Developmental Biology 323 (2008) 98–104

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/developmentalbiology

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

Qing Ma [a,b,1], Bin Zhou [a,b,1], William T. Pu [a,b,*]

[a] Department of Cardiology, Children's Hospital Boston, 300 Longwood Ave, Boston, MA 02115, USA
[b] Harvard Stem Cell Institute, 42 Church Street, Cambridge MA 02138, USA

ARTICLE INFO

Article history:
Received for publication 15 June 2008
Revised 8 August 2008
Accepted 9 August 2008
Available online 22 August 2008

Keywords:
Fate map
Cre reporter
Secondary heart field
Cardiogenesis
Cardiac progenitor

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

* Corresponding author. Department of Cardiology, Children's Hospital Boston, 300 Longwood Ave, Boston, MA 02115, USA. Fax: +1 617 730 0140.
E-mail address: wpu@enders.tch.harvard.edu (W.T. Pu).
[1] These authors contributed equally to this work. Their authorship positions are interchangeable.

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doi:10.1016/j.ydbio.2008.08.013

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,

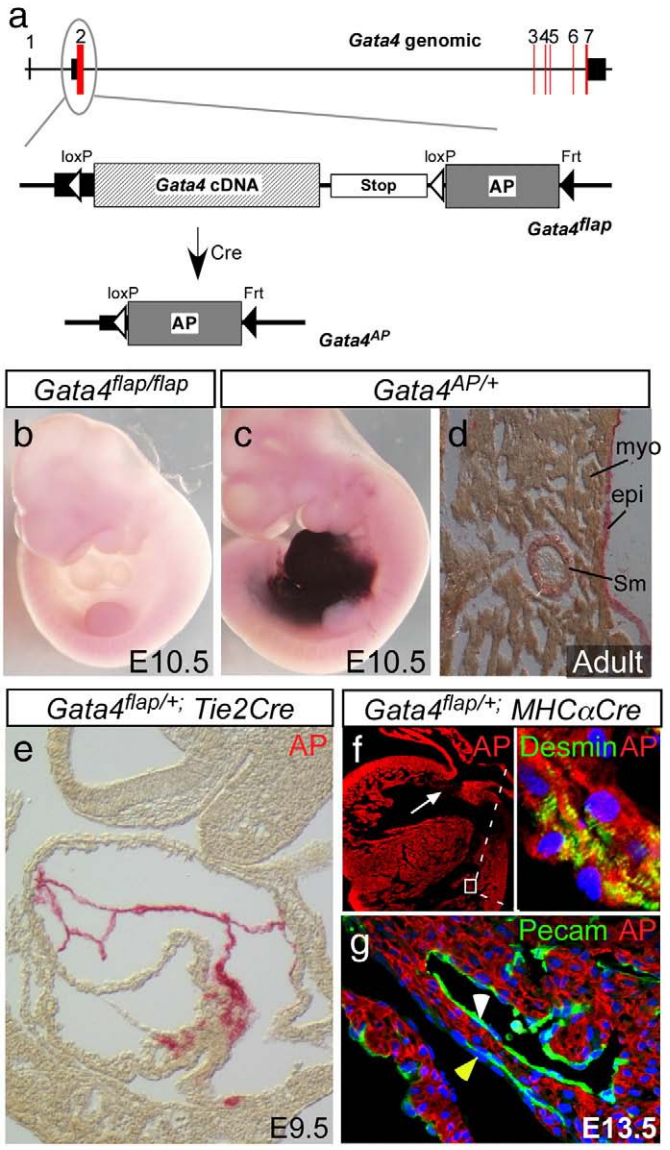

Fig. 1. *Gata4*^{flap}, a reporter of cardiac Cre activity. (a) Schematic depicting the structure of the *Gata4* genomic locus, and the knockin *Gata4*^{flap} allele. Red boxes indicate coding regions, and black boxes untranslated regions. AP, human placental alkaline phosphatase. (b) Lack of AP activity 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.