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Forkhead transcription factors, *Foxc1* and *Foxc2*, are required for the morphogenesis of the cardiac outflow tract

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Abstract

Previous studies have shown that *Foxc1* and *Foxc2*, closely related Fox transcription factors, have interactive roles in cardiovascular development. However, little is known about their functional overlap during early heart morphogenesis. Here, we show that *Foxc* genes are coexpressed in a novel heart field, the second heart field, as well as the cardiac neural crest cells (NCCs), endocardium, and proepicardium. Notably, compound *Foxc1*; *Foxc2* mutants have a wide spectrum of cardiac abnormalities, including hypoplasia or lack of the outflow tract (OFT) and right ventricle as well as the inflow tract, dysplasia of the OFT and atrioventricular cushions, and abnormal formation of the epicardium, in a dose-dependent manner. Most importantly, in the second heart field, compound mutants exhibit significant downregulation of *Tbx1* and *Fgf8*/*10* and a reduction in cell proliferation. Moreover, NCCs in compound mutants show extensive apoptosis during migration, leading to a failure of the OFT septation. Taken together, our results demonstrate that *Foxc1* and *Foxc2* play pivotal roles in the early processes of heart development, especially acting upstream of the Tbx1-FGF cascade during the morphogenesis of the OFT.

Keywords: Heart development; Second heart field; Outflow tract; Neural crest; Epicardium; Cushion mesenchyme; Forkhead

Introduction

The mammalian heart is composed of four chambers with multiple cell types such as myocardial and endocardial cells, heart-valve mesenchyme, and endothelial and smooth muscle cells of coronary blood vessels (Hutson and Kirby, 2003; Kirby, 2002; Reese et al., 2002). Heart morphogenesis begins with the formation of the primitive tubular heart by fusion of the cardiac crescent, which contains myocardial and endocardial progenitor cells. Until recently, it was believed that the cardiac crescent (the first heart field) gives rise to the myocardium and endocardium of the entire heart tube. However, compelling evidence has recently demonstrated the identification of a novel heart field, the second heart field, thereby modifying the classical concept of heart formation (Buckingham et al., 2005). In addition, it is known that two types of extracardiac cell populations, cardiac neural crest cells (NCCs) and epicardium-derived cells (EPDCs), significantly contribute to the morphogenesis of the developing heart (Hutson and Kirby, 2003; Kirby, 2002; Olivey et al., 2004; Wada et al., 2003). Compelling evidence demonstrates that signaling pathways such as BMP and FGF regulate early heart development (Brand, 2003; Buckingham et al., 2005; Kelly and Buckingham, 2002; Kirby, 2002; Yutzey and Kirby, 2002) and that the combined activities of different transcription factors modulate gene expression in this process (Cripps and Olson, 2002; Kelly, 2005). Here we show that the murine Foxc1 and Foxc2, closely related forkhead/Fox transcription factors, play important roles in early steps of the developing heart, including the formation of the OFT.

Recent studies have shown that the myocardium and endocardium of the OFT derive from a newly identified cell population, the anterior heart field (AHF) or secondary heart field (SHF) in the chick and mouse (Kelly et al., 2001; Mjaatvedt et al., 2001; Waldo et al., 2001). This cell population originates from the medial splanchnic mesoderm adjacent to the cardiac crescent and then extends progressively to the pharyngeal region anterior and dorsal to the arterial pole of the heart (Kelly and Buckingham, 2002; Kelly, 2005). Subsequently, it has been

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shown that expression of the *Isl1* transcription factor extends more posteriorly in addition to the AHF in the mouse, and cells expressing *Isl1* contribute to both the arterial and venous poles of the mouse heart (Cai et al., 2003). Retrospective clonal analysis in the mouse has further reinforced the two heart lineages with a common clonal origin of about 140 cardiac progenitor cells (Meilhac et al., 2004). Although the first and second lineages have, to a large extent, an overlapping contribution to the right ventricle (RV), atrioventricular canal, atria, and inflow tract (IFT) of the heart, the two lineages exclusively form the left ventricle (LV) and OFT, respectively. Notably, the second lineage gives rise to the second heart field as defined by derivatives of *Isl1*-positive cells.

The perturbation of the second heart field by disrupting the functions of signaling pathways or cardiac transcription factors leads to conotruncal defects such as persistent truncus arteriosus, as well as myocardial dysfunction (Kelly, 2005). For example, BMP and FGF signaling is involved in the development of the OFT through the recruitment and differentiation of myocardial precursors derived from the anterior part of the second heart field (Kelly and Buckingham, 2002; Kirby, 2002). Mice lacking both *Bmp4* and *Bmp7* have a shortened OFT with hypoplastic cushions (Liu et al., 2004), whereas hypomorphic mutants for *Fgf8* have defects in the OFT septation along with abnormal formation of the forth pharyngeal arch artery (PAA) and increased apoptosis of migrating NCCs (Frank et al., 2002).

Analysis of *Isl1*-deficient mice lacking the OFT and RV suggests that *Isl1* regulates proliferation, migration, and survival of the second heart field-derived cells through FGF and BMP signaling (Cai et al., 2003). *Myocyte enhancer factor 2c* (*Mef2c*) null mutants display lack of RV as well as hypolastic OFT and IFT (Lin et al., 1997). Importantly, activity of *Mef2c* enhancer, which directs its expression in the second heart field as well as OFT and RV, is dependent on *Gata-4* and *Isl1*, suggesting that *Mef2c* is a direct target of these transcription factors (Dodou et al., 2004). *Foxh1* mutant embryos fail to form the OFT and RV with significant downregulation of *Fgf8* and *Fgf10* in the second heart field-derived cells (von Both et al., 2004). *Tbx1*, a member of T-box transcription factor family, is a putative causative gene for DiGeorge syndrome associated with an interstitial deletion of chromosome 22q11 in human, and has been implicated in the development of the pharyngeal arch arteries and the OFT septation (Merscher et al., 2001; Vitelli et al., 2002). A recent study using conditional *Tbx1* mutants indicates that Tbx1 is required for cell proliferation in the second heart field through Fgf10 signaling (Xu et al., 2004).

Cardiac NCCs originating from rhombomeres 6, 7, and 8 in the dorsal neural tube have been shown to migrate into the heart through the third, fourth, and sixth pharyngeal arches. While this cell population forms the tunica media of all the great arteries, a subpopulation of this cell type further migrates into the OFT cushions by embryonic day 10.5 (E10.5) in the mouse and then participates in the septation of the OFT into the aorta and pulmonary trunk from E11.5 to E13.5 (Epstein et al., 2000; Gitler et al., 2003a; Hutson and Kirby, 2003). Cardiac NCCs are also indirectly involved in multiple processes of the developing heart (Hutson and Kirby, 2003; Jiang et al., 2000; Waldo et al., 1998). In particular, ablation of the cardiac neural crest in the chick results in primary myocardial dysfunction and the failure to elongate the OFT myocardium derived from the second heart field (Yelbuz et al., 2002, 2003), possibly because the cardiac neural crest alters the availability of FGF8 in the pharynx (Farrell et al., 2001). Recent studies further support the importance of the cardiac NCCs on the addition of the OFT myocardium, although they do not affect smooth muscle differentiation in the OFT (Waldo et al., 2005a,b).

The proepicardium (PE), another extracardiac cell population, is initially formed as a mesothelial primordium located on the pericardial side of the septum transversum, and cells from the PE migrate into the heart in which they form the epicardium. Some of the epicardial cells subsequently undergo a process of epithelial–mesenchymal transformation (EMT) to become epicardium-derived cells (EPDCs), which subsequently differentiate into endothelial and smooth muscle cells of coronary blood vessels and cardiac fibroblasts, and also provide signaling molecules to the ventricular myocardium (Kirby, 2002; Manner et al., 2001; Munoz-Chapuli et al., 2002; Olivey et al., 2004; Perez-Pomares et al., 2002a; Reese et al., 2002). For example, *WT* (*Wilms*' *tumor*)*-1* transcription factor is expressed in the epicardium and mutant mice for *WT-1* display abnormalities in the epicardium formation with a reduced number of subepicardial mesenchymal cells (Moore et al., 1999). However, the precise molecular and cellular mechanisms underlying epicardial EMT are still poorly understood.

We and others have previously shown that murine *Foxc1* and *Foxc2*, closely related forkhead/Foxc transcription factors, are implicated in cardiovascular development (Iida et al., 1997; Kume et al., 2001; Seo et al., 2006; Winnier et al., 1999; Yamagishi et al., 2003). *Foxc1* and *Foxc2* have similar expression patterns in many embryonic tissues, including endothelial and mesenchymal cells of the developing heart and blood vessels (Hiemisch et al., 1998; Iida et al., 1997; Kume et al., 2001; Seo et al., 2006; Winnier et al., 1999). *Foxc1* and *Foxc2* are also expressed in the ectomesenchyme and endocardial cushions derived from cardiac NCCs (Gitler et al., 2003a; Winnier et al., 1999). Moreover, we show in this paper that transcripts of *Foxc1* and *Foxc2* are detected in the second heart field at the cardiac crescent stage (E7.75 in mouse), as well as in the PE. Most compound *Foxc1*; *Foxc2* heterozygotes, as well as all single homozygotes, die pre- and perinatally with a similar spectrum of cardiovascular defects such as the interruption/coarctation of the aortic arch, ventricular septal defects (VSDs), hypoplasia of semilunar valves, and thin myocardium (Winnier et al., 1999). Compound *Foxc1*; *Foxc2* homozygous mutants die much earlier with much more severe cardiovascular defects than single *Foxc* null mutants (Kume et al., 2001), suggesting that they have overlapping, dose-dependent roles during cardiovascular development. However, cooperative functions of *Foxc* genes in the early development of the heart, including their role in the second heart field, remain to be elucidated. Here, we demonstrate that compound *Foxc1*; *Foxc2* mutant embryos have numerous cardiac abnormalities, including hypoplasia of the OFT. Therefore, we propose that