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An early requirement for *nkx*2.5 ensures the first and second heart field ventricular identity and cardiac function into adulthood

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ABSTRACT

Temporally controlled mechanisms that define the unique features of ventricular and atrial cardiomyocyte identity are essential for the construction of a coordinated, morphologically intact heart. We have previously demonstrated an important role for *nkx* genes in maintaining ventricular identity, however, the specific timing of *nkx*2.5 function in distinct cardiomyocyte populations has yet to be elucidated. Here, we show that heat-shock induction of a novel transgenic line, *Tg(hsp70l:nkx2.5-EGFP)*, during the initial stages of cardiomyocyte differentiation leads to rescue of chamber shape and identity in *nkx*2.5$^{-/-}$ embryos as chambers emerge. Intriguingly, our findings link an early role of this essential cardiac transcription factor with a later function. Moreover, these data reveal that *nkx*2.5 is also required in the second heart field as the heart tube forms, reflecting the temporal delay in differentiation of this population. Thus, our results support a model in which *nkx* genes induce downstream targets that are necessary to maintain chamber-specific identity in both early- and late-differentiating cardiomyocytes at discrete stages in cardiac morphogenesis. Furthermore, we show that overexpression of *nkx*2.5 during the first and second heart field development not only rescues the mutant phenotype, but also is sufficient for proper function of the adult heart. Taken together, these results shed new light on the stage-dependent mechanisms that sculpt chamber-specific cardiomyocytes and, therefore, have the potential to improve *in vitro* generation of ventricular cells to treat myocardial infarction and congenital heart disease.

Introduction

Mutations in *NKX*2-5 are associated with a myriad of congenital heart diseases (CHD) in humans (Benson et al., 1999; Elliott et al., 2003; Jay et al., 2003; McElhinney et al., 2003; Schott et al., 1998). Investigation of the underlying molecular and cellular basis of CHD in model systems has yielded insights into the functions of *Nkx*2-5 in progenitor specification in *Drosophila*, *Xenopus*, and mouse (Azpiazu and Frasch, 1993; Bodmer, 1993; Grow and Krieg, 1998; Prall et al., 2007) and in cardiac morphogenesis in mouse and zebrafish (Lyons et al., 1995; Prall et al., 2007; Tanaka et al., 1999a; Targoff et al., 2008; Tu et al., 2009). Through recent identification of null mutations in *nkx*2.5 and *nkx*2.7, two *Nkx*2-5 homologs expressed in zebrafish cardiomyocytes (Chen and Fishman, 1996; Lee et al., 1996), novel roles in maintaining cardiac chamber identity have also been revealed (Targoff et al., 2013). Furthermore, in postnatal hearts, transcriptional regulation by *Nkx*2-5 has been shown to be important in preserving highly differentiated cardiomyocyte properties and in controlling the cardiac gene program of the adult myocardium (Akazawa and Komuro, 2003, 2005; Takimoto et al., 2000). Despite an appreciation of these early and late roles of *Nkx* genes, their temporal requirement during cardiac development in safeguarding chamber-specific characteristics of differentiated cardiomyocytes has yet to be illuminated. Moreover, while the influence of specific signaling pathways during unique phases in cardiac morphogenesis has been uncovered (de Pater et al., 2009; Dohn and Waxman, 2012; Marques et al., 2008), rarely have the temporally coordinated functions of a cardiac transcription factor such as *nkx*2.5 been dissected with precision.

Innovative strategies for directing differentiation of pluripotent progenitors could benefit from insights regarding the timing of *nkx* genes in establishing specific ventricular and atrial cellular traits. Currently, protocols to convert ES and iPS cells into cardiomyocytes are being developed with improved rates of efficiency (Braam et al., 2009; Bu et al., 2009; Domian et al., 2009; Hansson et al., 2009; Lundy et al., 2013; Mercola et al., 2013; Murry and Keller, 2008; Yang et al., 2008). Yet, a central challenge for these techniques is the ability to favor differentiation of ventricular myocytes as opposed to

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mixed populations. Recently, novel approaches in regenerative medicine have enhanced the production of functional ventricular heart muscle through selection of progenitors expressing *Nkx*2-5 (Domian et al., 2009). Furthermore, there is evidence that Nkx2-5 participates in sub-type specific 'forward programming' of pluripotent stem cells towards a differentiated ventricular population (David et al., 2009). Given these recent advances, examination of the temporally controlled mechanisms mediated by *nkx* genes may help to generate improved protocols for *in vitro* production of ventricular cardiomyocytes for novel models of human cardiac disease and regeneration.

Timing is also relevant to our appreciation of the etiologies of congenital heart defects in humans given the importance of sequential differentiation of the first heart field (FHF) and the second heart field (SHF) (Bruneau, 2008; Nakano et al., 2008; Srivastava and Olson, 2000). *Nkx*2-5 is expressed in FHF and SHF of mouse and zebrafish embryos (Guner-Ataman et al., 2013; Stanley et al., 2002) and mutations in both lineages result in CHD (Lyons et al., 1995; Prall et al., 2007; Tanaka et al., 1999a). Different *Nkx*2-5 enhancer regions have also been shown to regulate gene expression in a temporally dynamic manner (Tanaka et al., 1999b). Furthermore, recent studies in mouse have highlighted the key roles of *Nkx*2-5 in orchestrating transitions between cardiac specification, proliferation, and morphogenesis in FHF and SHF populations (Prall et al., 2007). While *Nkx*2-5 expression begins in the cardiac progenitors of both heart fields and persists throughout embryogenesis into adulthood (Kasahara et al., 1998; Komuro and Izumo, 1993; Lints et al., 1993; Stanley et al., 2002), the specific temporally defined requirements of *Nkx*-dependent processes remain obscure. When are *Nkx* genes essential for developmental progression of cardiomyocyte fate and for insuring long-standing molecular signatures of the ventricle and atrium? Uncovering answers to these questions regarding the timing of *Nkx* gene function will enhance the improvement of therapeutic efforts *in vitro* and *in vivo*.

Our previous work in zebrafish revealed essential roles for *nkx*2.5 and *nkx*2.7 in limiting atrial cell number, promoting ventricular cell number, and preserving chamber-specific identity in differentiated myocardium (Targoff et al., 2013, 2008). From these studies, the initial manifestation of the *nkx*2.5$^{-/-}$;*nkx*2.7$^{-/-}$ phenotype following heart tube formation suggests a late requirement for *nkx* genes in chamber identity maintenance. Therefore, to dissect the early (prior to heart tube formation) and late (after heart tube formation) functions of *nkx* genes, we systematically evaluated the influence of timing of *nkx* expression on cardiac chamber formation and preservation of identity using a novel transgenic line, *Tg*(*hsp*70*l:nkx*2.5-*EGFP*). Remarkably, we found that *nkx*2.5 activity is necessary early during cardiac progenitor differentiation to maintain ventricular and atrial chamber morphology and cellular traits later in development. This newly defined temporal relationship broadens our appreciation of the initial roles of *nkx* genes, coupling an early necessity with a later function of chamber identity maintenance. Furthermore, we demonstrate that the temporal requirement for *nkx* genes in SHF cardiomyocytes is shifted later in development, emphasizing the delayed specification and differentiation of this population (de Pater et al., 2009; Hami et al., 2011; Lazic and Scott, 2011; Zhou et al., 2011). Interestingly, our studies also reveal that early re-expression of *nkx*2.5 in *nkx*2.5$^{-/-}$ embryos is adequate to maintain a functional cardiac rescue through adulthood. In summary, our data provide insights into the mechanisms responsible for initiation and maintenance of chamber identity *in vivo* which have the potential to translate into discoveries of novel paradigms for directed differentiation of ventricular and atrial cardiomyocytes *in vitro*, ultimately facilitating a greater understanding of congenital heart disease and myocardial repair.

Methods

Zebrafish

We used zebrafish carrying the following previously described mutations and transgenes: *nkx*2.5^{vu179} (Targoff et al., 2013), *nkx*2.7^{vu413} (Targoff et al., 2013), and *Tg*(-5.1*myl*7:*nDsRed*2)^{f2} (Mably et al., 2003). To produce a novel transgene expressing the fusion protein Nkx2.5-EGFP driven by a heat-shock promoter, we employed the Gateway system (Kwan et al., 2007; Villefranc et al., 2007). Through Tol2 transposase-mediated transgenesis (Fisher et al., 2006), we generated stably integrated transgenic lines carrying *Tg*(*hsp*70*l:nkx*2.5-*EGFP*). We examined 2 independent integrants and found functional rescue of the *nkx*2.5$^{-/-}$ phenotype in each case. Propagation of one line, *Tg*(*hsp*70*l:nkx*2.5-*EGFP*)^{fcu1}, was performed for future work. In this study, experiments were implemented with one transgenic fish per cross unless otherwise specified. For analyses of adult zebrafish, hearts were collected, dissected, and morphometric analysis was performed as previously described (Singleman and Holtzman, 2012). All zebrafish work followed Institutional Animal Care and Use Committee (IACUC)-approved protocols.

In situ hybridization

We conducted whole-mount *in situ* hybridization as previously described (Yelon et al., 1999) with the following probes: *myl*7 (ZDB-GENE-991019-3), *vmhc* (ZDB-GENE-991123-5), *amhc* (*myh*6; ZDB-GENE-031112-1), and *nkx*2.5 (ZDB-GENE-980526-321).

Immunofluorescence

Whole-mount immunofluorescence was performed with variations of a published protocol (Alexander et al., 1998), using primary monoclonal antibodies against sarcomeric myosin heavy chain (MF20) and atrial myosin heavy chain (S46). MF20 and S46 were obtained from the Developmental Studies Hybridoma Bank maintained by the Department of Biological Sciences, University of Iowa, under contract NO1-HD-2–3144 from the NICHD. In embryos, the secondary reagents, goat anti-mouse IgG1 Alexa Fluor 488 and goat anti-mouse IgG2b Alexa Fluor 568 (Invitrogen), were used to recognize MF20 and S46, respectively. In adults, zebrafish hearts were incubated in 10 ug/ml Proteinase K (Roche) and blocked overnight before proceeding with the standard immunofluorescence protocol.

For the developmental timing assay, a modified version of a previously described protocol was employed using embryos carrying *Tg*(-5.1*myl*7:*nDsRed*2) (de Pater et al., 2009). Sequential immunostaining was performed with S46 and goat anti-mouse IgG1 Alexa Fluor 488, then with MF20 and goat anti-mouse IgG Cy5 (Invitrogen). Visualization of DsRed was performed without immunofluorescence to detect transgenic expression levels.

Genotyping

PCR genotyping was performed on genomic DNA extracted from individual embryos following *in situ* hybridization, immunofluorescence, or live imaging. Detection of *nkx*2.5^{vu179} was executed using primers 5′–TCACCTCCACACAGGTGAAGATCTG-3′ and 5′–CAGAAAGATGAATGCTGTCGGT-3′ to generate a 443 bp fragment. Primer placement in the 3′–UTR was chosen specifically to amplify the endogenous *nkx*2.5 allele as opposed to the transgene, *Tg*(*hsp*70*l:nkx*2.5-*EGFP*). Digestion of the mutant PCR product with Hinf1 creates 207 bp, 162 bp, 49 bp, and 25 bp fragments. Analysis of *nkx*2.7^{vu413} was performed using primers 5′–CTTTTTCAGGCATGTGTCCA-3′ and 5′–AAAGCGTCTTTCCAGCTCAA-3′ to generate a 146 bp fragment. Digestion of the mutant PCR product with MseI

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