGFR inhibitor, PD173074, along with FGF10 resulted in a significant \( P<0.005 \) decrease in CFSE-labeled cells within the sub-epicardial space and compact myocardium, whereas treatment of explants with FGF9 had no effect on migration of CFSE-labeled epicardial cells (Fig. 6K-O). Taken together, these data demonstrate that FGF10 is sufficient to increase CFSE-labeled cell movement into the myocardium.

To determine whether FGF10 had an effect on EPDCs that had already migrated into the myocardium prior to CFSE labeling, the number of Wt1+, CFSE+ cells in FGF10-treated explanted hearts were counted. Consistent with a model in which FGF10 signals only to FGFR1b/FGFR2b in epicardial cells, there was no change in the number of Wt1+, CFSE+, EPDCs following FGF10 treatment (Fig. 6L-O).

**FGF10 regulates formation of cardiac fibroblasts**

During heart development, epicardial cells give rise to cardiac fibroblasts and smooth muscle cells that populate the compact myocardium. Under specialized conditions, such as following...
injury, epicardial cells may also give rise to cardiomyocytes and endothelial cells. To determine the fate of epicardial cells that respond to FGF10, explants labeled with CFSE and treated with FGF10 for 48 hours were sectioned and immunostained for markers of specific cardiac lineages, including myocytes, endothelial cells, smooth muscle cells and fibroblasts (Fig. 7). CFSE-labeled cells did not co-immunostain with antibodies to myocytes, endothelial cells or smooth muscle cells, but did co-label with an antibody to vimentin, a marker expressed on fibroblasts (Fig. 7D-F). These data suggest that FGF10 promotes formation and movement of EPDCs into the myocardium.

To determine whether FGF signaling could affect the motility of epicardial cells, live imaging was used to monitor movement of isolated epicardial cells from Fgfr2b−/− and wild-type hearts and hearts treated with vehicle or PD173074 (Fig. 8; see Movie 2 in the supplementary material). Epicardial cells from Fgfr2b−/− hearts showed a significantly (P<0.02) shorter displacement, but similar...
distance traveled when compared with wild-type epicardial cells. Consistent with this result, epicardial cells treated with PD173074 also showed a significantly ($P < 0.001$) shorter displacement and no change in the distance traveled. Addition of FGF10 to epicardial cell cultures did not increase epicardial cell motility (data not shown), indicating that FGF signaling was probably saturated in these cultures.

**DISCUSSION**

Epicardial-derived cells give rise to several cell types that populate the compact myocardium. These include interstitial fibroblasts, perivascular cells and smooth muscle cells. EPDCs also regulate growth of the myocardium but the factors that regulate their differentiation and their migration into the myocardium are poorly understood. We show that during late embryonic development, FGF10 signals to epicardial and epicardial-derived cells through FGFR2b to induce their migration into the myocardium.

In mice conditionally lacking *Fgfr1* and *Fgfr2* in epicardial cells, or lacking *Fgf10*, significantly fewer EPDCs were observed within the compact myocardium. Several mechanisms could result in this phenotype, including: defects in epicardial EMT; failure of EPDCs to migrate into the compact myocardium; or increased death of EPDCs. EMT is a complex process that requires the dissolution of cell-cell junctions, loss of apical-basal polarity and, finally, the modification of cytoskeletal proteins to a mesenchymal phenotype that permits, in the case of epicardial cells, movement into the subepicardial space (reviewed by Thiery et al., 2009). Although the precise signals and mechanisms governing epicardial EMT are not known, epicardial EMT requires β-catenin-dependent asymmetrical cell division (Wu et al., 2010) and Wt1-mediated repression of E-cadherin, and upregulation of Snail (Martinez-Estrada et al., 2010). In mice that lack FGF10 or epicardial FGFR1/2, epicardial EMT appears to occur normally, as proliferation and Wt1, Snail and β-catenin expression were not changed in epicardial cells.
Furthermore, increased apoptosis of EPDCs, which could also explain fewer of these cells within the myocardium, is also unlikely, as staining for activated caspase 3 revealed no increase in cell death in Fgfr1/2Wt1-Cre and Fgf10–/– hearts.

Following epicardial EMT, EPDCs migrate further into the myocardium and differentiate into either smooth muscle cells or cardiac fibroblasts. The mechanisms that direct EPDCs into the compact myocardium are not known; however, our data suggest that FGF10/FGFR2b signaling may regulate epicardial movement into the myocardium. In primary epicardial cell cultures that lack Fgfr2b or are treated with FGFR kinase inhibitors, we observed a reduction in cell displacement. These results could be explained by FGF10 functioning as a chemotactic factor or regulating cell motility (displacement in epicardial cultures). In vivo, FGF10 is unlikely to regulate directional migration of EPDCs in the heart because of its diffuse expression throughout the compact myocardium. By contrast, in the lung, Fgf10 is expressed focally in mesenchyme, where it functions to induce epithelial branching and migration towards the source of FGF10 (Weaver et al., 2000). In addition, in heart explants, addition of FGF10 protein to the media induced EPDC migration into the compact myocardium, suggesting that focal expression of FGF10 is not required. Recently, it was demonstrated that FGF-regulated increases in cell motility could have net positive effects on directional cell movements required for embryonic axis elongation (Benazeraf et al., 2010). It is, thus, possible that FGF10 regulated cell motility could account for the specific influx of cardiac fibroblasts into the compact myocardium. Other factors such as PDGFRβ and Alk5, which regulate either epicardial migration or EMT, have been found to specifically affect vascular smooth muscle cell recruitment or differentiation, but do not have reported effects on cardiac fibroblasts (Mellgren et al., 2008; Sridurongrit et al., 2008). We posit that FGF10 preferentially regulates migration of cardiac fibroblasts and PDGFβ preferentially regulates migration of vascular smooth muscle cells.

FGFs often signal bi-directionally during organogenesis, for example in limb bud and lung development (Yang, 2009; Zeller et al., 2009; Morrisey and Hogan, 2010). During midgestation heart development, communication between the epicardium and
myocardium appears necessary to regulate the ultimate size of the heart. Although reciprocal FGF signaling between mesenchymal and epicardial tissues is important for heart development, other signaling molecules, direct cell-cell contact and physiological factors are likely to interact with FGF signaling to coordinate heart size with growth of the embryo and its physiological requirements.

Multiple signals regulate growth of the myocardium at different stages of development (Sucov et al., 2009). Of these, epicardial derived FGF9 and FGF16 are factors that directly signal to FGF receptors expressed in cardiomyocytes during midgestation. Although myocardial proliferation is reduced in mice lacking FGF9 (Lavine et al., 2005) or FGF16 (Hotta et al., 2008), proliferation is clearly not arrested. This indicates that other factors must act in parallel to FGF9/16 to regulate myocardial proliferation. Recently, Igf2 has been identified as an epicardial-derived factor that regulates myocardial growth (Li et al., 2011).

Another pathway that regulates cardiomyocyte proliferation during late gestation is through direct contact with cardiac fibroblasts, which directly induce myocardial proliferation through a mechanism involving collagen/tegrin signaling (Ieda et al., 2009). The major source of embryonic cardiac fibroblasts during development is EPDCs. In our model, inactivation of FGF signaling in EPDCs leads to a decrease in EPDCs that specifically give rise to cardiac fibroblasts within the compact myocardium. Interestingly, we also observed a coincident decrease in myocardial proliferation and a reduction in heart size. We posit that decreased myocardial proliferation in hearts lacking epicardial FGF signaling could result indirectly from consequences of decreased numbers of interstitial cardiac fibroblasts. This is consistent with small heart size phenotypes that result from other mutations that disrupt pro-epicardial migration: defects in epicardial EMT and EPDC migration into the myocardium (Rhee et al., 2009; Martinez-Estrada et al., 2010; Wu et al., 2010).

In the studies presented here, the Fgfr2b heterozygote hearts appear to have a more severe (smaller heart) phenotype than Fgfr1/2Wt1-Cre hearts. Fgfr2b is a germline knockout; therefore, deletion of Fgfr2b is complete and can act over a longer period of time compared with a conditional knockout. In addition, other developmental defects could indirectly contribute to the cardiac phenotype in Fgfr2b heterozygote hearts.

Understanding mechanisms that regulate myocardial growth have historically been the focus of much research because of the importance of the epicardio-myoctye to heart homeostasis and response to injury. One of the challenges that have slowed advances in the treatment of the injured heart is the limited ability of adult cardiomyocytes to proliferate. Recent studies on epicardial cells and their ability to differentiate into various cell types and communicate with cardiac myocytes have suggested new therapeutic targets to treat heart disease. Future studies are needed to determine whether FGF10/FGF2b signaling occurs in the adult heart under homeostatic or pathological conditions and whether this signaling pathway could be therapeutically manipulated to promote cardiac protection or regeneration.

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Competing interests statement
The authors declare no competing financial interests.

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