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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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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.

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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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