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Developmental Biology 287 (2005) 134 – 145
DEVELOPMENTAL BIOLOGY
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The right ventricle, outflow tract, and ventricular septum comprise a restricted expression domain within the secondary/anterior heart field

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Received for publication 11 April 2005, revised 13 August 2005, accepted 29 August 2005
Available online 26 September 2005

Abstract

The vertebrate heart arises from the fusion of bilateral regions of anterior mesoderm to form a linear heart tube. Recent studies in mouse and chick have demonstrated that a second cardiac progenitor population, known as the anterior or secondary heart field, is progressively added to the heart at the time of cardiac looping. While it is clear that this second field contributes to the myocardium, its precise boundaries, other lineages derived from this population, and its contributions to the postnatal heart remain unclear. In this study, we used regulatory elements from the mouse *mef2c* gene to direct the expression of Cre recombinase exclusively in the anterior heart field and its derivatives in transgenic mice. By crossing these mice, termed *mef2c*-AHF-Cre, to Cre-dependent *lacZ* reporter mice, we generated a fate map of the embryonic, fetal, and postnatal heart. These studies show that the endothelial and myocardial components of the outflow tract, right ventricle, and ventricular septum are derivatives of *mef2c*-AHF-Cre expressing cells within the anterior heart field and its derivatives. These studies also show that the atria, epicardium, coronary vessels, and the majority of outflow tract smooth muscle are not derived from this anterior heart field population. Furthermore, a transgene marker specific for the anterior heart field is expressed in the common ventricular chamber in *mef2c* mutant mice, suggesting that the cardiac looping defect in these mice is not due to a failure in anterior heart field addition to the heart. Finally, the Cre transgenic mice described here will be a crucial tool for conditional gene inactivation exclusively in the anterior heart field and its derivatives.
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Keywords: Anterior heart field; Secondary heart field; Heart development; Outflow tract; *mef2c*; Cre; Neural crest; Mouse; Transgenic; Fate map; ROSA26R

Introduction

The mammalian heart initially arises from the fusion of bilateral regions of anterior mesoderm known as the cardiac crescent. During ventral folding and foregut invagination of the mammalian embryo, the two halves of this bilaterally symmetrical primary heart field meet at the midline to form a linear heart tube. As development proceeds, this linear tube is remodeled into a four chambered heart with the future atrial myocardium looping dorsal and anterior to the developing ventricles. Concurrently, the aorta and pulmonary artery arise from septation of the common outflow tract (Brand, 2003; Harvey, 2002).

Recent studies have demonstrated that the embryonic outflow tract and right ventricle are not derived from the primary heart field, but instead have their origins in a second population of cells known as the secondary or anterior heart field (Abu-Issa et al., 2004; Kelly and Buckingham, 2002; Yutzey and Kirby, 2002). The cells that comprise the anterior heart field reside in the splanchnic and pharyngeal mesoderm and appear to be progressively added to the arterial pole of the developing heart at the time of cardiac looping (Kelly et al., 2001; Mjaatvedt et al., 2001; Waldo et al., 2001). The identification of a second population of cardiogenic mesoderm that gives rise to the right ventricle and outflow tract provides a potential explanation for the observation that numerous genes and transgenes exhibit expression that is restricted to the outflow tract and right ventricle (Kelly and Buckingham, 2002; Schwartz and Olson, 1999). In addition, mutations in several key cardiac transcription factors, including *mef2c*, *Isl1*, *Nkx2.5*, *Hand2*, and *Foxh1*, appear to selectively affect the development of the right ventricle and outflow tract (Cai et al., 2003; Lin et al., 1997; Lyons et al., 1995; Srivastava et al., 1997; von

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0012-1606/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.ydbio.2005.08.041

Both et al., 2004). In each case, these mice have a single ventricular chamber, and the heart fails to undergo normal looping morphogenesis. However, it remains unclear whether addition of the anterior heart field occurs normally in these mice, and whether the observed cardiac phenotypes result from a defect in anterior heart field development.

While it is clear that the myocardium is derived from at least two mesodermal progenitor populations, the precise boundaries of the secondary/anterior heart field, the lineages derived from this population, and its contribution to the postnatal heart remain unclear. In the chick, fate mapping studies have revealed that the splanchnic mesoderm proximal to the developing outflow tract constitutes a secondary heart field that will contribute to the elongating outflow tract myocardium (Mjaatvedt et al., 2001; Waldo et al., 2001). Similarly, a population of cells in transgenic mice expressing *lacZ* under the control of *Fgf10* regulatory elements resides in the splanchnic and pharyngeal mesoderm before contributing to the myocardium at the arterial pole (Kelly et al., 2001). Other studies have suggested that the anterior heart field gives rise to the embryonic right ventricle as well as the outflow tract (Zaffran et al., 2004), or that the secondary/anterior heart field may have an even broader developmental potential (Cai et al., 2003; Laugwitz et al., 2005; Meilhac et al., 2004a). We have recently identified a promoter and enhancer from the mouse *mef2c* gene that is sufficient to direct expression exclusively to the anterior heart field during embryonic development (Dodou et al., 2004). This *cis*-acting regulatory module from *mef2c* directs expression to the anterior heart field beginning at cardiac crescent stage at 7.5 dpc, to the pharyngeal mesoderm and the arterial pole at the linear heart tube stage, and to the outflow tract and right ventricle during subsequent embryonic development. The enhancer is never active in the left ventricle or atria and is silent during adulthood (Dodou et al., 2004). The expression domain directed by the *mef2c* anterior heart field enhancer is likely a subfield of that described elsewhere as the second heart lineage (Meilhac et al., 2004a) or a more spatially restricted component of the *Isl1* expression domain (Cai et al., 2003).

In the present study, we examined the expression of *mef2c*-AHF-*lacZ*, which expresses *lacZ* under the control of the *mef2c* anterior heart field promoter and enhancer, in *mef2c* knockout mice. We show that the common ventricular chamber in *mef2c* knockout mice expresses *mef2c*-AHF-*lacZ*, suggesting that this chamber is composed of derivatives from both the primary and secondary heart fields. Interestingly, the *mef2c*-AHF-*lacZ* transgene is only expressed on the dorsal side of the common ventricular chamber, suggesting that these mice are not defective in the addition of the anterior heart field to the common ventricle, but rather in the patterning or looping of the ventricular chamber. We have also generated transgenic mice that express Cre exclusively within the anterior heart field and its derivatives in the right ventricle and outflow tract using this regulatory element from *mef2c*. We used these anterior heart field-specific Cre transgenic mice, termed *mef2c*-AHF-Cre, to generate a fate map of the embryonic, fetal, and postnatal heart. By crossing *mef2c*-AHF-Cre mice to ROSA26R Cre-dependent *lacZ* reporter mice (Soriano, 1999), we show that the outflow tract, right ventricle, and the ventricular septum are marked by the activity of the *mef2c*-AHF-Cre transgene.

In addition, the studies presented here demonstrate that cells marked by the *mef2c*-AHF-Cre transgene contribute to the endocardium of the right ventricle. We also show that within the outflow tract, the myocardium and endothelium are marked by the *mef2c*-AHF-Cre transgene, whereas the smooth muscle layer of the outflow tract is largely derived from neural crest with some anterior heart field contribution near the pulmonary trunk. These studies also demonstrate that the epicardium and coronary vessels have an embryonic origin distinct from the anterior heart field population marked by *mef2c*-AHF-Cre. Thus, these studies provide a fate map of a highly restricted subdivision within the secondary/anterior heart field in mice and describe the first transgenic mouse line with Cre activity restricted to the anterior heart field and its derivatives. These mice will be a crucial tool for examining the genetic pathways that control cardiac development by conditional gene inactivation exclusively in the anterior heart field and its derivatives in the outflow tract and right ventricle.

Materials and methods

Generation of anterior heart field-specific Cre transgene and mice

The *mef2c*-AHF-Cre transgene was generated by excising the 3970 bp *mef2c* anterior heart field enhancer and promoter as an *Xho*I fragment from plasmid *Mef2c*-F6/Frag3, which has been described previously (Dodou et al., 2004). This enhancer and promoter fragment was then cloned into a Cre expression plasmid containing the Cre cDNA and the SV40 splice and polyA signal sequence to create plasmid *mef2c*-AHF-Cre. The approximately 5.5 kb *mef2c*-AHF-Cre transgene fragment was then purified as a *Not*I fragment and injected into the male pronuclei of fertilized oocytes as described previously (Dodou et al., 2003). Cre positive founder mice were identified by Southern blot using a Cre-specific radiolabeled probe on genomic DNA isolated from tail biopsies. Male *Mef2c*-AHF-Cre transgenic mice were crossed to female ROSA26R Cre-dependent *lacZ* reporter mice (Soriano, 1999) to screen for Cre recombinase activity. *Wnt1*-Cre mice have been described (Danielian et al., 1998) and were used to fate map neural crest descendants in the outflow tract (Jiang et al., 2000). *Mef2c* knockout mice were kindly provided by John Schwarz (Albany), and have been described (Lin et al., 1997). *Mef2c*-AHF-*lacZ* mice, containing the *mef2c* anterior heart field enhancer directing expression of *lacZ*, have also been described previously as *mef2c* F6/Frag2-*lacZ* (Dodou et al., 2004). Mouse and embryo genotyping was performed by Southern blot using standard methods and probes specific for each allele. All experiments using animals complied with federal and institutional guidelines and were reviewed and approved by the UCSF Institutional Animal Care and Use Committee.

Analysis of Cre expression and recombination

Transgenic male founders or transgenic male offspring of female founders were crossed to female ROSA26R reporter mice (Soriano, 1999). Embryos were collected at 10.5 dpc and stained with 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal) to detect β-galactosidase activity, as previously described (Anderson et al., 2004). After establishing three transgenic lines that exhibited robust anterior heart field-specific expression, embryos, embryonic tissues collected at various time points, and neonatal tissues were X-gal stained. For analysis of sections from embryos younger than 14.5 dpc, representative X-gal stained embryos were prepared and stained as described previously (Anderson et al., 2004) and counterstained with Neutral Fast Red for better visualization of histology. Embryos older than 14.5 dpc and neonatal hearts

were fixed briefly in 2% paraformaldehyde, 0.2% glutaraldehyde, and embedded in OCT (Tissue Tek #4583) on dry ice. Frozen samples were then sectioned on a cryostat (Leica) at a thickness of 10 μm and allowed to dry overnight. Sections were fixed in 4% paraformaldehyde, rinsed in phosphate-buffered saline, pH 7.4 (PBS), and stained in X-gal solution. After adequate staining (6–16 h), sections were rinsed in PBS and counterstained in Neutral Fast Red.

Immunohistochemistry and in situ hybridization

Immunohistochemical detection of β-galactosidase and PECAM-1 was performed as described previously with minor modifications (De Val et al., 2004). Briefly, *mef2c*-AHF-Cre Tg/0; ROSA26R *lacZ*/+ embryos were collected at 10.5 dpc and fixed overnight in 4% paraformaldehyde in PBS, transferred into increasingly concentrated sucrose solutions from 0 to 30% in PBS, embedded in OCT on dry ice, and cryosectioned at a thickness of 14 μm. Sections were then analyzed for β-galactosidase expression by immunohistochemistry using rabbit anti-β-galactosidase (ICN) as primary antibody diluted 1 in 1000 in 3% normal goat serum. Primary antibody binding was detected using Alexa Fluor 488 goat anti-rabbit secondary antibody (Molecular Probes) diluted 1 in 300 in 3% normal goat serum. The endothelial cell marker PECAM-1 was detected using a rat anti-mouse PECAM-1 antibody (Pharmingen) diluted 1:300 in 3% normal goat serum, and the secondary antibody was Cy3-conjugated goat anti-rat (Jackson Immunoresearch) diluted 1 in 300 in 3% normal goat serum.

Whole mount in situ hybridization was performed as described previously (Wilkinson and Nieto, 1993). Briefly, embryos were fixed overnight in 4% paraformaldehyde and then washed twice with phosphate-buffered saline (PBS) and dehydrated through a series of PBT (1× PBS + 0.1% Tween20)-methanol washes. Embryos were then rehydrated through a reciprocal series of PBT-methanol washes and were treated at room temperature with 10 μg/ml proteinase K for varied times depending on age as described (Wilkinson and Nieto, 1993). After proteinase K treatment, embryos were rinsed with 2 mg/ml glycine in PBT followed by two successive washes in PBT at room temperature. Embryos were fixed in 4% paraformaldehyde and 0.2% glutaraldehyde for 20 min at room temperature, rinsed three times in PBT, and incubated in hybridization solution (50% formamide, 1% SDS, 5× SSC, 50 μg/ml yeast tRNA, 50 μg/ml heparin) for 16 h at 70°C.

For in situ hybridization on sections, embryos were fixed, embedded in paraffin, sectioned, and dewaxed as described previously (De Val et al., 2004). Sections were then digested for 8.5 min in 40 μg/ml proteinase K, fixed in 4% paraformaldehyde for 20 min, dehydrated through a series of ethanol washes, and allowed to dry. 50 μl of hybridization solution was added to each slide, and they were incubated at 70°C for 16 h. In situ hybridization was carried out with digoxigenin-labeled antisense or sense RNA probes at 100 ng/ml (whole mount) or 1 μg/ml (sections) in hybridization buffer. Following hybridization, embryos or slides were rinsed through a series of formamide and SSC washes and treated with RNaseA as described previously (Verzi et al., 2002). Signal was detected using an alkaline phosphatase-conjugated anti-digoxigenin antibody and BM Purple alkaline phosphatase substrate (Roche Pharmaceuticals). Following staining, slides were counterstained with Neutral Fast Red for visualization of embryonic structures. The *Isl1* in situ probe plasmid was generously provided by S. Evans (UCSD) and has been described (Cai et al., 2003). The *Fgf10* antisense probe was kindly provided by P. T. Chuang (UCSF) and has been described previously (Chuang et al., 2003).

Results

An anterior heart field-specific transgene is expressed in the common ventricular chamber in mef2c null mice

Coincident with ventral turning of an E8.5 mouse embryo is the rightward looping of the embryonic heart. Previous studies have identified a number of genes necessary for cardiac looping morphogenesis, including *Isl1*, *Foxh1*, *Hand2*, *Nkx2.5*, and *mef2c* (Cai et al., 2003; Lin et al., 1997; Lyons et al., 1995; Srivastava et al., 1997; von Both et al., 2004). Mice lacking these genes share a common phenotype: a generally hypoplastic heart with a single, linear ventricular chamber and death at midgestation. Interestingly, these genes also share a common expression pattern in the anterior heart field or in tissues derived from that progenitor population (Cai et al., 2003; Edmondson et al., 1994; Lints et al., 1993; Srivastava et al., 1995, 1997; von Both et al., 2004). It has been suggested that the migration of cells from the anterior heart field into the linear heart tube may direct looping morphogenesis, and that a lack of anterior heart field contribution to the common ventricular chamber may be the cause of the looping defect in these mutant mice (Yelbuz et al., 2002). It has also been suggested that the common ventricular chamber in these knockout mice lacks right ventricle identity, expressing primarily left ventricular markers (McFadden et al., 2000; Thomas et al., 1998).

To test whether markers of the anterior heart field are expressed in the single common ventricular chamber in *mef2c* mutant mice, we examined the expression of an anterior heart field-specific *lacZ* transgene, *mef2c*-AHF-*lacZ*, in *mef2c* knockout embryos (Fig. 1). Previous work has shown that this transgene is expressed exclusively in the anterior heart field and its descendants in the right ventricle and outflow tract during development (Dodou et al., 2004). *LacZ* expression in wild type and *mef2c* null animals appeared identical from 7.75 dpc until 8.25 dpc (Fig. 1, compare panels A, B to E, F). However, by 9.0 dpc, *mef2c* null animals began to show abnormal cardiac morphology (Figs. 1G, H). At this stage, anterior heart field marked cells were observed in the right ventricle and outflow tract of wild-type mice (Figs. 1C, D). Interestingly, *mef2c*-AHF-*lacZ* expression was also detected in the common ventricular chamber of *mef2c* null embryos (Figs. 1G, H). These results indicate that the *mef2c*-AHF-*lacZ* anterior heart field marker is expressed in the common ventricular chamber in *mef2c* mutants. In the unlooped heart of these mutant mice, the anterior heart field marked cells comprise the dorsal anterior region of the common ventricular chamber (Figs. 1G, H). This result suggests that the unlooped ventricle in *mef2c* mutants has progenitor cells from the anterior heart field, which should normally give rise to the right ventricle, even though this common ventricle lacks markers of right ventricular identity (McFadden et al., 2000). The expression of this anterior heart field marker in the hearts of *mef2c* mutant embryos strongly suggests that cells from the anterior heart field are added to the ventricle in *mef2c* null mice and possibly other looping mutants.

An anterior heart field-specific Cre transgene under the control of regulatory elements from mef2c

Anterior heart field marker expression in the hearts of *mef2c* mutant mice was unexpected and suggests that we do not have a full appreciation for the contribution or role of the anterior heart field in the developing mouse heart. Indeed, there are several discrepancies regarding contribution of the secondary/anterior heart field to the developing heart (Cai et al., 2003;

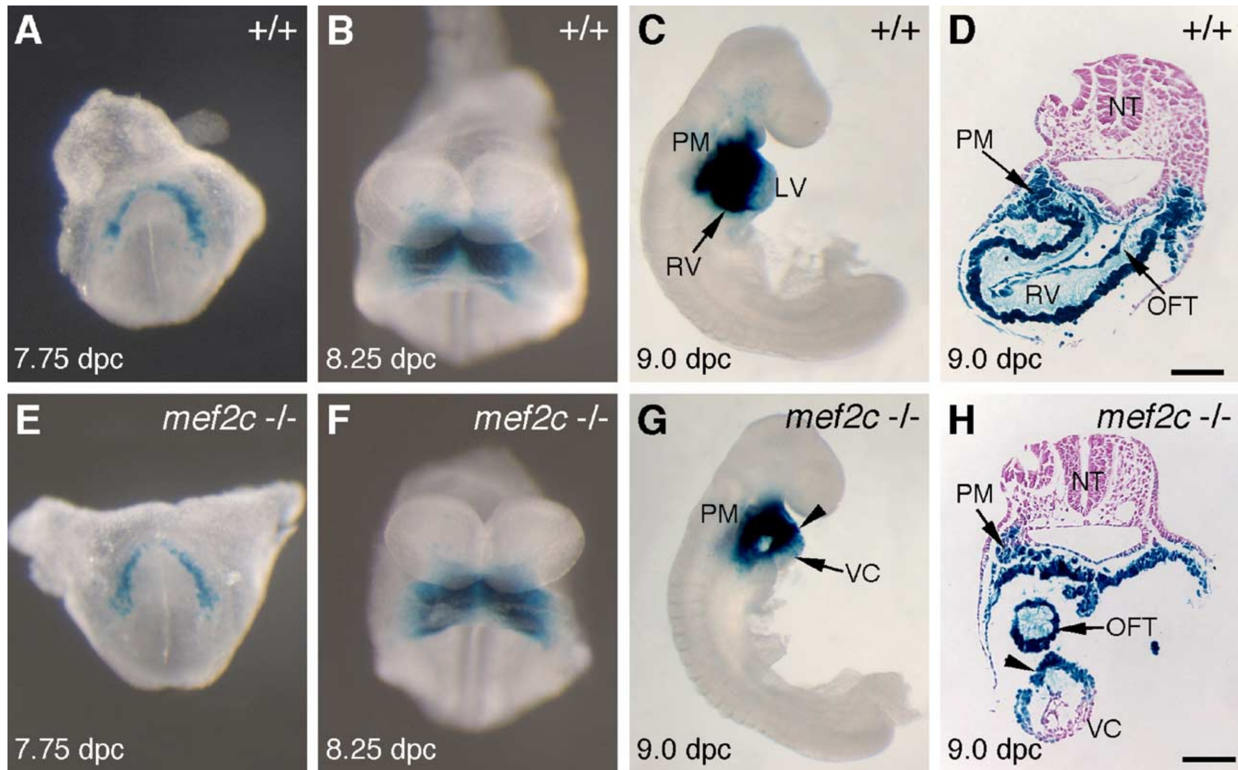

Fig. 1. The anterior heart field contributes to the common ventricular chamber in *mef2c* null embryos. Whole mount and sectioned embryos from wild-type (A–D) and *mef2c* knockout (E–H) embryos containing the *mef2c*-AHF-*lacZ* transgene were assayed for β-galactosidase activity. (A, E) Frontal view of presomite embryos collected at 7.75 dpc with β-galactosidase activity detected in the medial portion of the cardiac crescent, consistent with the anterior heart field expression. (B, F) Frontal view of 6 somite embryos collected at 8.25 dpc and stained with X-gal. (C, G) Lateral view (right side) of 16 somite embryos collected at 9.0 dpc and stained with X-gal. Notice that, in the mutant (G), the unlooped heart is stained in the dorsal portion of the common ventricular chamber, whereas staining in the wild-type embryo (C) is restricted to the right side of the ventricle (bulbus cordis). (D, H) Transverse sections through 16 somite embryos. Staining in the knockout is observed in the outflow and dorsal portion of the common ventricle (H). Arrowheads mark the dorsal anterior portion of the common ventricular chamber in *mef2c* mutant mice. LV, left ventricle; NT, neural tube; OFT, outflow tract; PM, pharyngeal mesoderm; RV, right ventricle; VC, common ventricular chamber. Scale bar is equal to 100 μm.

Kelly and Buckingham, 2002; Yutzey and Kirby, 2002), and many questions remain regarding the boundaries of anterior heart field-derived cell populations. In addition, the contribution of the secondary/anterior heart field to the postnatal heart has yet to be determined in detail. To address these issues, we used an anterior heart field-specific promoter and enhancer from *mef2c* to direct expression of Cre recombinase in transgenic mice (Fig. 2A). We crossed these mice, termed

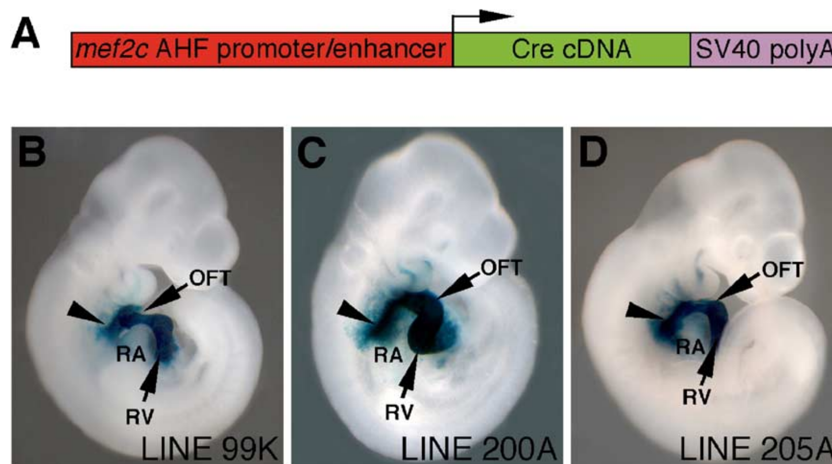

Fig. 2. Generation of an anterior heart field-specific Cre transgenic mouse line. (A) Schematic representation of the *mef2c*-AHF-Cre transgene. The *mef2c* anterior heart field promoter and enhancer were subcloned upstream of the cDNA encoding Cre recombinase and the SV40 splice and polyadenylation signal sequence. The resulting construct was used to generate transgenic mice. (B–D) Three different *mef2c*-AHF-Cre transgenic founders were crossed to ROSA26R Cre-dependent *lacZ* reporter mice, and embryos were collected at 10.0 dpc. Cre activity was readily apparent in the pharyngeal mesoderm (arrowheads), outflow tract (OFT), and future right ventricle (RV). No expression was observed in the atria. RA, right atrium.

mef2c-AHF-Cre, to ROSA26R *lacZ* reporter mice (Soriano, 1999) to determine the contribution of this population of cells to the developing mouse heart. As shown here and in previous work (Dodou et al., 2004), the *mef2c* anterior heart field enhancer is specific to the population of cells that make up the anterior heart field and its derivatives in the outflow tract and right ventricle. Expression directed by the enhancer is tightly restricted to these tissues and is never observed in the left ventricle or atria (Dodou et al., 2004).

Mef2c-AHF-Cre positive transgenic founder mice were screened by crossing to ROSA26R *lacZ* reporter mice, which activate *lacZ* expression in a Cre-dependent manner (Soriano, 1999). We screened six *mef2c*-AHF-Cre founder lines that were positive for the transgene by Southern blot. Among these, four had a pattern of β-galactosidase activity consistent with the anterior heart field and its derivatives, whereas the other two lines had no detectable X-gal staining (not shown). In the *lacZ* expressing lines, β-galactosidase activity was detected in the right ventricle, outflow tract, and pharyngeal mesoderm at 10.0 dpc, consistent with an anterior heart field origin (Fig. 2). Three *mef2c*-AHF-Cre transgenic lines were characterized in detail for Cre expression (Figs. 2B–D), and one of these lines, 200A, was chosen for further analysis.

The mef2c-AHF-Cre transgene is active at early cardiac crescent stage and its expression overlaps with markers of the secondary/anterior heart field

To verify that Cre activity in *mef2c*-AHF-Cre mice was specifically marking the anterior heart field, we compared β-galactosidase expression in *mef2c*-AHF-Cre Tg/0; ROSA26R lacZ/+ embryos with the expression of *Isl1* and *Fgf10*, which are early markers of the secondary/anterior heart field in mice. *Fgf10* was the first anterior heart field marker described (Kelly et al., 2001), while *Isl1* is an essential gene required for secondary heart field development (Cai et al., 2003) and has been shown to directly activate *mef2c* transcription in the anterior heart field (Dodou et al., 2004). β-galactosidase activity was first observed in *mef2c*-AHF-Cre Tg/0; ROSA26R *lacZ*/+ embryos in the cardiac crescent at 7.5 dpc (Fig. 3A). By 8.0 dpc, Cre activity was strongly apparent in the cardiac crescent as ventral folding was occurring to bring the two lateral fields of splanchnic mesoderm cells together in the dorsal anterior domain characteristic of the anterior heart field (Fig. 3B). This pattern of expression overlapped the expression of *Fgf10* and *Isl1*, which are expressed in the early anterior heart field but are also expressed broadly outside this domain

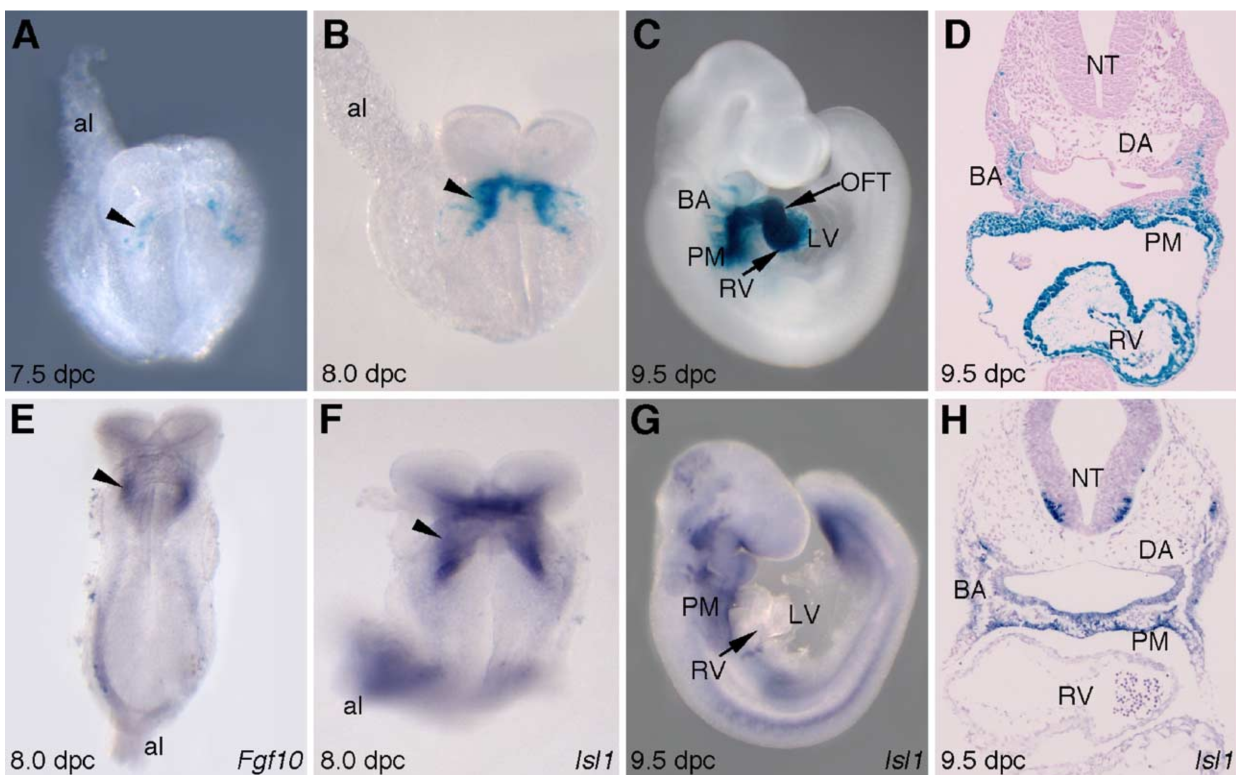

Fig. 3. The *mef2c*-AHF-Cre fate map overlaps with the expression of anterior heart field markers during early cardiac development. (A–D) Embryos from *mef2c*-AHF-Cre transgenic mice crossed to ROSA26R Cre-dependent *lacZ* reporter mice exhibit β-galactosidase activity exclusively in the anterior heart field and its derivatives. Cre activity directed by the *mef2c*-AHF-Cre transgene is first detectable in the dorsomedial region of the cardiac crescent at 7.5 dpc (A). By 8.0 dpc (B), *mef2c*-AHF-Cre expresses Cre in the migrating cells of the anterior heart field (arrowhead). Expression of Cre at 8.0 dpc overlaps with the expression of the anterior heart field markers *Fgf10* (E) and *Isl1* (F) as shown by in situ hybridization. Expression of *Fgf10* and *Isl1* is considerably more broad than the anterior heart field; arrowheads point to the anterior heart field stained cells in panels E and F. By 9.5 dpc (C), Cre activity directed by the Cre transgene is detectable throughout the right ventricle (RV) and outflow tract (OFT), as well as in the pharyngeal mesoderm (PM) component of the branchial arches (BA) and in the outlet region of the future left ventricle (LV). Cre expression overlaps the expression of *Isl1* in the pharyngeal mesoderm (G). Transverse sections of embryos collected at 9.5 dpc show similar expression patterns between *mef2c*-AHF-Cre marked cells (D) and *Isl1* expressing cells (H) in the pharyngeal mesoderm (PM). al, allantois; DA, dorsal aorta; NT, neural tube.

(Figs. 3E, F). These results demonstrate that *mef2c*-AHF-Cre specifically marks cells only within the anterior heart field and is robustly active even at early stages in cardiac development. Expression of β-galactosidase at 9.5 dpc strongly marked cells within the anterior heart field and its derivatives, including the pharyngeal mesoderm, outflow tract, and right ventricular myocardium (Figs. 3C, D). Similar histological sections of E9.5 embryos showed overlapping expression between *mef2c*-AHF-Cre marked cells and *Isl1* mRNA in the pharyngeal mesoderm and in the dorsal wall of the pericardium (Figs. 3D, H). Taken together, these results show that expression of Cre in *mef2c*-AHF-Cre transgenic mice marks the anterior heart field.

Mef2c-AHF-Cre expressing cells and their descendants contribute to the right ventricle, outflow tract, and ventricular septum

While previous work has suggested that anterior heart field-derived cells give rise to a number of cardiac tissues in the embryo, none have examined the contribution to the fetal or postnatal heart in detail. Furthermore, some discrepancies exist regarding the boundaries of secondary/anterior heart field contribution to the mature heart (Cai et al., 2003; Meilhac et al., 2004a,b; Waldo et al., 2001; Zaffran et al., 2004). To define the contribution of the *mef2c*-AHF-Cre anterior heart field marked population to the developing and postnatal heart, we crossed these mice to ROSA26R Cre-dependent *lacZ* reporter mice to generate a fate map (Fig. 4). We compared the Cre-dependent *lacZ* expression in these crosses to the expression of *lacZ* directly under the control of the *mef2c* anterior heart field enhancer using *mef2c*-AHF-*lacZ* transgenic mice (Dodou et al., 2004). The goal of these comparisons was to identify regions of the heart that were marked by the Cre-derived fate map but that never showed direct *lacZ* expression in *mef2c*-AHF-*lacZ* transgenic hearts. This would indicate regions of the heart that had embryological origins derived from the *mef2c*-AHF-Cre defined anterior heart field and its descendants. In addition, we sought to identify regions of the heart that were not marked by the *mef2c*-AHF-Cre fate map, which would indicate regions of the heart that were not derived from the *mef2c*-AHF-Cre expressing population.

At 9.5 dpc, the *mef2c* anterior heart field enhancer was clearly active in the future right ventricle and outflow tract, as well as in the pharyngeal mesoderm (Figs. 4A, I). However, no activity was observed in the future left ventricle in *mef2c*-AHF-*lacZ* transgenic embryos (Figs. 4A, I). The Cre-derived fate map also marked the right ventricle, outflow tract, and pharyngeal mesoderm at 9.5 dpc (Figs. 4E, M). β-galactosidase activity in *mef2c*-AHF-Cre embryos was also observed in the future left ventricle at this stage (Figs. 4E, M), indicating that cells marked at an earlier time by the *mef2c* enhancer contribute to the future left ventricle since the enhancer itself is never active in the left ventricle (Figs. 4A, I). The *mef2c* anterior heart field enhancer continued to be active in the outflow tract and right ventricle at 12.0 dpc (Figs. 4B, J). Again, the Cre-derived fate map also marked these regions and additionally marked the developing ventricular septum and proximal regions of the left ventricle at this stage (Figs. 4F, N). By comparison, the activity of the *mef2c* anterior heart field enhancer itself, as indicated in *mef2c*-AHF-*lacZ* transgenic animals, was restricted to the right ventricle and outflow tract and did not cross the ventricular septum at this or any other stage of development (Figs. 4B, J).

By 14.5 dpc, the once common outflow vessel has been distinctly divided into the pulmonary artery and aorta, and by this stage, the activity of the *mef2c* anterior heart field enhancer itself was largely restricted to a region of the right ventricle near the pulmonary trunk (Fig. 4C). There was also some weak, residual β-galactosidase activity in right ventricular myocardium near the ventricular chamber in *mef2c*-AHF-*lacZ* transgenic hearts (Figs. 4C, K). By contrast, β-galactosidase expression in *mef2c*-AHF-Cre; ROSA26R embryos was observed throughout the right ventricle, ventricular septum, the left ventricle proximal to the septum, and in both outflow vessels at this stage (Figs. 4G, O). In *mef2c*-AHF-*lacZ* neonatal hearts, β-galactosidase activity continued to be observed weakly in the right ventricular wall especially near the pulmonary trunk (Figs. 4D, L). In *mef2c*-AHF-Cre; ROSA26R transgenic mice, β-galactosidase activity was observed throughout the right ventricle, proximal aorta and pulmonary artery, and throughout the ventricular septum, while essentially no expression was observed in the atria or most of the left ventricle (Figs. 4H, P). It is notable that no *mef2c*-AHF-Cre marked cells were detected in the majority of the left ventricle or in the atria at this or any other developmental stage (Figs. 4E–H, M–P).

Taken together, the data presented in Fig. 4 demonstrate that the outflow tract and right ventricle are marked by the activity of the *mef2c*-AHF-Cre transgene, and that the ventricular septum and proximal portions of the left ventricle are derived from *mef2c*-AHF-Cre expressing cells in the anterior heart field and their descendants. These data also demonstrate that the majority of the left ventricle and the atria are not derived from *mef2c*-AHF-Cre expressing cells in the anterior heart field and pharyngeal mesoderm. This suggests that the *mef2c* anterior heart field expression domain represents a subpopulation of the larger secondary heart field in the mouse, which is marked by the *Isl1*-Cre fate map (Cai et al., 2003).

Right ventricular and outflow tract endothelial cells are marked by the mef2c-AHF-Cre transgene

The endocardium is the endothelial cell lining of the heart and plays an important role in heart function and in reciprocal signaling with the myocardium (Brutsaert, 2003). Despite the importance of the endocardium, its developmental origins remain to be defined in detail. Previous studies have indicated that some endocardial cells are derived from the secondary heart field, although the extent of this contribution remains unclear (Cai et al., 2003). In crosses of *mef2c*-AHF-Cre transgenic mice with ROSA26R Cre-dependent reporter mice, we observed that the endothelial cells of outflow tract and right ventricle were positively marked by X-gal staining (Fig. 4M). To examine *mef2c*-AHF-Cre directed *lacZ* expression in the endothelium in greater detail, we also assayed β-galactosidase

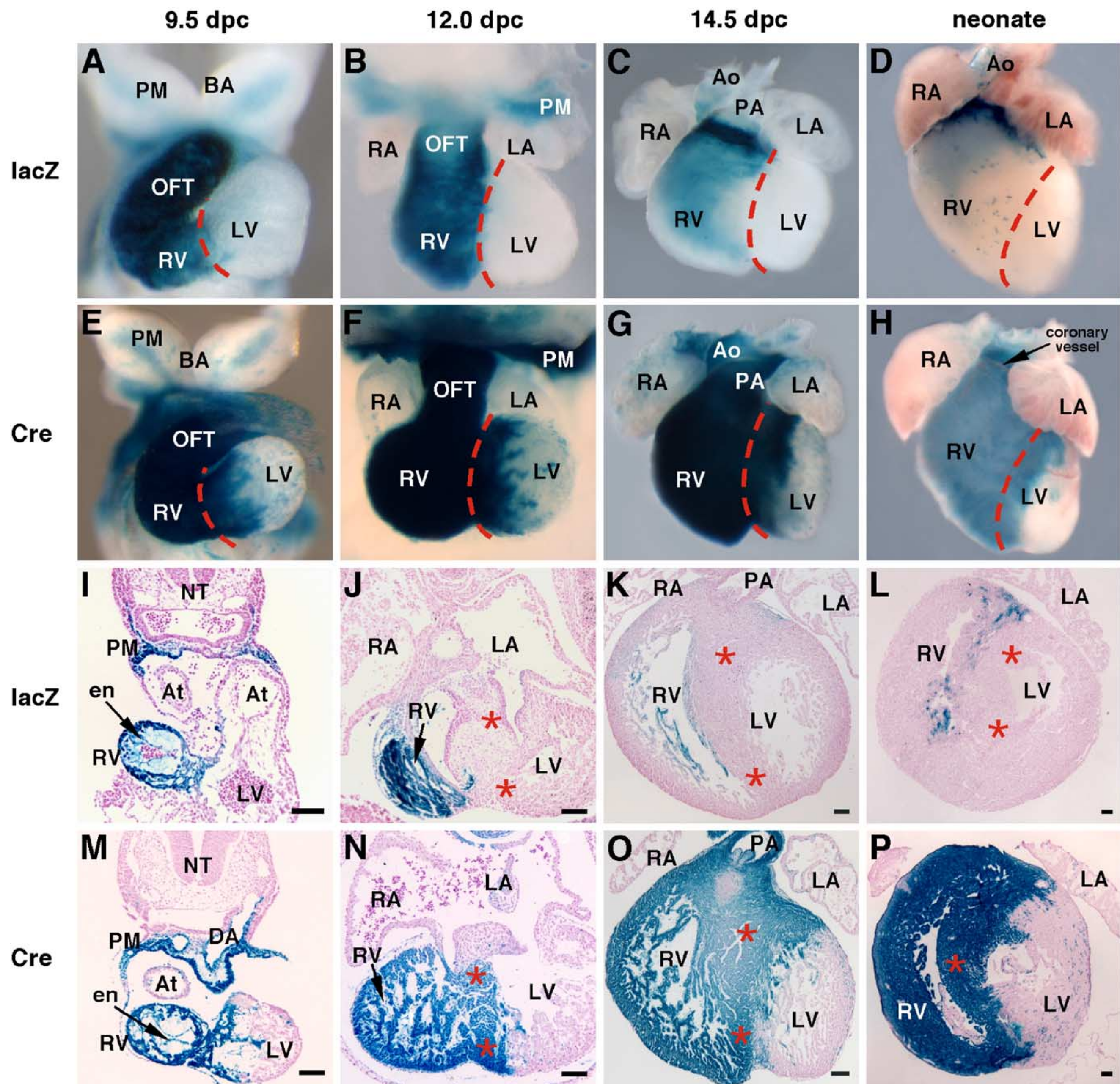

Fig. 4. The right ventricle, outflow tract, and ventricular septum are composed of *mef2c*-AHF-Cre expressing derivatives. Hearts collected from either *mef2c*-AHF-*lacZ* (A–D, I–L) or *mef2c*-AHF-Cre Tg/0; ROSA26R *lacZ*/+ mice (E–H, M–P) were stained for β-galactosidase activity with X-gal. In panels I–K and M–O, whole embryos were stained with X-gal and then cut in transverse sections. In panels L and P, dissected hearts were cut in transverse sections and then X-gal stained. At 9.5 dpc, *mef2c*-AHF-*lacZ* directed expression was detected in the right ventricle (RV), outflow tract (OFT), and the pharyngeal mesoderm (PM) of the branchial arches (BA) (A, I). *Mef2c*-AHF-Cre Tg/0; ROSA26R *lacZ*/+ mice exhibited a similar pattern of expression in these tissues, but the expression was broader and extended into the right-most portion of the left ventricle (LV) (E, M). X-gal staining was also detected in the endocardium (en) at this stage; no expression was observed in the common atrial chamber (At) at this stage. *Mef2c*-AHF-Cre marked cells in hearts collected at 12.0 dpc (F, N) were present in the same domains as in *mef2c*-AHF-*lacZ* hearts (B, J), but extended throughout the right ventricle, through the developing ventricular septum (asterisk), and into the left ventricle. Note the lack of staining in the right and left atria (RA and LA, respectively) and the absence of staining in the epicardium. At 14.5 dpc, *mef2c*-AHF-*lacZ* expression was more restricted and less intense (C, K), with no staining in the pulmonary artery or ventricular septum (asterisk). *Mef2c*-AHF-Cre fate mapped cells were evident in the pulmonary artery (PA) as well as the right ventricle and the portion of the left ventricle proximal to the septum (G, O). No staining was observed in the atria. In the postnatal heart from *mef2c-AHF-lacZ* mice (D, L), positive cells were primarily found only at the base of the pulmonary trunk and scattered within the right ventricle and aorta. In *mef2c*-AHF-Cre hearts (H, P), positive cells were detected in the right ventricle, outflow vessels, and throughout the entire ventricular septum (asterisk). The atria, the majority of the left ventricle, and the coronary vessels were not marked by the *mef2c*-AHF-Cre transgene. Red dashed lines demark the position of the ventricular septum in panels A–H. Ao, aorta; DA, dorsal aorta; NT, neural tube. Scale bar equals 100 μm.

expression directly by immunohistochemistry using an anti-β-galactosidase antibody (Fig. 5A). We compared β-galactosidase expression to the expression of the endothelial cell marker PECAM (Fig. 5B), and observed that β-galactosidase protein was co-expressed with PECAM in the right ventricle and outflow tract at 10.5 dpc (Fig. 5C). Taken together, the results

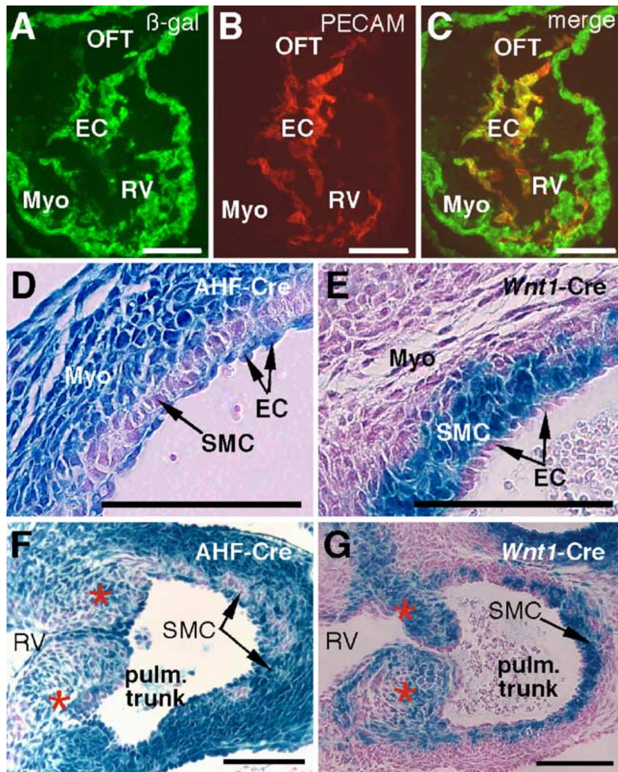

Fig. 5. The right ventricular endocardium and the endothelium and myocardium of the outflow tract are derived from *mef2c*-AHF-Cre progenitors, and the smooth muscle of the outflow tract is largely neural crest-derived. (A) Immunohistochemical detection of β-galactosidase (β-gal) protein in a transverse section through the right ventricle (RV) and outflow tract (OFT) of a representative *mef2c*-AHF-Cre Tg/0; ROSA26R *lacZ*/+ transgenic heart collected at 10.5 dpc. (B) PECAM staining to detect endothelial cells in the same section shown in panel A. (C) Merged image of panels A and B showing overlap of β-galactosidase and PECAM in the endocardium (EC), but not in the myocardium (Myo), which was only marked by β-galactosidase. (D) A representative transverse section through the pulmonary artery of a *mef2c*-AHF-Cre Tg/0; ROSA26R *lacZ*/+ transgenic embryo at 14.5 dpc. Cre activity was evident in the myocardial (myo) and endothelial cell (EC) layers of the outflow tract, while no activity was observed in the smooth muscle cell (SMC) layer. (E) Section through the pulmonary artery of a *Wnt1*-Cre Tg/0; ROSA26R *lacZ*/+ embryo at 14.5 dpc. Cre activity was evident in the smooth muscle layer, but not in endothelial cells or the myocardial layer of the outflow tract. Representative parafrontal sections through the pulmonary artery (pulm. trunk) at the junction with the right ventricle from a *mef2c*-AHF-Cre Tg/0; ROSA26R *lacZ*/+ embryo (F) or a *Wnt1*-Cre Tg/0; ROSA26R *lacZ*/+ embryo (G) at 14.5 dpc. *Mef2c*-AHF-Cre marked cells were detected in the myocardial components of the vessel, as well as in some of the cells subadjacent to the endothelium, presumably smooth muscle, and in cells of the pulmonary valve leaflets (asterisks). *Wnt1*-cre activity was detected in the smooth muscle cell layer of the pulmonary artery and aorta, the leaflets of the pulmonary valves (asterisks), and in the conotruncal (CT) septum. Scale bar is equal to 100 μm.

from Figs. 4 and 5 demonstrate that the endothelial cells of the proximal outflow tract and right ventricle are marked by the *mef2c*-AHF-Cre transgene.

Epicardium, coronary vessels, and the majority of outflow tract smooth muscle are not derived from mef2c-AHF-Cre expressing cells in the anterior heart field

Our work presented here, as well as the work of others (Cai et al., 2003; Kelly et al., 2001; Mjaatvedt et al., 2001; Waldo et al., 2001), demonstrates that anterior heart field-derived cells contribute to the myocardial component of the outflow vessels of the heart (Fig. 4). Interestingly, previous studies have shown that the cardiac neural crest also contributes to the outflow vessels, giving rise to the smooth muscle of the tunica media (Epstein et al., 2000; Jiang et al., 2000; Waldo et al., 1999). Very recently, a study performed in chick showed that the smooth muscle of the most proximal outflow tract is derived from secondary heart field rather than cardiac neural crest (Waldo et al., 2005b). To address which cells of the outflow vessels are derived from each specific developmental field in the mouse, we compared ROSA26R directed β-galactosidase expression in the outflow tract from transgenic mice expressing *mef2c*-AHF-Cre and *Wnt1*-Cre (Danielian et al., 1998). *Wnt1*-Cre marks all neural crest cells and their descendants, including the cardiac neural crest and its derivatives in the outflow tract (Danielian et al., 1998; Jiang et al., 2000). *Mef2c*-AHF-Cre-derived cells comprised the endothelial lining and outer myocardial component of the aorta and pulmonary artery, but did not appear to contribute to the smooth muscle cells in the more distal regions of the outflow tract (Fig. 5D). Instead, neural crest-derived cells comprised the smooth muscle layer within the more distal outflow tract (Fig. 5E). Neural crest descendants were not observed within the endothelial lining of the outflow tract or in the muscular myocardial portion of the outflow vessels (Fig. 5E). Near the base of the heart, both Wnt1-Cre and *mef2c*-AHF-Cre-derived cells contributed to the smooth muscle cells of the pulmonary trunk and to the cells of the pulmonary valve leaflets (Figs. 5F, G). Thus, it appears that the cardiac neural crest and the anterior heart field each give rise to smooth muscle cells of the outflow tract near the base of the heart but have largely non-overlapping contributions to the more distal regions of the outflow vessels (Figs. 5D–G).

Previous studies have suggested that the coronary vessels and epicardium have a common developmental origin in the proepicardial organ (septum transversum) (Olivey et al., 2004). We examined whether these tissues might also be derived in part from the anterior heart field, particularly for the epicardium and vessels overlying the right ventricle (Fig. 6). Our results clearly show that the epicardium lying directly adjacent to the positively marked right ventricular myocardium was negative for β-galactosidase activity, indicating that the epicardium is not derived from *mef2c*-AHF-Cre expressing cells (Fig. 6A). Similar to the epicardium, whole mount staining of neonatal hearts, or sections through coronary vessels showed little or no investment of cells marked by the *mef2c*-AHF-Cre transgene (Figs. 6B, C). Occasionally, *mef2c*-AHF-Cre marked cells were observed within coronary vessels (Fig. 6C) suggesting the possibility that some smooth muscle, or possibly endothelial, cells of anterior heart field origin might contribute or be added to the coronary vessels. Overall, however, we conclude that the coronary vessels and the epicardium have developmental origins that are largely distinct from the anterior heart field population marked by *mef2c*-AHF-Cre, which is consistent with a common developmental origin for these structures and the addition of these cells after the

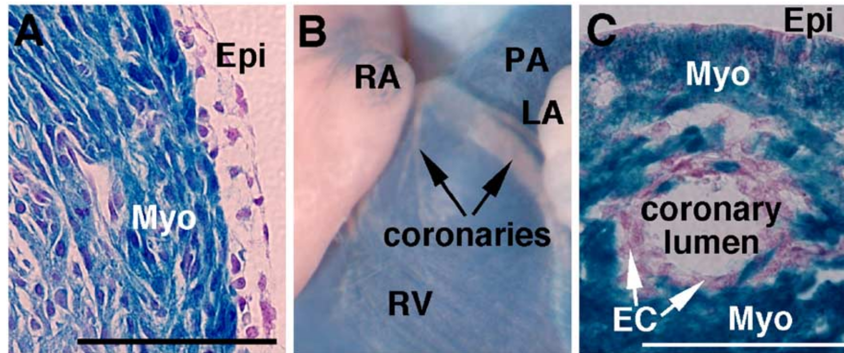

Fig. 6. The epicardium and coronary vessels are not derivatives of the anterior heart field. (A) Representative section from a *mef2c*-AHF-Cre Tg/0; ROSA26R *lacZ*/+ heart collected at 14.5 dpc shows strong β-galactosidase activity in the myocardium (Myo), and negative staining in the adjacent epicardium (Epi). The coronary vessels of the neonatal heart were largely negative for β-galactosidase activity as seen in whole mount (B), or transverse sections (C). Notice also that the epicardium of the neonatal heart is negative for β-galactosidase staining. EC, endothelial cells; LA, left atrium; PA, pulmonary artery; RA, right atrium; RV, right ventricle. Scale bar is equal to 100 μm.

addition of the anterior heart field to the heart (Olivey et al., 2004).

Discussion

It has only recently become clear that the arterial portion of the developing heart arises from a discrete population of cells called the secondary or anterior heart field (Kelly et al., 2001; Mjaatvedt et al., 2001; Waldo et al., 2001). Understanding the developmental potential of this novel population of cells is required for future studies focused on the role of the anterior heart field in cardiac development and its involvement in congenital heart disease. In the present study, we have described a Cre transgenic mouse line, *mef2c*-AHF-Cre, which exhibits activity that is tightly restricted spatially to a subset of cells within the secondary/anterior heart field and its descendants. We used these mice to follow the contribution of this population of cells within the anterior heart field to the embryonic and postnatal heart. These studies demonstrate that the myocardium of the outflow tract and right ventricle is marked by *mef2c*-AHF-Cre expression and that the ventricular septum and proximal portions of the left ventricle are derived from *mef2c* expressing cells within the anterior heart field. This work also shows that the endocardium of the right ventricle and proximal outflow vessels are marked by the *mef2c*-AHF-Cre transgene, while the epicardium and coronary vessels do not share a common origin with this population of anterior heart field-derived cells. Importantly, we also show that this population of cells within the anterior heart field does not contribute to the majority of the left ventricle or the atria.

Previous studies have come to differing conclusions when defining the boundaries of secondary/anterior heart field contribution to the heart. Using retrospective fate mapping, a clonal boundary was observed between the left and right ventricles, with the ventricular septum arising from both right and left ventricular myocyte clones, suggesting a dual origin for the septum (Meilhac et al., 2004b). The *mef2c*-AHF-Cre generated fate map described here spans this clonal boundary, suggesting that the entire ventricular septum is derived from this anterior heart field population of cells (Fig. 4). Indeed, more recent retrospective clonal analyses suggest that myocyte clones can span the septal boundary and further suggested that the outflow tract is derived exclusively from secondary heart field, while the right ventricle and atria are derived from progenitors from both the primary and secondary heart fields (Meilhac et al., 2004a). Other recent work defines the secondary heart field as *Isl1* expressing progenitors that give rise to the outflow tract and right ventricle, as well as the majority of the cells in the left ventricle and developing atrium (Cai et al., 2003). By contrast, the fate map generated by *mef2c*-AHF-Cre described here has more restricted spatial boundaries and does not include the atria or the majority of the left ventricle. One possible explanation for this discrepancy is that *Isl1* is expressed in the precardiac mesoderm prior to the partitioning of the primary and secondary heart fields, and therefore *Isl1* may mark descendants of both heart fields (Abu-Issa et al., 2004; Meilhac et al., 2004a). *Mef2c*-AHF-Cre may be activated in the precardiac mesoderm after the secondary heart field has a distinct identity from the primary heart field (Fig. 3) and, therefore, marks only the secondary/anterior heart field. This notion is consistent with fate mapping studies done in chick embryos where the secondary heart field appears to contribute only to the arterial pole of the developing heart (Mjaatvedt et al., 2001; Waldo et al., 2001). Alternatively, the *mef2c*-AHF-Cre-derived fate map may represent a restricted subdomain of the secondary heart field with the *mef2c* enhancer marking only a portion of the larger *Isl1* expression domain, which is a notion that is also consistent with previous studies (Cai et al., 2003; Meilhac et al., 2004a).

Genetic fate mapping has several advantages over other fate mapping approaches, which rely on embryological manipulations and ex vivo culture systems. A Cre-based, genetic fate mapping approach, such as the one utilized here, provides nearly exact spatial resolution and does not require any perturbations to the developing embryo, thus allowing an embryonic fate map to be extended into adulthood. The disadvantage of the fate map presented here is that it does not provide a strict temporal boundary from which to map the fate of cells. By comparing the activity of the *mef2c* enhancer driving *lacZ* expression directly to the enhancer generated fate

map, we were able to identify regions of the heart that did not exhibit enhancer activity at any time during development yet were marked by the fate map. This allowed us to use *mef2c*-AHF-Cre to identify the contribution of this population of cells within the anterior heart field to the postnatal heart, and importantly, to identify regions of the heart that lacked contribution from anterior heart field cells marked by the *mef2c*-AHF-Cre transgene. The use of multiple fate mapping methods, including genetic and other approaches, is essential for a detailed understanding of the developmental potential of the secondary/anterior heart field, including the subpopulations and expression domain gradients within this progenitor pool.

Anterior heart field contribution in mutant mice with defective heart looping

Knockout studies in mice have shown that several cardiac transcription factors are required for cardiac development. Mice with mutations in *mef2c*, *Hand2*, *Nkx2.5*, *Isl1*, and *Foxh1* exhibit defects in looping morphogenesis and have only a single hypoplastic common ventricular chamber, and each of these genes is strongly expressed in the anterior heart field (Cai et al., 2003; Lin et al., 1997; Lyons et al., 1995; Srivastava et al., 1997; von Both et al., 2004). It has been proposed that the common ventricular chamber in mice with mutations in these genes may lack right ventricular identity, which is consistent with a failure of the anterior heart field to be added to the developing heart (McFadden et al., 2000; Srivastava et al., 1997). We examined the expression of a transgenic marker of the anterior heart field, *mef2c*-AHF-*lacZ*, in *mef2c* knockout embryos. Our results show that this marker of the anterior heart field is expressed in the common ventricular chamber of *mef2c* null embryos, suggesting that the looping defects seen in *mef2c*, and possibly other mutant mice, are not due to failure of anterior heart field migration into the ventricular chamber. Interestingly, however, the *mef2c*-AHF-*lacZ* anterior heart field marker is misexpressed in *mef2c* knockout embryos in a dorsal rather than a right-sided pattern. These observations suggest that the failure in looping morphogenesis in *mef2c* knockout animals may be due to a failure of anterior heart field-derived cells to properly differentiate, migrate in an appropriate pattern, or communicate with adjacent tissues. A failure of anterior heart field cells to differentiate properly is consistent with previous observations of the *Hand2* cardiac enhancer in *mef2c* null mice, which suggested that the common ventricular chamber lacked cells with right ventricular identity (McFadden et al., 2000). Future studies will explore the role of the anterior heart field in cardiac morphogenesis.

Interaction of the anterior heart field with the cardiac neural crest and other tissues

The studies presented here have focused primarily on the expression of a transgenic marker of the anterior heart field and the embryological contributions of that population of cells to the heart; however, it is clear that anterior heart field-derived components of the heart do not function autonomously and must integrate with several other lineages. We have described that the epicardium and coronary vessels come from a developmental origin distinct from the anterior heart field, consistent with addition to the heart from the proepicardial organ after addition of the anterior heart field (Kirby, 2002). It will be interesting to determine how anterior heart field-derived cardiomyocytes create a permissive environment for subsequent addition of these tissues to the heart. The data presented here demonstrate that the majority of the left ventricle and the atria are not derived from the regions of anterior heart field marked by the activity of the *mef2c*-AHF-Cre transgene. It remains to be determined how the primary and secondary heart fields are merged and whether reciprocal interactions between these two cardiogenic populations control boundary formation between the ventricles.

The cardiac neural crest is required for proper outflow tract development (Hutson and Kirby, 2003), and it has been shown to contribute to the smooth muscle of the outflow vessels (Epstein et al., 2000; Jiang et al., 2000; Waldo et al., 1999). Before septation, the outflow tract is primarily a myocardial tissue of secondary heart field origin. As the outflow tract shortens, the myocardial component is reduced, potentially by programmed cell death (Sugishita et al., 2004; Watanabe et al., 1998) or by transdifferentiation of myocardium to smooth muscle cells (Ya et al., 1998). Concurrent with outflow tract shortening, cardiac neural crest cells migrate into the outflow tract and contribute to the developing aorticopulmonary septum as well as to the tunica media of the developing great vessels (Jiang et al., 2000).

The smooth muscle cells of the outflow tract are positioned between the anterior heart field-derived myocardial and endocardial components of these vessels, and anterior heart field progenitors in the pharyngeal mesoderm are juxtaposed with neural crest-derived mesenchyme in the branchial arches. The close proximity of these two lineages and their eventual co-contribution to the outflow tract suggests the possibility of an intricate reciprocal signaling relationship between the secondary/anterior heart field and the cardiac neural crest. Indeed, in animals lacking cardiac neural crest cells, secondary heart field myocardium fails to be added properly to the arterial pole of the embryonic heart (Waldo et al., 2005a; Yelbuz et al., 2002). It will be interesting to determine if the neural crest also requires signaling from the anterior heart field for proper addition to the outflow tract and valves. The *mef2c*-AHF-Cre transgenic mouse line will allow the ability to ablate genes specifically in the anterior heart field and its derivatives, which will permit a more detailed study of reciprocal signaling interactions in the outflow tract.

Implications of an anterior heart field-specific Cre transgenic mouse line

Several common congenital anomalies result from outflow tract defects, including tetralogy of Fallot, double outlet right ventricle, persistent truncus arteriosus, and transposition of the great arteries (Srivastava, 2003; Winlaw et al., 2005). Because the outflow tract is derived from at least two distinct

embryological origins, the anterior heart field and the neural crest, it will be important to define the role of each of these lineages in congenital malformations.

Conditional gene inactivation is an integral approach to understanding developmental processes such as cardiac development. In order to understand the function of genes specifically within the anterior heart field, lineage restricted Cre mice must be developed. Currently, the only existing Cre line described for this lineage is *Isl1*-Cre, which is expressed broadly in the embryo, including the secondary heart field (Srinivas et al., 2001). *Isl1*-Cre has the advantage of being expressed earlier than the *mef2c*-AHF-Cre transgene described here (Cai et al., 2003). Indeed, ISL1 binding is required for *mef2c* anterior heart field enhancer activation (Dodou et al., 2004). In addition, an inducible *Isl1*-Cre transgenic mouse has been developed and has the advantage of temporal control of Cre activity (Laugwitz et al., 2005). Furthermore, *Isl1* is not expressed in many of the anterior heart field-derived cells that comprise the developing myocardium and outflow tract (Cai et al., 2003). However, *Isl1* is expressed in many developing tissues and lineages outside of cardiac progenitor populations, including motor neurons, oral epithelium, cranial sensory ganglia, inner ear, thalamus, pituitary, pancreatic endothelium and mesenchyme, and distal tubular cells of the kidney (Ahlgren et al., 1997; Dong et al., 1991; Mitsiadis et al., 2003; Nakagawa and O'Leary, 2001; Pfaff et al., 1996; Radde-Gallwitz et al., 2004). Consistent with the broad expression of *Isl1*, the *Isl1*-Cre transgene is also broadly expressed during development and is clearly expressed outside cardiac progenitor populations. Indeed, the *Isl1*-Cre transgenic mouse line was first utilized to mark cells of motor neurons and dorsal root ganglia (Srinivas et al., 2001). In contrast, expression of the *mef2c*-AHF-Cre transgene described in the present study is highly restricted to the anterior heart field and its derivatives in the developing heart, and this novel reagent will allow for conditional inactivation of genes exclusively in the anterior heart field and its derivatives. This will be particularly relevant since many genes expressed in the anterior heart field are also expressed in the primary heart field or in the neural crest, and conditional inactivation approaches will be necessary to determine specific functions of these genes in the anterior heart field, right ventricle, and outflow tract independent of other lineages. These types of studies and the use of multiple distinct Cre lines will provide important insights into the mechanisms underlying the congenital anomalies that appear to impact the secondary/anterior heart field and its derivatives in the outflow tract.

Acknowledgments

We are grateful to Sylvia Evans (UCSD) and Pao-Tien Chuang (UCSF) for providing in situ probes and John Schwarz (Albany) for *mef2c* knockout mice. We thank Shan-Mei Xu and Eric Ho for technical assistance with these studies, and Jim Martin and Jim Bristow for providing comments on the manuscript. MPV was supported by a Howard Hughes Medical Institute Predoctoral Fellowship and SDV was supported in part by a fellowship from the American Heart Association, Western States Affiliate. This work was supported by grant HL64658 from the National Heart, Lung, and Blood Institute to BLB.

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