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Proliferation of embryonic cardiomyocytes in zebrafish requires the sodium channel *scn5Lab*

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Summary

In mice, homozygous deletion of the cardiac sodium channel *Scn5a* results in defects in cardiac morphology and embryonic death before robust sodium current can be detected. In zebrafish, morpholino knockdown of cardiac sodium channel orthologs *scn5Laa* and *scn5Lab* perturbs specification of pre-cardiac mesoderm and inhibits growth of the embryonic heart. It is not known which developmental processes are perturbed by sodium channel knockdown and whether reduced cell number is from impaired migration of cardiac progenitors into the heart, impaired myocyte proliferation, or both. We found that embryos deficient in *scn5Lab* displayed defects in primary cardiogenesis specific to loss of *nkx2.5*, but not *nkx2.7*. We generated kaede reporter fish and demonstrated that embryos treated with anti-*scn5Lab* morpholino showed normal secondary differentiation of cardiomyocytes at the arterial pole between 30 and 48 hours post-fertilization. However, while proliferating myocytes were readily detected at 48 hpf in wild type embryos, there were no BrdU-positive cardiomyocytes in embryos subjected to anti-*scn5Lab* treatment. Proliferating myocytes were present in embryos injected with anti-*tnnt2* morpholino to phenocopy the *silent heart* mutation, and absent in embryos injected with anti-*tnnt2* and anti-*scn5Lab* morpholinos, indicating cardiac contraction is not required for the loss of proliferation. These data demonstrate that the role of *scn5Lab* in later heart growth does not involve contribution of the secondary heart field, but rather proliferation of cardiomyocytes, and appears unrelated to the role of the channel in cardiac electrogenesis.

Keywords

heart; development; sodium channel; myocardium

INTRODUCTION

Voltage-gated sodium channels are key regulators of cardiac electrical activity. In addition, a body of evidence strongly supports the notion that these channels play an indispensable role in cardiac development. In mice, homozygous deletion of the cardiac sodium channel gene *Scn5a* results in death at 10.5 days of embryonic gestation, before robust sodium current can be detected (Davies et al., 1996; Papadatos et al., 2002). These embryos have small ventricles, lacking trabeculation, but with intact endocardial tissues and vasculature (Papadatos et al., 2002).

The zebrafish genome includes two *Scn5a* orthologs, with similar sequence homology to *Scn5a*: *scn5Laa* (*scn12aa*) and *scn5Lab* (*scn12ab*) (Novak et al., 2006; Chopra et al., 2010). Development of the zebrafish heart begins early in embryogenesis, as fate-mapping reveals regions of blastomeres destined to the cardiac lineage as early as 5 hours post-fertilization (hpf) (Keegan et al., 2004). By 12 hpf, the cardiac-specification gene *nkx2.5* identifies bilateral stripes of cardiac progenitors in the anterior lateral plate mesoderm (Chen and Fishman, 1996). Zebrafish in which either *scn5Laa* or *scn5Lab* has been knocked down exhibit defects in initial specification of pre-cardiac mesoderm, shown by marked reduction in early expression of *nkx2.5* (Chopra et al., 2010). The NK2 homeobox genes *nkx2.5* and *nkx2.7* (Lee et al., 1996) have similar but not completely redundant functions in zebrafish heart tube elongation and ventricular growth. Other studies have demonstrated morpholino knockdown of *nkx2.5* alone has the mild phenotype of a slightly smaller ventricular chamber (Targoff et al., 2008), while knockdown of *nkx2.7* results in failure of the developing heart to loop correctly (Tu et al., 2009). Both studies observed dramatic phenotype on double knockdown, including reduced ventricular chamber size, impaired looping and weaker contractions (Targoff et al., 2008; Tu et al., 2009). It is unknown whether *nkx2.7* is affected by *scn5Lab* knockdown.

In a wildtype zebrafish, the bilateral stripes of *nkx2.5*⁺ precursors fuse along the midline and will form a beating heart tube at 24 hpf (Stainier, 2001), and by 36 hours post-fertilization, the atrium and ventricle can be easily distinguished by light microscopy (Westerfield, 2000; Stainier, 2001). The heart tube in zebrafish deficient in *scn5Lab* has significantly fewer cardiomyocytes than in the wildtype zebrafish at 24 hpf (Chopra et al., 2010). As development proceeds, morphant zebrafish possess visibly smaller hearts, fewer ventricular cardiomyocytes, and the heart fails to loop and develop properly (Chopra et al., 2010). The embryos develop pericardial edema and die by 5 days post-fertilization (dpf) (Chopra et al., 2010).

The initial cardiomyocyte population of the heart tube at 24 hpf differentiates from mesodermal precursors in the lateral plate mesoderm (Serbedzija et al., 1998; Stainier, 2001). Recent work has identified a second heart field in zebrafish (Hami et al., 2011), containing an initially extra-cardiac population of atrial and ventricular myocyte progenitors. The venous and arterial poles of the heart are largely comprised of cardiomyocytes from this late-differentiating (24-36 hpf) population of precursors, which expresses the early heart-field marker *nkx2.5* (Zhou et al., 2011) as well as other cardiac-specific genes (Lazic and Scott, 2011). Among other pathways, Fgf signaling within this region has been demonstrated as critical to the differentiation and development of outflow tract myocardium (de Pater et al., 2009). By 48 hpf, differentiation of myocardium is largely complete, and further increases in cardiomyocyte number are a result largely of cellular proliferation (Chernyavskaya et al., 2012).

Our group has previously shown that *scn5Lab* is required for early specification of pre-cardiac mesoderm, and that this effect appears unrelated to the channel's role in electrogenesis. During the early stages of cardiac development, expression of *scn5Lab* has been detected by *in situ* hybridization (Novak et al., 2006; Chopra et al., 2010), but has been demonstrated to not contribute to action potential generation (Chopra et al., 2010). Here, we test the competing hypotheses that impaired cardiac growth and decreased cardiomyocyte number in *scn5Lab*-deficient embryos are due to impaired secondary differentiation, or arrested proliferation and growth of previously-differentiated cardiomyocytes.

RESULTS

***scn5Lab* morphant hearts acquire reduced numbers of cardiomyocytes**

Zebrafish deficient in *scn5Lab* develop with visibly smaller, un-looped hearts, slow heart rates, and pericardial edema. To determine if reduced heart size resulted from fewer cells, we counted cardiomyocytes at each stage of early heart development. Beginning with initial peristaltic contractions of the wildtype heart tube at 24 hpf, uninjected and morpholino-injected embryos were formaldehyde-fixed at 12 hour-increments and immunofluorescence was performed to identify dsRed⁺ cardiomyocytes. Compared to uninjected wildtype embryos, zebrafish deficient in *scn5Lab* (morphants) had significantly reduced numbers of cardiomyocytes at 24 hpf (Fig. 1a,e,i; 129.2 ± 2.4 vs. 83.6 ± 6.1 , $n=6,7$). Following initial differentiation and establishment of the heart tube, wildtype zebrafish embryos continued to add cardiomyocytes throughout the second day (24-48 hpf) of development (Fig. 1a-c,i). During this same period, however, morphant hearts also continued to grow (Fig. 1e-g,i), with a similar increase in cardiomyocyte number as the uninjected embryos. After 48 hpf, uninjected and morphant hearts again diverged in cardiomyocyte number, as wildtype hearts continued to acquire dsRed⁺ cells (Fig. 1d,i), while morphant hearts failed to add a similar number of cardiomyocytes (Fig. 1h,i). Additionally, wildtype hearts underwent normal cardiac looping (Fig. 1d) while adding myocytes, whereas morphant hearts remained un-looped or partially looped (Fig. 1g,h).

***scn5Lab* is required for specific expression of *nkx2.5* but not *nkx2.7* in primary cardiac differentiation**

We have previously demonstrated a requirement for voltage-gated sodium channels in expression of *nkx2.5* in the anterior lateral plate mesoderm (Chopra et al., 2010). However the observed cardiac phenotype in *scn5Lab*-morphant embryos is more suggestive of the double *nkx2.5/nkx2.7* knockdown. To investigate the specificity of the role for *scn5Lab* in expression of selected cardiac transcription factors and determine if other cardiac specification pathways were affected by loss of *scn5Lab*, we performed *in situ* hybridization for *nkx2.5* and *nkx2.7* in 12 hpf embryos. At this stage, *nkx2.5* expression in the anterior lateral plate mesoderm is nearly absent in *scn5Lab*-morphant embryos (Chopra et al., 2010) compared to wildtype (Fig. 2a-b). However, embryos from the same clutch showed normal *nkx2.7* expression in both wildtype and morpholino-injected embryos (Fig. 2c-h). This finding suggests the role for *scn5Lab* in expression of early cardiac transcription factors is specific for *nkx2.5*, or its upstream mediators, rather than playing a more general role in which many cardiac transcription factors would be affected by its absence. Additionally, differentiated cardiomyocytes in the heart tube at 24 hpf displayed normal expression of *nkx2.5* (Fig. 2c-d), suggesting the role for *scn5Lab* in expression of *nkx2.5* is limited to the specification of pre-cardiac mesoderm. Given the severe phenotype suggestive not only of loss of *nkx2.5*, but of additional gene expression defects, we attempted to rescue the morphant phenotype with microinjection of *nkx2.5* mRNA. Embryos injected with mRNA alone developed with slightly enlarged hearts (Fig. 3a-c), as expected from previous reports (Chen and Fishman, 1996). However, embryos injected with *scn5Lab* active morpholino and *nkx2.5* mRNA (Fig. 3f) displayed no rescue of the morphant phenotype (Fig. 3d,e), suggesting other cardiac specification genes are affected by *scn5Lab* knockdown. Thus, while *nkx2.5* is an important readout of impaired cardiac specification in *scn5Lab* morphants, other unidentified pathways contribute to the phenotype as well.

Secondary cardiomyocyte differentiation is normal in *scn5Lab* morphant embryos

Loss of gastrula-stage expression of *nkx2.5* helps to explain a decrease in differentiated cardiomyocytes in the zebrafish heart tube at 24 hpf. To determine if *scn5Lab* is required for late differentiation of myocardium, we generated the Tg(*cmlc2:kaede*) line as previously

reported (de Pater et al., 2009); this line and others used in these studies are described in further detail in Methods below. *Cmlc2*⁺ myocytes (positive for the cardiac marker *cmlc2*) expressing the green fluorescent Kaede at 30 hpf were permanently photo-converted to red fluorescence, marking the initial phase of cardiac differentiation. As embryos continued to develop, both primary and secondary cardiomyocytes expressed green (new) Kaede, marking primary cardiomyocytes as both red and green fluorescent, while secondary myocytes expressed green Kaede only. Using this approach, we found that the extent of secondary differentiation at the arterial pole between 30 and 48 hpf was similar in uninjected wildtype (Fig. 4a) and *scn5Lab* morphant (Fig. 4b) embryos. Photo-conversion at 30 hpf followed by imaging at 48 hpf displayed in clustering of FITC⁺, CY3⁻ myocytes at the ventricular outflow tract in wildtype, as expected based on previous studies of secondary differentiation (de Pater et al., 2009; Lazic and Scott, 2011; Zhou et al., 2011), and this was unchanged in morphants.

Morphant embryos add normal number of cells to the arterial pole between 30 and 48 hpf

To test the hypothesis that differentiation of outflow tract myocardium proceeds normally in embryos deficient in *scn5Lab*, we quantitated secondary differentiation in wildtype and morphant ventricles. Stable transgenic zebrafish tg(*UAS:Kaede*) were crossed with a cardiac-specific driver line tg(*cmlc2:Gal4*) to generate robust expression of Kaede protein. Embryos were photo-converted as above at 30 hpf and then allowed to grow to 48 hpf and fixed. Embryos were stained with the nuclear marker TO-PRO-3-Iodide to quantitatively assay secondary cardiac differentiation at the arterial pole (Fig. 5a-e). There was no difference in the number of arterial pole myocytes differentiating between 30 and 48 hpf in wildtype versus morphant embryos (34.8 ± 1.3 vs. 33.1 ± 1.6 , $n = 8$, $p > 0.2$, Fig. 5f). The overall average number of new myocytes (34.1 ± 1.0 , $n = 18$) observed at this stage was consistent with previously published counts (Lazic and Scott, 2011). Thus, despite impaired early specification and differentiation of pre-cardiac mesoderm, *scn5Lab*-morphant embryos exhibited a normal secondary phase of cardiac differentiation. This result indicates perturbation of the second heart field is not responsible for sub-physiological number of ventricular myocytes in the embryos.

Ventricular cardiomyocyte proliferation is impaired in morphant embryos at 48 hpf

After 48 hpf, cardiac differentiation in the zebrafish is largely complete (Reifers et al., 2000; de Pater et al., 2009), and ventricle size and cell number increases predominantly through growth and proliferation of myocardium (Mably et al., 2006; Qu et al., 2008; Chernyavskaya et al., 2012). One potential contributor to the continued cardiomyocyte deficit in morphant embryos after initial heart tube formation is the loss of ability of differentiated cardiomyocytes to divide. To examine proliferation in the embryonic heart, we examined embryos exposed to BrdU for a five-hour window at 48-53 hpf, a time point reflecting completed secondary differentiation of myocardium. Embryos were formaldehyde-fixed, and immunofluorescence was performed, to evaluate mismatch and active morpholino-injected embryos for altered cellular proliferation. While both wildtype (5.8 ± 0.6 , $n = 14$) and mismatch (4.0 ± 0.9 , $n = 11$) (Fig. 6a-f, Fig. 7) hearts showed double positive (BrdU⁺, dsRed⁺) cardiomyocytes, there were no proliferating cardiomyocytes ($n = 12$, Kruskal-Wallis test $p < 10^{-5}$) from 48-53 hpf in morphant embryos (Fig. 6g-i, Fig. 7). The results indicate that proliferation of post-differentiation myocardium requires the voltage-gated sodium channel *scn5Lab*.

Impaired cardiac contractility is not responsible for impaired myocardial proliferation in *scn5La* morphants

To evaluate the role of cardiac contraction in growth and proliferation of cardiomyocytes, we phenocopied the *silent heart* mutant zebrafish line using previously described

morpholino against cardiac troponin (*tnnt2-MO*) (Arnaout et al., 2007). Despite complete loss of cardiac contraction, the hearts appeared to develop normally through 60 hpf development. Using morpholinos in the Tg(*cmlc2:dsRed-nuc*) line, we injected BrdU into the pericardium of the *tnnt2-MO*, *tnnt2-MO* + *scn5Lab*-mismatch (MIS), and *tnnt2-MO* + *scn5Lab*-morpholino (MO) embryos, and counted proliferating cardiomyocytes (Fig. 8). Proliferation appeared slightly reduced in *tnnt2-MO* compared to wildtype embryos (2.1 ± 0.5 vs 1.1 ± 0.3 , $n = 24, 22$, $p = 0.08$). Co-injection of *scn5Lab*-MIS with *tnnt2-MO* appeared to have no effect on proliferation vs. *tnnt2-MO* alone ($p = 0.69$); however, the effect of co-injection of active *scn5Lab* morpholino was identical to that observed in the non-silent heart fish: there was nearly complete absence of proliferation (1.2 ± 0.3 vs. 0.1 ± 0.1 , $n = 12, 10$, $p = 0.007$).

DISCUSSION

***scn5Lab* is required for zebrafish cardiogenesis at distinct phases of differentiation and proliferation**

The voltage-gated sodium channel *scn5Lab* is required for normal development of the heart in embryonic zebrafish. Development of the embryonic zebrafish heart, as with much of vertebrate organogenesis, undergoes distinct phases of specification, differentiation and proliferation to form and grow the functional organ (Shu et al., 2002; Tao and Peng, 2009). Prior studies provided evidence that *scn5Lab* is required for expression of *nkx2.5* in the specification of pre-cardiac mesoderm. Here we provide additional evidence that the morphant defect is specific to *nkx2.5* and not *nkx2.7*. The phenotype resulting from loss of *scn5Lab* during cardiac development is similar to that seen in the loss of both *nkx2.5* and *nkx2.7* (Tu et al., 2009). Altered anterior lateral plate mesoderm gene expression resulting from loss of *scn5La* may be a direct effect of *scn5La* on activating transcription factors of *nkx2.5*, or through a yet-undefined network regulating cardiac specification, of which *nkx2.5* is a small readout. Failure of *nkx2.5* mRNA to rescue the morphant phenotype, with normal expression of *nkx2.7*, suggests that *scn5Lab* is required for the function of other critical cardiac specification pathways earlier during development. In addition to a role for sodium channels in specification of pre-cardiac mesoderm, data here implicate *scn5Lab* in a later role in cardiac development, being required for proliferation of differentiated cardiomyocytes. We also demonstrate that late-differentiation of cardiomyocytes from an extra-cardiac second heart field-like population is unaffected by morpholino knockdown on *scn5Lab*.

***Scn5Lab* is required for development of normal numbers of primary but not secondary cardiac cardiomyocytes in the zebrafish**

Cardiomyocytes expressing markers of terminal differentiation form the heart tube at 24 hpf, completing the primary phase of cardiac differentiation. As we have previously shown and demonstrate here, embryos deficient in *scn5Lab* possess a significant deficit in cardiac cell number at this stage. A second phase of differentiation, induced by a separate set of controlling transcription factors, increases cardiac cell number by adding cells from the second heart field to the atrial and ventricular (outflow tract) poles. We initially hypothesized that impaired differentiation from the second heart field could be responsible for impaired ventricular growth, both in myocyte number and function (de Pater et al., 2009; Hami et al., 2011). This hypothesis was supported by prior evidence that *scn5Lab* is required for physiologic differentiation of the cardiac tube (Chopra et al., 2010). Interestingly, despite cardiac defects in primary differentiation, and later defects in cardiac growth, adequate secondary cardiomyocytes were added to the heart by 48 hpf by morphant embryos. In addition, the second heart field-derived myocytes were added to the distal third of the ventricle, appearing to form a normal functional outflow tract, as previously reported for

normal cardiogenesis (de Pater et al., 2009; Lazic and Scott, 2011). While *scn5Lab* is required for specification of cardiomyocytes forming the initial heart tube at 24 hpf, different factors likely govern the differentiation of later (second heart field) cardiomyocytes, a mechanism that is unaffected by morpholino knockdown of the channel.

Scn5Lab is required for proliferation of cardiomyocytes and maintenance of physiological ventricular cell number

By 36 hpf, both early and late differentiation are largely complete, and morphant and control embryos have added cardiomyocytes at the arterial pole of the ventricle. Evidence suggests the remaining cardiac growth occurs primarily through hypertrophy (Mably et al., 2006; Lin et al., 2012) and phases of increased and decreased proliferation (Qu et al., 2008; Chernyavskaya et al., 2012) contributing to looping and completing the form of the larval heart. At this stage, from 48 hpf onward, an additional deficit in cardiac cell number is revealed in morphant embryos, which are unable to continue cardiac growth. The data we present here support the hypothesis that loss of myocardial proliferation is an important contributing mechanism to an increasing cardiomyocyte deficit in morphant embryos. While adult myocardium is not considered a proliferative tissue at baseline, proliferation is an important facet of the development of the embryonic heart (Qu et al., 2008; Chernyavskaya et al., 2012), and regeneration of adult zebrafish cardiac tissue (Jopling et al., 2010). Importantly, proliferation and early growth of the heart does not appear dependent on myocardial contraction, as silent heart (*tnnt2*) mutants are able to grow normally in the absence of a heart beat (Sehnert et al., 2002), obtaining adequate perfusion of tissues through diffusion of oxygen from its aquatic environment.

A mutant zebrafish line with a silent ventricle, *tell tale heart* (Rottbauer et al., 2006) was reported to have identical cardiomyocyte numbers to wildtype fish through 72 hpf (Rottbauer et al., 2001). Data presented here suggest that while proliferation may be decreased in silent hearts, BrdU-positive cardiomyocytes can easily be detected at 48 hpf in *tnnt2*-MO injected embryos. By contrast, embryos injected with *scn5Lab*-MO showed a nearly total absence of detectable myocardial proliferation. This experiment provides further evidence that impaired growth of the zebrafish heart due to sodium channel knockdown is not simply a secondary phenotype from impaired cardiac contraction, but rather a defect specific to sodium channel knockdown. In addition, the observed morphant phenotype, including impaired heart tube differentiation and myocardial proliferation, occurs before observed phenotypes in the *silent heart* phenocopy line, suggesting the requirement for *scn5Lab* in heart development precedes the requirement for cardiac function in maturation of the myocardium. Action potentials in the developing heart of both zebrafish and mice are supported by depolarizing current through calcium channels, with voltage-gated sodium channel activity contributing only later in development (Davies et al., 1996; Rottbauer et al., 2001; Chopra et al., 2010). Whether or not the morphant phenotype arises from a loss of sodium ion permeation through *scn5Lab*, it is unlikely to be due to the role of Na_v1.5 in promoting action potentials responsible for cardiac contractility.

scn5Lab may influence cardiac growth independently of sodium ion permeation

Thus, the data we present above suggest the role for *scn5Lab* in cardiac development of zebrafish may be independent of its role in sodium permeation and action potential generation. Ion flux-dependent and independent roles ion channels in zebrafish have already been described (Kessler et al., 2012). Calcium ion signaling through the L-type calcium channel is critical for cardiac development in zebrafish independent of cardiac function (Rottbauer et al., 2001). Mutations in potassium channels on the other hand, present with an electrophysiology phenotype with no morphologic abnormalities (Kessler et al., 2012). Here, we have demonstrated a requirement for the voltage-gated sodium channel *scn5Lab* in

both specification of pre-cardiac mesoderm, and for proliferation and growth of the developing heart. Both developmental processes occur well before sodium ion influx has been observed to be required for action potential generation and propagation, at 4-5 dpf (Chopra et al., 2010). While the mechanism of the requirement for *scn5Lab* in proliferation of embryonic myocardium is unknown, evidence cited above indicates that this function is likely independent of the channel's contribution to the cardiac action potential, as even asystolic hearts display normal early development (Sehnert et al., 2002; Rottbauer et al., 2006). An alternative role promoting cellular proliferation is not without precedent for voltage-gated sodium channels. Ion channels are increasingly becoming implicated in tumor initiation and progression (Fiske et al., 2006). mRNA for Nav1.5 was recently shown to be significantly increased in some human ovarian cancers, with protein expression possibly correlated to grade and metastasis status of the tumor (Gao et al., 2010). Additionally, protein shown to bind to Nav1.5 in some cell lines, the sigma-1 receptor, has been shown to promote cancer proliferation and invasiveness (Balasuriya et al., 2012). Voltage-gated sodium channels are an integral component of the mature cardiomyocyte and as such could serve non-electrogenic roles such as acting as a critical checkpoint for myocardial division.

Conserved domains of the voltage-gated sodium channels may provide insight to its role in cellular proliferation

Voltage-gated sodium channels interact with multiple protein partners, and the c-terminus, highly conserved across orthologs including *scn5Lab*, is especially rich with predicted protein-binding motifs. The channels are well-characterized in their associations with β -subunits, molecules which not only modulate gating properties of sodium permeation, but serve cell-adhesion functions (Isom, 2001). A possible mechanism for the involvement in development is that through protein binding-partners, *scn5Lab* interacts with integral structural components of cardiomyocytes. For example, disrupted association of the SCN5A complex with the sarcomere protein syntrophin has been implicated in a variant of long QT syndrome (Ueda et al., 2008). Another possible mechanism involves participation, either directly or indirectly, of *scn5Lab* in signaling pathways critical to cardiac development. This is not unprecedented, as the C-terminus of the L-type voltage-gated calcium channel has been shown to function directly as a transcription factor in function and differentiation for neuronal cells (Gomez-Ospina et al., 2006). The zebrafish cardiac sodium channel *scn5Lab* possesses putative interaction domains for FHF1b, Nedd4-like ubiquitin ligases, and calmodulin, as well as an EF hand Ca^{2+} -binding motif (Abriel, 2010). Electrochemical signaling has also been demonstrated to activate transcription factors directly. For example, CREB (cAMP response element-binding protein) has been shown in neurons to be directly activated by calcium ion waves, rather than through calmodulin translocation to the nucleus (Hardingham et al., 2001). This mechanism seems unlikely, as defects in zebrafish and in mice appear to arise earlier than channel expression or function can be detected. Further, specific blockers of sodium permeation have no effect on lateral plate mesodermal expression of *nkx2.5* and early cardiac growth (Chopra et al., 2010).

Limitations

Zebrafish possess two isoforms of cardiac sodium channels, *scn5Laa* and *scn5Lab*. While these studies focus on the role for *scn5Lab* in cardiac development, there is evidence that *scn5Laa* is also important for cardiac development, in particular for expression of *nkx2.5* in the lateral plate mesoderm (Chopra et al., 2010). Just what role each channel plays, and why knockdown of either channel results in a similar early phenotype is a question that remains to be answered. It is possible that perturbed expression of one channel affects the expression of the other channel, making it difficult to elucidate the exact mechanisms through which these genes promote cardiac development. To date, expression of the full-length channel (nearly 2,000 amino acids) *in vivo* has not been accomplished, so rescue of the morphant

phenotype has yet to be accomplished using co-injection of mRNA. While mounting evidence suggests the role for *scn5Lab* (and *scn5Laa*) is independent of the channels' function in action potential propagation, mRNA rescue using a non-permeant mutated channel, could definitively answer the question.

Conclusion

We demonstrate that the cardiac sodium channel *scn5Lab* modulates the proliferation of the ventricle following the completion of differentiation, and this effect occurs before the channel assumes a prominent role in cardiac electrogenesis. Interestingly, despite a demonstrated requirement for *scn5Lab* in the expression of *nkx2.5* in primary specification pre-cardiac mesoderm, differentiation from the second heart field proceeds as normal in morphant embryos.

METHODS

Morpholinos

Previously validated (Chopra et al., 2010) active morpholino (AGTTTGTGCTGACCGGTGGTCTGGG, Gene Tools LLC) targeting the exon 25 splice site of *scn5Lab* was microinjected (1.8 ng) into the yolk of fertilized ova. Control experiments were conducted by injecting a 5-base mismatch (AcTTTcTGCTGAGCGGTGcTCTGcG), at the same injection volume (140 μ m droplet) and concentration (150 μ M) as active morpholino. Mismatch-morpholino injection had no detectable effect on cardiac development. Injections of antisense oligonucleotides can produce non-specific developmental effects, which can be largely mitigated using co-injection of anti-p53 morpholino (Robu et al., 2007). Accordingly, to limit toxicity, both active and mismatch morpholinos were co-injected with standard p53 morpholino (4.5 ng, Gene Tools LLC). As previously described, injection of the p53 morpholino had no detectable effect on morphant cardiac phenotype (Chopra et al., 2010). Translation of zebrafish cardiac troponin (*tnnt2*) was inhibited using previously validated morpholino (CATGTTTGTCTGATCTGACACGCA), at a dose of 4ng (Sehnert et al., 2002).

Zebrafish Lines Used

For counting and proliferation studies, we used zebrafish with a transgene for the red-fluorescent protein dsRed localized to the nucleus and driven by the cardiac myosin light chain promoter (*cmhc2:dsRed2-nuc*) (Mably et al., 2003). For qualitative secondary differentiation studies, we generated a line of zebrafish expressing Kaede under control of the cardiac-specific *cmhc2* promoter. The *Kaede* gene from *Trachyphyllia geoffroyi* is a photo-convertible fluorescent protein (), which upon exposure to ultraviolet (420nm) irradiation permanently converts from green (native) to red fluorescence (Ando et al., 2002; Sato et al., 2006). The previously identified (Huang et al., 2003) promoter region for cardiac myosin light chain (*cmhc2/myl7*) was first isolated using standard PCR protocols. The region was then subcloned into the 5E-MCS element of the Tol2Kit (Kwan et al., 2007), and combined with a middle element containing the sequence for the photo-convertible Kaede to generate an insertion vector. Single-cell embryos were injected with the vector, selected for transient Kaede expression. Adult zebrafish were then test-crossed to verify germline insertion, and bred to homozygosity, and multiple lines were crossed to maximize fluorescence. In addition, we utilized the Gal4-UAS transactivator system to increase the cardiac expression of Kaede so that a more accurate quantitative evaluation of secondary differentiation could be performed. The cardiac myosin light chain (*cmhc2*) promoter (Huang et al., 2003) was cloned upstream of *Gal4* transactivator sequence in a Tol2 based vector (kind gift from M.Nonet, Wash U St. Louis). This construct was then utilized to create a stable transgenic line that had cardiac restricted expression of the Gal4 protein. This

line (*cmlc2:Gal4*) was then crossed with the previously described *tg(UAS:Kaede)^{s1999t}* line (Scott et al., 2007). Resulting double transgenic embryos had robust cardiac restricted expression of Kaede, which maintained red (CY3+) and green (FITC+) fluorescence after formaldehyde fixation and processing.

Cardiomyocyte counting

To count cardiomyocytes, zebrafish with nuclear cardiomyocyte fluorescence *cmlc2:dsRed2-nuc* were grown to specific time-points during development, and fixed for 1 hour in 4% formaldehyde/PBST, followed by dechoriation and 10 minute fixation in acetone. Fixed embryos were then permeabilized with Triton X (0.5% in PBS) and washed in blocking buffer (2mg/ml BSA, 2% normal goat serum, 1% DMSO). Sequential primary (anti-dsRed antibody, 1:300, Rockland) and secondary (goat-anti-Rabbit Alexa Fluor 568, 1:200, Invitrogen) antibody washes were followed by partial dissection and embedding in mounting medium on bridge slides. Hearts were imaged with an Olympus FluoView 1000 confocal microscope, and nuclei of differentiated cardiomyocytes were counted using ImageJ software (Abramoff et al., 2004) (<http://rsbweb.nih.gov/ij/>).

In situ hybridization

In situ hybridizations were performed using a standard protocol (Westerfield, 2000) with digoxigenin-labeled probes. Embryos at appropriate stages were fixed in 4% PBST/ formaldehyde, and incubated with previously-published antisense probes *nkx2.5* (Chopra et al., 2010), or cloned using published gene sequences (*nkx2.7*, NM_131419). Probes were detected with mouse-anti-DIG antibody conjugated to alkaline phosphatase (Roche, 1:5000), and developed in BM Purple AP substrate (Roche). Except where otherwise noted, embryos were examined throughout development, and fixed at identical time points. ‘Hours post-fertilization’ refers to the developmental stage of the wildtype embryo. For embryos fixed during somitogenesis, individual somites were counted for each group to ensure identical developmental stages.

Assessment of secondary differentiation of ventricular cardiomyocytes

Qualitative evaluation of secondary differentiation of cardiomyocytes was performed on embryos with FITC+ hearts from the *tg(cmlc2:Kaede)* zebrafish described above. As previously described by others (de Pater et al., 2009), wildtype and morpholino-injected embryos were exposed to UV laser irradiation at 30 hpf, to allow cardiomyocytes of the heart tube (primary cardiac differentiation) to produce sufficient Kaede protein. Embryos were visualized as UV laser irradiation (330-385 nm) photo-converted cardiomyocytes from green (FITC+ fluorescence) to red (CY3-positive fluorescence), and focused along the z axis until complete throughout the entire heart (approximately 30 seconds per embryo). Embryos then were allowed to develop to 48 hpf, anesthetized and mounted in 1% methyl cellulose. Hearts were imaged in live anesthetized embryos on an Olympus FluoView 1000 confocal microscope, using FITC (488nm, green) and CY3 (568nm, red) lasers to detect primary (red, green) and secondary (green only) cardiomyocytes.

Quantification of secondary differentiation

The reporter line described above *tg(UAS:Kaede)^{s1999t}* (Scott et al., 2007) was crossed to the *tg(cmlc2:Gal4)* driver line to increase expression of Kaede protein in *cmlc2*-expressing cardiomyocytes. Wildtype and morpholino-injected embryos were exposed to UV irradiation at 30 hpf, then grown to 48 hpf and fixed in 4% paraformaldehyde. Embryos were then permeabilized using 0.5% Triton-X in PBS, and washed in blocking buffer containing 1μM TO-PRO-3-Iodide (Invitrogen). Samples were partially dissected to expose the heart, and placed in mounting medium on bridge slides and imaged on an Olympus

FluoView 1000 confocal microscope. Nuclei of secondary-differentiated cardiomyocytes were identified as FITC+ (expressing Kaede) and CY3- (not expressing Kaede before 30 hpf) and were counted using the ImageJ software (Abramoff et al., 2004).

Quantification of Cardiomyocyte Proliferation

BrdU injection to 48 hpf embryos was performed as previously described (Ribeiro et al., 2007; Qu et al., 2008). Briefly, tg(*cmhc2:mCherry*) embryos were injected with 2nL (1mg/mL) BrdU into the pericardiac region, and incubated for five hours. Embryos were then fixed in 4% paraformaldehyde and immunofluorescence was performed with mouse-anti-BrdU (Developmental Studies Hybridoma, 1:300) and rabbit-anti-dsRed (Rockland, 1:300). Embryos were then stripped of their heads, mounted on bridge slides, and imaged on an Olympus FluoView 1000 confocal microscope. Images were analyzed with ImageJ software package (Abramoff et al., 2004), using the cell counting feature to identify and mark proliferating cardiomyocytes.

Drug delivery to zebrafish circulation

Modulators of voltage-gated sodium channel function were delivered directly to the circulation of 48 hpf zebrafish. Embryos were lightly anesthetized with tricaine methanesulfonate to inhibit movement without affecting heart rate, and placed in 1.5% methyl cellulose in zebrafish egg water (Westerfield, 2000). Borosilicate needles were used to deliver 2 nL (25ng/mL in H₂O) anemone toxin (ATX II, Sigma) directly into the left common cardinal vein (Isogai et al., 2001) to avoid volume-related disruption of cardiac function. Phenol red dye was used to evaluate for efficient delivery of the toxin. Embryos were then immediately transferred to egg water without anesthesia and evaluated for altered cardiac rhythm. The L-type calcium channel blocker nifedipine (2nL, 15mM) and H₂O (2nL) were used as positive and negative controls, respectively.

***nkx2.5* mRNA Injections**—A zebrafish cDNA library was used to isolate and clone *nkx2.5* using primers aligned to the published sequence (NCBI: NM_131421.1) into the pcGlobin2 vector (Ro et al., 2004). mRNA was transcribed *in vitro* using the T7 mMessage mMachine kit (Ambion). Transcript was purified and verified on 1% agarose gel, and 75 or 150 pg was microinjected into single cell embryos, as previously described (Lee et al., 1996).

Statistical Methods Used

Data are presented as mean $\pm$ standard error. Pair-wise comparisons of normally-distributed data were performed using Student's *t*-test. For grouped comparisons of non-parametric data, the Kruskal-Wallis test was used, followed by Mann-Whitney U test for pair-wise comparisons between individual groups. Statistically significant differences ($p < 0.05$) are noted with asterisks.

Animal Care

All organisms were cared for in accordance with guidelines set by the Vanderbilt University Institutional Animal Care and Use Committee (IACUC) and the Office of Animal Welfare Assurance (OAWA).

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Fig. 1.

Throughout development, morpholino-injected embryos have fewer *cmhc2*+cardiomyocytes than uninjected embryos. At 24 hpf (**a,e,i**), the number of cells in the initial heart tube is dramatically decreased in morphant embryos (129.2 ± 2.4 vs 83.6 ± 6.1 , $n=6,7$, $p<.001$). As development progresses, both morphant and wildtype embryos continue to add cardiomyocytes. At 36 hpf (**b,f,i**), uninjected embryos continue to have a significantly greater number of cardiomyocytes (182 ± 4.2 vs 143.5 ± 3.0 , $n=6$, $p<.001$). Between 48 hpf (**c,g,i**: 198.5 ± 5.2 vs. 162.5 ± 2.5 , $n=6$, $p<.001$) and 60 hpf (**d,h,i**: 219.8 ± 3.8 vs. 170.5 ± 4.2 , $n=7,6$, $p<.001$) wildtype embryos continue to add cardiomyocytes to the hearts, while morphant embryos have slowed in growth. A = atrium, V = ventricle, W = wildtype, M = morphant.

Fig. 2.

Embryos injected with active morpholino show impaired cardiac specification. In situ hybridization for the cardiac-specification transcription factor *nkx2.5* (a-b) shows markedly reduced expression in the anterior lateral plate mesoderm. Expression of the related homeobox gene *nkx2.7* (e-j) was unaffected in embryos from the same injection clutch. Cardiomyocytes at 26 hpf (c,d) have restored expression of *nkx2.5*, within the smaller heart tube. MO = morphants, WT = uninjected

Fig 3.
nkx2.5 mRNA is insufficient to rescue loss of *scn5Lab*: Wildtype embryos (a), or embryos injected with 150pg *nkx2.5* mRNA (b) or mRNA with *scn5Lab*-MIS morpholino (c) show no gross morphological phenotype. *scn5Lab*-MO injected embryos (d,e) are not rescued by co-injection of *nkx2.5* (f).

Fig. 4. (a) Wildtype (WT) and (b) morpholino-injected embryos (MO) were exposed to UV irradiation at 30 hpf and allowed to develop until 48 hpf. Stacked confocal imaging shows photoconverted primary (yellow) cardiomyocytes, and new (green) cardiomyocytes. Between 30 and 48 hpf, secondary differentiation of cardiomyocytes occurred exclusively at the arterial pole outflow tract (OT) of the ventricle in wildtype and morphant embryos. Both groups appeared to efficiently add cells between 30 and 48 hpf.

Fig. 5.

Formaldehyde-fixed *tg(cmlc2:gal4, UAS:kaede)* embryos maintain green (a) and red (b) fluorescence after processing. Topro3-I marks nuclei (c) without affecting identification of secondary cardiomyocytes. Secondary cardiomyocytes (d) form the arterial pole of the ventricle, clustering at the outflow tract and appearing green with blue nuclei (arrow), while older, primary cardiomyocytes appear as yellow with white nuclei (arrowhead). At high-powered magnification, primary (arrow) and secondary cardiomyocytes can be identified and counted. Wildtype (34.8 ± 1.3 , $n=10$) and morpholino-injected (33.1 ± 1.6 , $n=8$) embryos (e) add equal numbers of cells to the arterial pole between 30 and 48 ($p>0.2$).

Fig. 6. BrdU-injected tg(cmlc2:dsRed) embryos are positive for proliferating cardiomyocytes in wildtype(a-c), 5 base mismatch morpholino-injected (d-f), and morphant (g-i) embryos. Embryos injected with BrdU at 48 hpf, and incubated for 5 hours, show colocalization (arrows: c,f) of the BrdU (green) and dsRed (red) signals. Embryos injected with active morpholino do not show any colocalization of the two signals (i). Despite having no proliferation in the myocardium, morphant embryos do appear to have normal proliferation in other tissues (arrowheads).

Fig 7.

Quantification of BrdU-positive cardiomyocytes suggests embryos deficient in *Scn5Lab* have a proliferation defect. Embryos were imaged and sections hyperstacked in ImageJ. Cells positive for both BrdU and dsRed were counted. Embryos injected with active morpholino had no evidence of myocardial proliferation (0 double-positive cells, n=12) compared to uninjected (5.8 ± 0.6 , n=14) and mismatch-injected (4 ± 0.9 , n=11) hearts. *= pairwise significance $p < 0.001$

Fig 8.

Impaired cardiac contraction is not responsible for loss of proliferation in *scn5Lab*-MO injected embryos. WT, *tnnt2*-MO, *tnnt2*-MO+*scn5Lab*-MIS embryos all have detectable proliferation at 48 hpf, while *scn5Lab*-MO embryos had none (Kruskal Wallis test $p=0.004$). Mann-Whitney U test identified proliferation as significantly decreased in *tnnt2*-MO/*scn5Lab*-MO co-injected embryos vs. *tnnt2*-MO/*scn5Lab*-MIS (1.1 ± 0.3 vs 0.1 ± 0.1 , $n=12,10$; $p=0.007$) and wildtype (2.2 ± 0.5 , $n=10,24$; $p=0.001$) hearts. These four sets of experiments were performed at the same time.