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Reassessment of *Isl1* and *Nkx2-5* cardiac fate maps using a *Gata4*-based reporter of Cre activity

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ABSTRACT

Isl1 and *Nkx2-5*-expressing cardiovascular progenitors play pivotal roles in cardiogenesis. Previously reported Cre-based fate-mapping studies showed that *Isl1* progenitors contribute predominantly to the derivatives of the second heart field, and *Nkx2-5* progenitors contributed mainly to the cardiomyocyte lineage. However, partial recombination of Cre reporter genes can complicate interpretation of Cre fate-mapping experiments. We found that a *Gata4*-based Cre-activated reporter was recombined by $Isl1^{Cre}$ and $Nkx2\text{-}5^{Cre}$ in a substantially broader domain than previously reported using standard Cre-activated reporters. The expanded *Isl1* and *Nkx2-5* cardiac fate maps were remarkably similar, and included extensive contributions to cardiomyocyte, endocardial, and smooth muscle lineages in all four cardiac chambers. These data indicate that *Isl1* is expressed in progenitors of both primary and secondary heart fields, and that *Nkx2-5* is expressed in progenitors of cardiac endothelium and smooth muscle, in addition to cardiomyocytes. These results have important implications for our understanding of cardiac lineage diversification in vivo, and for the interpretation of Cre-based fate maps.

Introduction

The mature heart is mainly comprised of cells belonging to cardiomyocyte, endothelial, and smooth muscle lineages. The diversification of these lineages from progenitor cells has become an area of intensive investigation (reviewed in Bruneau and Black, 2007). Progress has been driven by two major approaches. One approach has been to determine the developmental fate of progenitor cells in vivo. In mammals this is most commonly achieved by expression of Cre recombinase in progenitor cells. Cells descended from these progenitors are heritably and irreversibly marked by recombination of Cre-activated reporter genes (Soriano, 1999). Using this approach, *Isl1* progenitors were found to contribute to cardiomyocyte, smooth muscle cells (SMCs), and endothelial cells (ECs) (Moretti et al., 2006). *Isl1*-marked cells contributed extensively to right ventricle (RV), outflow tract (OT), and atria, with a reduced contribution to left ventricle (LV) (Cai et al., 2003; Yang et al., 2006; Sun et al., 2007). This has been a key observation supporting the existence of two distinct cardiac progenitor populations, one that gives rise to left ventricle (first heart field or FHF), and one that gives rise to right ventricle (RV), outflow tract (OT), and atria (second heart field or SHF) (Buckingham et al., 2005). Using a similar fate-mapping strategy, *Nkx2-5*-expressing progenitors were shown to primarily give rise to the cardiomyocyte lineage, with an additional but infrequent contribution to the endothelial lineage (Moses et al., 2001; Stanley et al., 2002).

A second approach has been to analyze the in vitro differentiation potential of cardiac progenitors, isolated by their expression of marker genes such as *Isl1* or *Nkx2-5*. Consistent with in vivo fate mapping, these in vitro studies showed that $Isl1^{+}$ progenitors differentiated into cardiomyocyte, SMC, and EC lineages (Moretti et al., 2006), and that $Nkx2\text{-}5^{+}$ progenitors differentiated into cardiomyocytes (Wu et al., 2006). However, gaps between in vivo fate-mapping and in vitro differentiation studies remain. Fate-mapping studies did not demonstrate descent of most LV cardiomyocytes from $Isl1^{+}$ progenitors. *Nkx2-5*-expressing progenitors differentiated into SMCs in addition to cardiomyocytes in vitro (Wu et al., 2006), but an SMC fate for *Nkx2-5* cells has not been noted in vivo.

Different floxed loci exhibit differential susceptibility to Cre recombination (Novak et al., 2000; Vooijs et al., 2001). Incomplete recombination of Cre-dependent reporters has the potential to significantly influence Cre-based fate-mapping experiments. We recently described a *Gata4*-based reporter, $Gata4^{flap}$, that was more susceptible to Cre recombination than a *Rosa26*-based reporter (Zhou et al., 2008). Because *Gata4* is expressed in the major lineages of the developing and mature heart (Fig. 1 and Heikinheimo et al., 1994), this *Gata4*-based reporter can be used to report on the cardiac fates of Cre-expressing precursors. Using $Gata4^{flap}$, we showed that *Isl1* and *Nkx2-5*-expressing progenitors contribute extensively to the

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proepicardium. Here, we use this reporter to reassess the cardiac fates of *Isl1* and *Nkx2-5* progenitors. We found that the cardiac fate maps of *Isl1* and *Nkx2-5* are significantly broader that previously reported (Moses et al., 2001; Stanley et al., 2002; Cai et al., 2003; Yang et al., 2006; Sun et al., 2007). These results have important implications for our understanding of lineage diversification in the developing heart, and for the interpretation of recombinase-based fate-mapping experiments.

Materials and methods

Mice

Gata4^{flox}, *Gata4*^{flap}, *Rosa26*^{fsLz}, *EIIaCre*, *Tie2Cre*, *Nkx2-5*^{Cre}, *MHCαCre*, and *Isl1*^{Cre} mice were described previously (Lakso et al., 1996; Agah et al., 1997; Mao et al., 1999; Kisanuki et al., 2001; Moses et al., 2001; Pu et al., 2004; Yang et al., 2006; Zhou et al., 2008). We note that the *Rosa26*^{fsLz} mouse line of Mao et al. is distinct from a similar line described in Soriano (1999). Mice were used according to protocols approved by the Institutional Animal Care and Use Committee.

Histological analysis

Detection of β-galactosidase and human placental alkaline phosphatase (AP) was performed as described (Lobe et al., 1999). AP activity was visualized with either BCIP/NBT (purple; Roche) or Permanent Red (red; Dako). For fluorescent imaging, Permanent Red was detected with Cy5 filters. Isl1, Tnnt, desmin, smooth muscle actin, and PECAM antibodies were from Iowa Developmental Hybridoma Bank, Neomarkers, Biomedia, Sigma, and BD Biosciences, respectively.

Results

We previously described generation of a *Gata4*-based Cre reporter, *Gata4*^{flap}, in which Cre-mediated *Gata4* inactivation is coupled with expression of the reporter gene alkaline phosphatase (AP) (Zhou et al., 2008). *Gata4*^{flap} contains a loxP-*Gata4* cDNA-transcriptional stop-loxP cassette followed by an AP cDNA at the endogenous *Gata4* start codon (Fig. 1a). Prior to Cre-mediated recombination, endogenous *Gata4* regulatory elements drive transcription of *Gata4* cDNA. *Gata4*$^{flap/flap}$ mice were viable and fertile, indicating that the *Gata4* cDNA functionally replaced *Gata4* expression from the native gene. Cre recombinase excises the *Gata4* cDNA and transcriptional stop signal, permitting expression of AP under control of endogenous *Gata4* regulatory elements. In the absence of Cre, no AP activity was detected (Fig. 1b). Germline Cre recombination by *EIIaCre* generated *Gata4*^{AP} mice, which expressed AP in most cells of the developing and adult heart, including cardiomyocytes, SMCs, and ECs (Figs. 1c–d). Cardiac and extracardiac AP expressions (Figs. 1 and S1–S2) were consistent with previously reported expression of *Gata4* (Arceci et al., 1993; Heikinheimo et al., 1994; Rivera-Feliciano et al., 2006), indicating that *Gata4*^{AP} faithfully reports on *Gata4* expression. Because *Gata4* is expressed in most cells of the heart, within this domain *Gata4*$^{flap/+}$ can be used to report on tissue-specific Cre activity. Tissue specific expression of Cre recombinase by *Tie2Cre*, *MHCαCre*, and *cTNTCre* transgenes selectively activated *Gata4*^{flap} in ECs and cardiomyocytes, respectively, matching previously reported patterns of Cre activity driven by these transgenes within the heart (Figs. 1e–g and Zhou et al., 2008). Collectively, these data validate the *Gata4*^{flap} allele.

Different genetic loci are known to vary widely in their susceptibility to Cre-mediated recombination (Novak et al., 2000; Vooijs et al., 2001). We found that *Gata4*^{flap} was more susceptible to Cre recombination than *Rosa26*^{fsLz} (Fig. S3; $p<0.001$). This increased sensitivity permitted detection of an expanded contribution of *Isl1*$^{+}$ and *Nkx2-5*$^{+}$ progenitors to the proepicardium (Zhou et al., 2008). We therefore investigated whether or not the *Gata4*^{flap}-based cardiac fate maps of *Isl1*^{Cre} or *Nkx2-5*^{Cre} would differ from those previously reported (Moses et al., 2001; Stanley et al., 2002; Cai et al., 2003; Yang et al., 2006; Sun et al., 2007).

Extensive contribution of *Isl1*$^{+}$ progenitors to FHF and SHF derivatives of the fetal and postnatal heart

We used *Gata4*^{flap} to determine the sites where *Isl1*^{Cre} recombined *Gata4*, comparing the results to a second Cre-activated reporter, *Rosa26*^{fsLz} (Mao et al., 1999). Consistent with previous reports (Cai et al., 2003; Moretti et al., 2006), *Isl1*^{Cre} efficiently recombined *Rosa26*^{fsLz} in cardiomyocytes, ECs, and endocardial-cushion mesenchyme of OT and RV, but did not efficiently recombine *Rosa26*^{fsLz} in LV or atrioventricular (AV) endocardial cushions (Fig. 2a; Table 1). However,

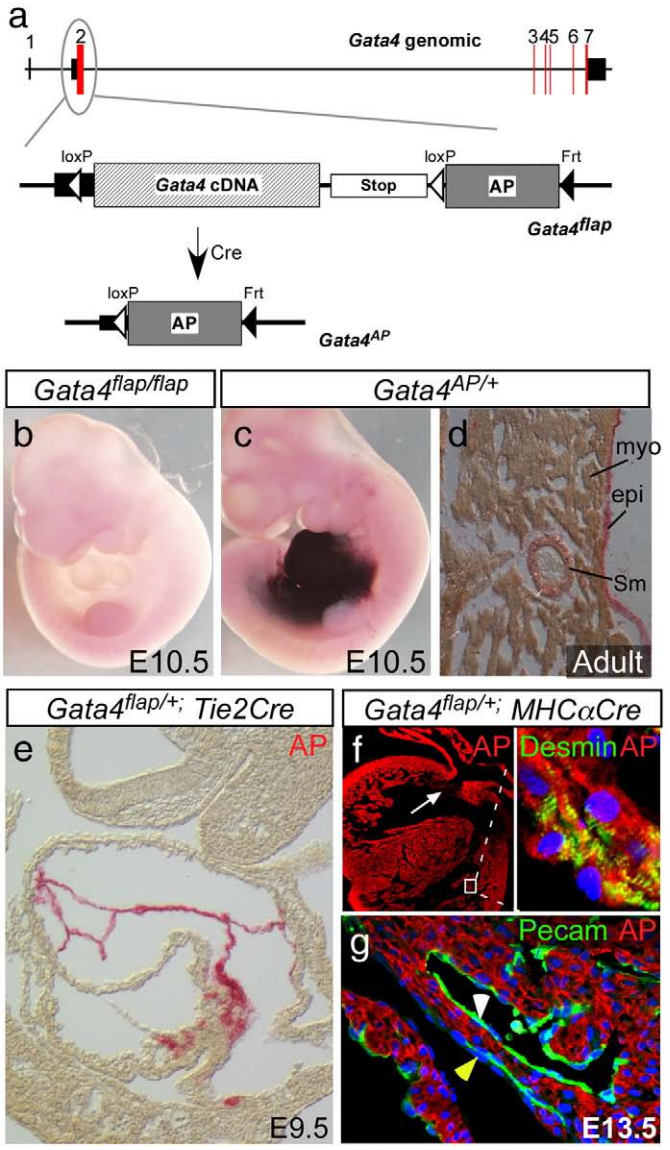

Fig. 1. *Gata4*^{flap}, a reporter of cardiac Cre activity. (a) Schematic depicting the structure of the *Gata4* genomic locus, and the knockin *Gata4*^{flap} allele. Red boxes indicate coding regions, and black boxes untranslated regions. AP, human placental alkaline phosphatase. (b) Lack of AP activity in Gata4$^{flap/flap}$ embryos. (c) AP activity in *Gata4*- embryo. (d) AP expression in *Gata4*$^{AP/+}$ adult heart. (e) *Tie2Cre* activated *Gata4*^{flap} in the endocardium but not myocardium. (f–g) *MHCαCre* recombined *Gata4*^{flap} selectively in cardiomyocytes (Myo). Cells of the endocardium (white arrowhead, g), epicardium (yellow arrowhead, g) and endocardial cushions (white arrow, f) were not recombined. AP was detected with BCIP/NBT in panels b, c and Permanent Red in panels d–g.

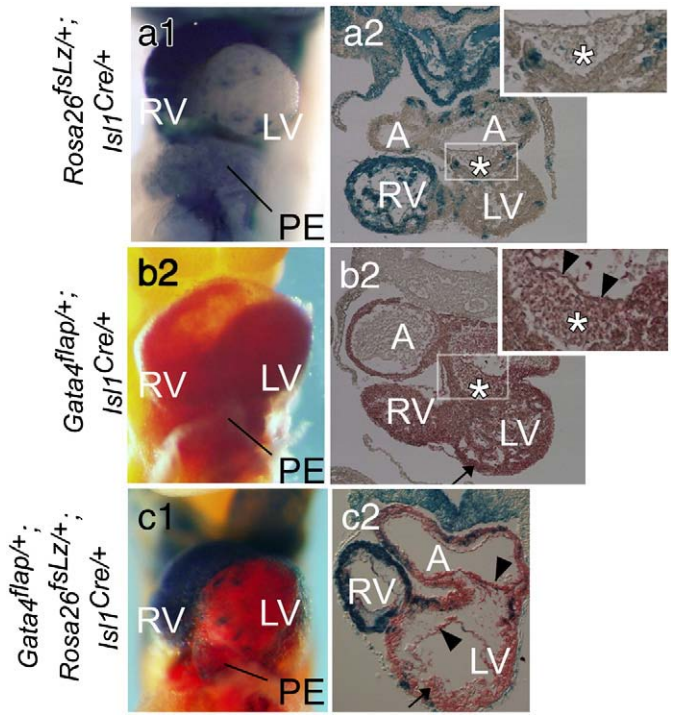

Fig. 2. Developmental fates of *Isl1*$^{+}$ progenitors in the developing heart. E9.5 *Rosa26*$^{fsLz/+}$; *Isl1*$^{Cre/+}$ (a), *Gata4*$^{flap/+}$; *Isl1*$^{Cre+}$ (b), and *Gata4*$^{flap/+}$; *Rosa26*$^{fsLz/+}$; *Isl1*$^{Cre/+}$ (c) embryos were stained for β-galactosidase (blue, a), AP (red, b), or both (blue and red, c; overlap appears as dark blue). Embryos were then transversely sectioned. Note that *Gata4*^{flap} was activated much more extensively than *Rosa26*^{fsLz} in LV and AV cushion (asterisk and inset, a2 vs b2). Black arrowheads indicate endocardium marked by *Isl1*^{Cre}. Arrows indicate LV myocardium, which strongly expressed AP but only expressed LacZ in a mosaic pattern. Abbreviations are as in Fig. 1.

the *Gata4*^{flap}-based fate map was strikingly different (Fig. 2b; Table 1). *Isl1*^{Cre} efficiently recombined *Gata4*^{flap} in LV, as well as in RV and OT. In addition, *Isl1*^{Cre} efficiently recombined *Gata4*^{flap}, but not *Rosa26*^{fsLz}, in proepicardium and in ECs and mesenchymal cells of the AV endocardial cushions (Figs. 2a–b; Table 1).

We considered the possibility that genetic differences, such as *Gata4* genotype or strain background, may have led to divergent results. We compared *Isl1*^{Cre} fate maps for *Gata4*^{flap} and *Rosa26*^{fsLz} reporters in the same embryo (Fig. 2c). The differences between the two reporters persisted within the same embryo, excluding genetic differences as the cause of divergent *Gata4*^{flap} and *Rosa26*^{fsLz} fate maps.

We also considered whether differences in reporter gene promoter activity or AP versus LacZ detection thresholds led to the more extensive *Gata4*^{flap} fate map. Cre recombination acts as a binary on/off switch, while the reporter gene promoter determines the strength of reporter gene expression. Therefore, both possibilities can be tested by examining reporter gene expression after germline reporter gene activation by *EIIaCre* (Lakso et al., 1996). Germline recombination of both *Gata4*^{flap} and *Rosa26*^{fsLz} resulted in strong

Table 1
Recombination domains of *Isl1*^{Cre} and *Nkx2-5*^{Cre}, as determined by *Rosa26*^{fsLz} and *Gata4*^{flap} Cre-activated reporters

Structure	Location	Lineage	*Isl1*^{Cre}		*Nkx2-5*^{Cre}	
			Rosa26^{fsLz}	*Gata4*^{flap}	*Rosa26*^{fsLz}	*Gata4*^{flap}
Myocardium	A	CM	+	++++	++++	++++
		EC	+/−	++++	−	++++
	LV	CM	+	++++	++++	++++
		EC	+/−	++++	−	++++
	RV	CM	++++	++++	++++	++++
		EC	+++	++++	−	++++
AV Canal		CM	+/−	++++	++++	++++
		EC	+/−	++++	−	++++
		Mes	+/−	++++	−	++++
Outflow Tract		CM	++++	++++	++++	++++
		EC	+++	++++	−	++++
		Mes	++++	++++	−	++++
Proepicardium			+	++++	+	++++
Coronary		SMC	ND	+++	ND	+++
		EC	ND	+++	ND	++

A, atria; CM, cardiomyocyte; LV, (left ventricle) RV, (right ventricle) Mes, mesenchyme. Frequency of Cre-marked cells was qualitatively scored from "−" (no contribution detected) to "++++" (nearly all cells marked). ND, not determined.

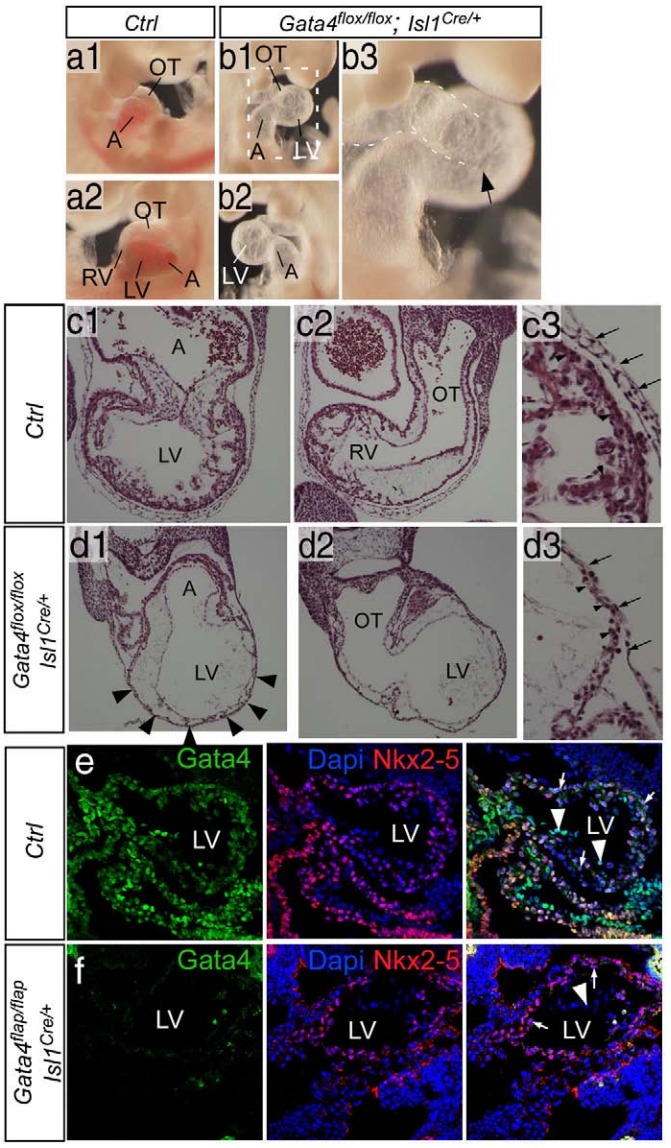

Fig. 3. Inactivation of *Gata4* by *Isl1*^{Cre}. (a–b) Gross morphology of control and mutant embryos at E10.5. Mutant embryos were growth retarded and often had large pericardial effusions (not shown). Removal of the pericardial sac showed a bulbous and translucent appearing LV, and absence or severe hypoplasia of the RV (arrow, b3). (c–d) Histological transverse sections of E10.5 control and mutant embryos. The mutant embryos had severe thinning of the LV myocardium and absence of myocardial trabeculation (arrowheads, d1). The morphological RV was severely hypoplastic (not shown) to absent (d2). *c3–d3* show higher magnification of LV myocardium. Arrows indicate pericardium, arrowheads LV myocardium. A, atrium. LV and RV, left and right ventricle. OT, outflow tract. (e–f) Loss of *Gata4* immunoreactivity in *Isl1*^{Cre}-recombined mutant LV. Sagittal frozen sections of control and *Gata4*$^{flap/flap}$; *Isl1*$^{Cre/+}$ mutant E9.5 littermate hearts. *Gata4* was expressed in cardiomyocytes (arrows) and endothelium (arrowheads) of control LV. In *Gata4*$^{flap/flap}$; *Isl1*$^{Cre/+}$ hearts, *Gata4* immunoreactivity was not detected in either cardiomyocytes or endothelium of LV. *Nkx2-5* expression was unchanged in the mutant, demonstrating preservation of antigen integrity in mutant as well as control samples.

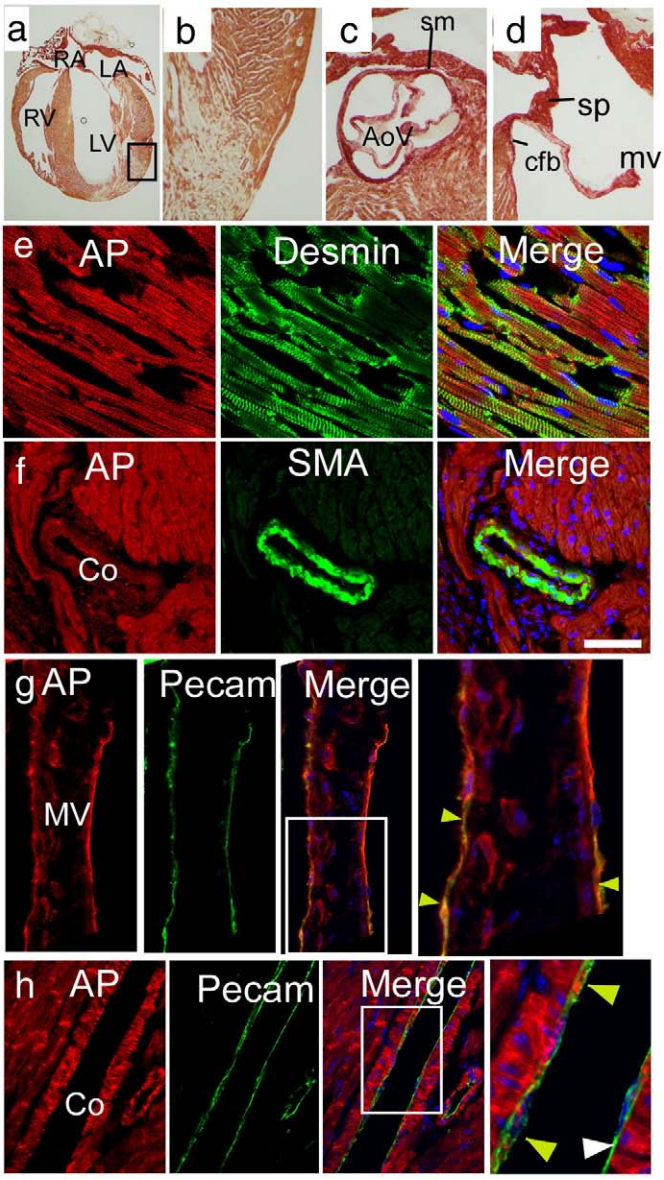

Fig. 4. Developmental fates of *Isl1* progenitors in the postnatal heart. Frozen sections of postnatal *Gata4*$^{flap/+}$; *Isl1*$^{Cre/+}$ hearts, stained for AP activity (red) and lineage markers. *Isl1*^{Cre} activated *Gata4*^{flap} in cardiomyocytes (a–b), in the base of the aorta and in the semilunar valves (c), and in derivatives of the AV endocardial cushions (d). AP activity colocalized with markers of cardiomyocytes (desmin, e), coronary SMCs (smooth muscle α-actin (SMA), f), and valve and coronary ECs (Pecam, yellow arrowheads, g and h). Coronary ECs were mosaic for AP staining (white arrowheads indicate Pecam$^+$ cells that were AP negative). AoV, aortic valve. cfb, central fibrous body. co, coronary. mv, mitral valve. sm, smooth muscle. sp, atrial septum primum.

expression of both reporters throughout the heart, including LV (Fig. S4) (Mao et al., 1999; Soriano, 1999; Stanley et al., 2002). These data indicate that when activated both reporters provide a robust signal throughout the heart.

The divergent fate maps reported by *Rosa26*^{fsLz} and *Gata4*^{flap} led us to examine the fate of *Isl1*$^+$ progenitors with a third Cre-activated reporter, Z/Red (Vintersten et al., 2004), in which the strong CAG promoter drives expression of red fluorescent protein (RFP) after Cre recombination. *Isl1*^{Cre} efficiently activated RFP expression in OT myocardium, less efficiently in RV myocardium, and rarely in LV myocardium (Fig. S5). Thus, each of the three Cre reporters yielded distinct *Isl1* fate maps. These data reinforce the conclusion that recombinase-based fate maps require a nuanced interpretation that considers the properties of the Cre-dependent reporter used (see Discussion).

Having established that *Gata4*^{flap} is extensively recombined throughout the heart by *Isl1*^{Cre}, we next examined the phenotype resulting from *Gata4* knockout by *Isl1*^{Cre}. *Isl1*^{Cre} inactivation of *Gata4*^{flap} or an independently constructed *Gata4* conditional allele, *Gata4*^{flox} (Pu et al., 2004), resulted in similar phenotypes (Figs. 3 and S6). These embryos had markedly hypoplastic to absent RVs (Figs. 3a–b) and severe thinning of the LV myocardium (Figs. 3c–d). There was a paucity of myocardial trabeculation. This phenotype was highly reminiscent of hearts in which *Gata4* was inactivated throughout the myocardium by *Nkx2-5*^{Cre} (Zeisberg et al., 2005). Consistent with extensive *Gata4* recombination by *Isl1*^{Cre} in LV, Gata4 protein was not detectable in *Gata4*$^{flap/flap}$; *Isl1*$^{Cre/+}$ LV endocardium or cardiomyocytes (Figs. 3e–f). *Gata4* loss of function did not lead to ectopically increased *Isl1*^{Cre} activity in LV, because *Rosa26*^{fsLz} remained largely inactive in *Gata4*$^{flap/flap}$; *Rosa26*^{fsLz}; *Isl1*$^{Cre/+}$ LV (Fig. S7). In contrast to *Gata4* inactivation by *Isl1*^{Cre}, a different, SHF-restricted Cre transgene based on a Mef2c enhancer selectively inactivated *Gata4* in RV and OFT (Rojas et al., 2008). This impaired RV/OFT development, but spared the LV, suggesting that abnormal LV development following *Gata4* inactivation by *Isl1*^{Cre} was unlikely to be an indirect consequence of *Gata4* inactivation in SHF. Together, these data indicate that *Isl1*^{Cre} efficiently knocked out *Gata4* throughout the derivatives of both primary and secondary heart fields and suggest that caution should be exercised when using *Isl1*^{Cre} to achieve gene inactivation restricted to the second heart field.

Given the expanded fate map of *Isl1*$^+$ progenitors in the embryonic heart demonstrated by *Gata4*^{flap}, we re-examined the fate map of *Isl1*$^+$ progenitors in the postnatal heart (Fig. 4; Table 1). In the absence of Cre, we did not detect AP activity in postnatal *Gata4*$^{flap/+}$ hearts (data not shown). In *Gata4*$^{flap/+}$; *Isl1*$^{Cre/+}$ hearts, most cardiomyocytes were marked by *Isl1*^{Cre}, although there was a region of LV apex that was mosaic for AP expression (Figs. 4a–b). This was not due to mosaic *Gata4* promoter activity, because *Gata4*^{AP} was expressed by all cardiomyocytes (data not shown). Most likely, *Isl1*-driven Cre expression in progenitors of these cells was insufficient for recombination of *Gata4*^{flap}.

Isl1^{Cre} labeled smooth muscle at the base of the aorta (Fig. 4c) and in the coronary arteries (Fig. 4f). Septum primum, the crux of the heart, and portions of the heart valves, all derivatives of the

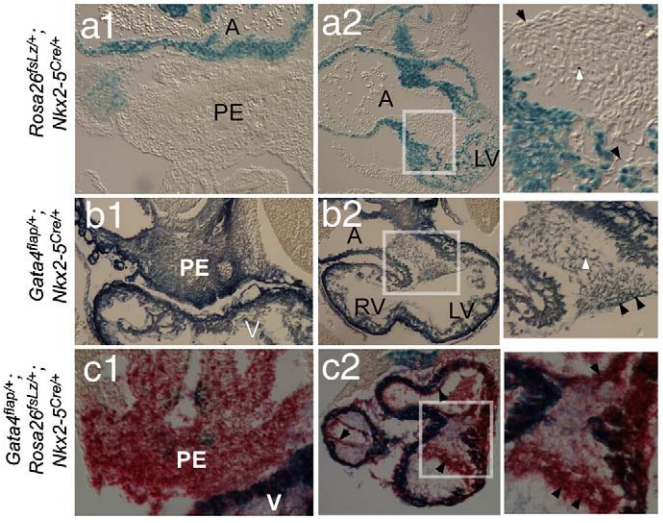

Fig. 5. Developmental fates of *Nkx2-5*$^+$ progenitors in the developing heart. Transverse paraffin sections of embryos (a–b, E10.0; c, E9.5) stained in whole mount for LacZ (blue) or AP (BCIP/NBT in b, Permanent Red in c). Boxed regions of AV endocardial cushions are enlarged in right panels. *Nkx2-5*^{Cre} activated both reporters in cardiomyocytes (dark blue). *Nkx2-5*^{Cre} activation of *Gata4*^{flap} was markedly more efficient than *Rosa26*^{fsLz} in endocardium (black arrowhead), endocardial-cushion mesenchyme (white arrowhead), and proepicardium (PE).

endocardial cushions, were efficiently recombined by *Isl1*^{Cre} in the mature heart (Figs. 4c–d), consistent with *Isl1*^{Cre} labeling of the cushions of the fetal heart (Fig. 1). Chamber and valve endocardium were likewise marked by *Isl1*^{Cre} (Fig. 4g), while coronary ECs expressed AP in a mosaic pattern (Fig. 4h).

Nkx2-5 progenitors contribute extensively to cardiomyocyte, smooth muscle, and endothelial lineages

Nkx2-5 has also emerged as an important marker of multipotent cardiac progenitor cells (Kattman et al., 2006; Moretti et al., 2006; Wu et al., 2006). Consistent with previous reports (Moses et al., 2001; Stanley et al., 2002), *Nkx2-5*^{Cre} activated *Rosa26*^{fsLz} predominantly in cardiomyocytes (Fig. 5a; Table 1). *Rosa26*^{fsLz} was not efficiently activated in endocardium or endocardial-cushion mesenchyme. However, studies have suggested that *Nkx2-5* progenitors have the potential to differentiate into a broader set of lineages than reported by Cre labeling using *Rosa26*^{fsLz} (Moretti et al., 2006; Wu et al., 2006). Therefore, we used *Gata4*^{flap} to reassess the fate of *Nkx2-5*-expressing cells. *Gata4*^{flap} revealed a considerably broader *Nkx2-5*^{Cre} fate map. *Nkx2-5*^{Cre} efficiently recombined *Gata4*^{flap} in endocardium and endocardial-cushion mesenchyme, as well as in cardiomyocytes (Fig. 5b; Table 1). *Nkx2-5*^{Cre} also recombined *Gata4*^{flap}, but not *Rosa26*^{fsLz}, in proepicardium (Figs. 5a–b). These findings were not due to genetic differences, because *Gata4*^{flap} and *Rosa26*^{fsLz} reporters yielded divergent results within the same embryo (Fig. 5c).

Next, we reanalyzed the contribution of *Nkx2-5*$^{+}$ progenitors to lineages of the adult heart. (Fig. 6; Table 1). *Nkx2-5*^{Cre} marked virtually all cardiomyocytes (Figs. 6a–e). Smooth muscle of the coronary arteries and the base of the aorta were labeled (Figs. 6c and 6f). Chamber and valve endocardium and coronary endothelium were also marked by *Nkx2-5*^{Cre} (Figs. 6g–h). The heart valves and endocardial-cushion-derived portions of the atrial and ventricular septae were labeled (Figs. 6c–d and 6g), consistent with the extensive contribution of *Nkx2-5*$^{+}$ progenitors to the fetal endocardial cushions (Figs. 5b–c). These data indicate that *Nkx2-5*$^{+}$ progenitors contribute to cardiomyocyte, SMC, and EC lineages of the developing and postnatal heart.

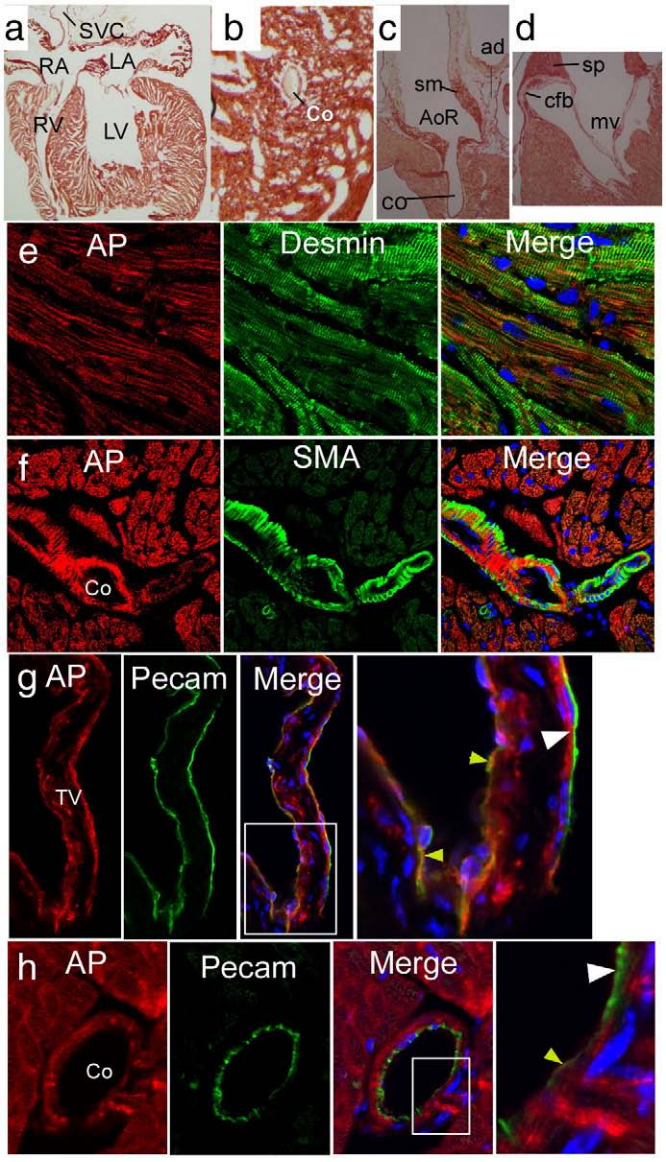

Fig. 6. Developmental fates of *Nkx2-5*$^{+}$ progenitors in the postnatal heart. *Gata4*^{flap} fate map of *Nkx2-5* in adult heart. Frozen sections were stained for AP activity (red) and lineage markers. *Nkx2-5*^{Cre} activated *Gata4*^{flap} in cardiomyocytes (a–b), in the base of the aorta and in the semilunar valves (c), and in derivatives of the AV endocardial cushions (d). AP activity colocalized with markers of cardiomyocytes (e), coronary smooth muscle (f), valve endocardium (yellow arrowheads, g), and coronary ECs (yellow arrowheads, h). Endothelium was mosaic for AP staining (white arrowheads indicate Pecam^{+} cells that were AP negative). ad, adventitial tissue around aortic root, AoR, aortic root, tv, tricuspid valve. Other abbreviations as in Fig. 4.

Discussion

Interpretation of recombinase-based fate-mapping experiments

Recombinase-based fate mapping has become an important strategy for defining progenitor–descendant relationships in mammalian development. However, interpretation of such experiments is complex. Our data demonstrate that susceptibility of the Cre-dependent reporter to recombination significantly influences Cre-based fate-mapping results. While Cre is expressed in temporally and spatially graded patterns, activation of a Cre-dependent reporter is a binary readout in which progenitors surpassing a Cre exposure threshold become activated. The specific threshold depends on the Cre-dependent reporter and the cellular context. The importance of this thresholding effect on Cre-dependent reporter readouts is illustrated in Fig. S8. Reporters that are more susceptible to recombination reveal a broader fate map that includes progenitors with lower level or transient Cre expression, while less sensitive reporters reveal a more restricted fate map that corresponds to progenitors with higher level or duration of Cre expression. An important implication is that lack of Cre reporter activation must be interpreted carefully, because this does not exclude Cre expression in progenitors at levels below the threshold required for reporter recombination. Conversely, the thresholding that occurs in Cre fate-mapping experiments can obscure biologically relevant differences in the level of gene expression, and this may become more problematic with extremely sensitive Cre-dependent reporters.

Our fate-mapping results with *Isl1*^{Cre} and *Z/Red*, *Rosa26*^{fsLz}, and *Gata4*^{flap} reporters illustrate the importance of the Cre reporter in determining the fate map. A likely explanation to the divergent fate maps obtained with these reporters is that more sensitive reporters are activated by lower levels or shorter durations of Cre exposure. In LV progenitors, where *Isl1* expression is likely most transient, only *Gata4*^{flap} was efficiently activated. In RV progenitors, with likely intermediate duration of *Isl1* expression, *Gata4*^{flap} and *Rosa26*^{fsLz} were efficiently activated, while *Z/Red* was not. In OT, with the greatest duration of *Isl1* expression (Sun et al., 2007), all three reporters were efficiently activated. In this model, each reporter delineates the fate of *Isl1*-expressing progenitors, but the fate maps differ by the threshold level of *Isl1* expression that triggered a positive readout. While the variable readout of different Cre-activated reporters has been noted

previously (Novak et al., 2000; Vooijs et al., 2001), our data emphasize the substantial impact this may have on the outcome of fate-mapping experiments.

Contribution of Isl1$^+$ and Nkx2-5$^+$ progenitors to the developing heart

Isl1 and *Nkx2-5* have emerged as important markers of cardiac progenitor cells (Bruneau and Black, 2007). The contribution of these progenitors to the developing heart has been determined primarily by fate mapping using Cre-activated reporters. We reassessed the fate map of *Isl1* and *Nkx2-5*-expressing progenitors using a Cre-activated reporter based on *Gata4*, a gene expressed natively in the principal lineages of the heart. The *Gata4^{flap}*-based fate maps, summarized in Table 1, demonstrated that *Isl1* and *Nkx2-5* progenitors make substantially greater contributions to the developing and postnatal heart than previously described: (1) *Isl1$^+$* progenitors contribute extensively to cardiomyocytes, SMCs (base of aorta; coronaries), and ECs (coronary, chamber, and valve endothelium) of FHF as well as SHF derivatives; (2) *Nkx2-5$^+$* progenitors contribute significantly to SMCs (base of aorta; coronaries) and ECs (coronary, chamber, and valve endothelium), in addition to cardiomyocytes; (3) *Isl1$^+$* and *Nkx2-5$^+$* progenitors contribute extensively to development of both the AV and OT cushions, and to the mature heart valves; (4) *Isl1$^+$* and *Nkx2-5$^+$* progenitors contribute extensively to proepicardium and its derivatives, the coronary vasculature. These fate-mapping data reconcile differences between in vivo fate-mapping and in vitro differentiation studies, putting the in vitro findings in the context of heart development in the intact embryo.

The fate map of *Isl1$^+$* progenitors was initially studied with an *Isl1$^{IRES\text{-}Cre}$* knockin, and showed substantial contribution of *Isl1$^+$* progenitors to RV, OT, and atria, with a minor contribution to LV (Cai et al., 2003). Subsequent reanalysis using *Isl1^{Cre}* (no IRES) and *Isl1^{MerCreMer}* demonstrated somewhat increased contribution of *Isl1$^+$* progenitors to LV, but was interpreted to be consistent with the *Isl1$^{IRES\text{-}Cre}$* fate map (Yang et al., 2006; Sun et al., 2007). These data suggested that FHF and SHF progenitors differ by their expression of *Isl1*, supporting the hypothesis that FHF and SHF progenitors are distinct. On the other hand, previous reports have described *Isl1* expression in precardiac mesoderm (Yuan and Schoenwolf, 2000; Brade et al., 2007; Prall et al., 2007), the presumptive location of FHF progenitors. However, at present *Isl1* expression in FHF progenitor cells cannot be definitively demonstrated, because specific markers of this population are lacking. Our fate-mapping data, based on *Isl1^{Cre}* (no IRES) and *Gata4^{flap}*, resolve this discrepancy between *Isl1* expression and *Isl1* fate, and indicate that LV progenitors, like RV/OT/atrial progenitors, express *Isl1*. The phenotype of *Isl1* null embryos, which have severe underdevelopment of the residual (left) ventricle in addition to aberrant RV/OT morphogenesis (Cai et al., 2003), suggests that *Isl1* expression in LV precursors is functionally significant. Whether the heart originates from two progenitor pools with distinct molecular signatures, or a single progenitor pool that differentiates in temporally and spatially distinct manners into right and left heart structures, remains controversial (Abu-issa et al., 2004; Moorman et al., 2007). Our results indicate that Isl1 is expressed in both left and right heart precursors, and therefore Isl1 expression does not qualitatively distinguish SHF and FHF progenitors.

Precardiac mesoderm contains precursors of both endocardial and myocardial lineages (Linask and Lash, 1993; Sugi and Markwald, 1995). Whether these lineages arise from a common precursor in vivo is not known. In vitro studies identified a multipotent progenitor marked by expression of *Isl1* and *Nkx2-5* that could differentiate into cardiomyocyte, endothelial, and smooth muscle lineages (Moretti et al., 2006; Wu et al., 2006). Our data are consistent with these studies, and indicate that most endocardial and myocardial cells of the developing heart arise from progenitors that express *Nkx2-5* and *Isl1*. The contribution of *Isl1*-or *Nkx2-5*-expressing progenitors to the endothelial lineage was noted previously (Stanley et al., 2002; Moretti et al., 2006; Sun et al., 2007), although the extent of contribution appeared to be less than noted in this study. Transient *Nkx2-5* expression in endocardial progenitors might be functionally significant for endocardial differentiation, as suggested by the failure of *Nkx2-5* knockout endocardium to subspecialize to form endocardial cushions (Tanaka et al., 1999).

We found that smooth muscle at the base of the aorta, and in the wall of some large and small coronary vessels, derives from *Nkx2-5$^+$* and *Isl1$^+$* progenitors. *Isl1$^+$* progenitors were previously noted to contribute to the smooth muscle lineage in vivo (Moretti et al., 2006; Sun et al., 2007). Most smooth muscle within the myocardium originates from precursors located in the proepicardium (Wilm et al., 2005). Because prior fate-mapping studies did not detect *Isl1^{Cre}* marking of the proepicardium, it was concluded that the *Isl1^{Cre}*-marked smooth muscle population arose from a distinct source. However, our fate-mapping studies indicate that *Isl1^{Cre}* efficiently labels the proepicardium (Fig. 2 and Zhou et al., 2008), suggesting that the *Isl1*-derived smooth muscle likely arises from a proepicardial intermediate. A smooth muscle fate of *Nkx2-5$^+$* progenitors was not previously noted in vivo, but is consistent with the differentiation potential of *Nkx2-5$^+$* progenitors observed in vitro (Wu et al., 2006). However, not all coronary endothelium or smooth muscle was labeled by *Nkx2-5^{Cre}* or *Isl^{1Cre}*. Whether this mosaicism reflects heterogeneous origins of coronary vascular cells, or incomplete Cre-marking, is unclear.

We observed a broad overlap of *Nkx2-5* and *Isl1* fate maps. This extensive overlap was reflected in the similarity of mutant embryos in which *Gata4* was inactivated by *Nkx2-5^{Cre}* or *Isl1^{Cre}*. The overlapping fate maps suggest that most cells of the mature heart traverse a developmental pathway that includes at least transient expression of both *Nkx2-5* and *Isl1*. Based on these data, one might speculate that the multipotent *Isl1$^+$/Nkx2-5$^+$* progenitor recently identified by Moretti et al. (2006) represents a common cardiac progenitor of cardiomyocyte, SMC, and EC lineages in all four cardiac chambers.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2008.08.013.

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