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A Molecular Pathway Including *Id2, Tbx5,* and *Nkx2-5* Required for Cardiac Conduction System Development

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SUMMARY

The cardiac conduction system is an anatomically discrete segment of specialized myocardium that initiates and propagates electrical impulses to coordinate myocardial contraction. To define the molecular composition of the mouse ventricular conduction system we used microdissection and transcriptional profiling by serial analysis of gene expression (SAGE). Conduction-system-specific expression for *Id2,* a member of the *Id* gene family of transcriptional repressors, was identified. Analyses of *Id2*-deficient mice demonstrated structural and functional conduction system abnormalities, including left bundle branch block. A 1.2 kb fragment of the *Id2* promoter proved sufficient for cooperative regulation by *Nkx2-5* and *Tbx5* in vitro and for conduction-system-specific gene expression in vivo. Furthermore, compound haploinsufficiency of *Tbx5* and *Nkx2-5* or *Tbx5* and *Id2* prevented embryonic specification of the ventricular conduction system. We conclude that a molecular pathway including *Tbx5, Nkx2-5,* and *Id2* coordinates specification of ventricular myocytes into the ventricular conduction system lineage.

INTRODUCTION

The conduction system is a specialized structure within the heart responsible for establishing and maintaining electrophysiological activities. Disorders of the cardiac conduction system occur commonly, either in isolation or in the context of generalized heart disease, and often produce life-threatening arrhythmias. Medical treatments for conduction system disease are limited and primarily involve antiarrhythmic medications and pacemaker implantation.

The anatomic structures of the mammalian conduction system were first described 100 years ago (Tawara, 1906). Atrial components of this complex structure include the sinoatrial and atrioventricular nodes; these have been conserved throughout vertebrate development (reviewed in Moorman et al., 1998). Ventricular components have evolved more recently (Sedmera et al., 2003b) and in mammals include an atrioventricular bundle and the left and right bundle branches. The ventricular conduction system accounts for rapid spread of electrical impulses from the atrioventricular node at the atrial base to the ventricular apex, which is essential for apical contraction initiation. Coordinated cardiac contraction shifts from a linear pattern to an apex-initiated pattern between E11 and E14 (Morley and Vaidya, 2001). The transition to a mature pattern of cardiac contraction is coincident with the differentiation of myocytes in the ventricular conduction system lineage, including the onset of molecular conduction system marker expression (e.g., *minK*), implying establishment of a formal ventricular conduction system (Gourdie et al., 1995; Cheng et al., 1999; Moorman et al., 1998; Kondo et al., 2003). Whether the ontogeny of the atrial and ventricular conduction components requires the same molecular pathways or whether they are independently generated and physically coupled is unknown.

Tbx5 and *Nkx2-5,* two transcription factor genes expressed broadly in the heart, have roles in the

maintenance of conduction system structure and function. Dominant mutations in these genes cause congenital heart defects and conduction system abnormalities, most notably atrioventricular block, in adult humans and mice (Basson et al., 1994; Li et al., 1997; Schott et al., 1998, Benson et al., 1999; Bruneau et al., 2001; Prall et al., 2002; Jay et al., 2004; Moskowitz et al., 2004; Pashmforoush et al., 2004). Because electrophysiological defects can occur in the absence of cardiac structural malformations (Basson et al., 1994; Benson et al., 1999; Schott et al., 1998, Pashmforoush et al., 2004), *Tbx5* and *Nkx2-5* are hypothesized to have roles in conduction system development independent of their roles in cardiac morphogenesis. Consistent with this model, *Tbx5* and *Nkx2-5* are transcribed at higher levels in the ventricular conduction system than in surrounding myocardium (Thomas et al., 2001; Moskowitz et al., 2004; Harris et al., 2006). However, more precise understanding of the role of *Tbx5*, *Nkx2-5,* and other transcription factors in ventricular conduction system development is lacking.

Neither a molecular description of conduction system myocytes nor a definition of the features that distinguish these from nonconduction myocytes has been described, in part due to the difficulty of isolating pure conduction system tissue, given its complex three-dimensional structure and intimate association with nonconduction myocytes. We developed and verified a ventricular conduction system microdissection technique and defined the transcriptome of the left bundle branch by serial analysis of gene expression (SAGE). The ventricular conduction system shared RNAs expressed in both myocyte and neuron transcriptomes. SAGE identified *Id2*, a member of the *Id* family of transcriptional repressors, as having conduction-system-specific expression. We characterized *Id2* expression and evaluated the consequences of *Id2* deficiency on conduction system function. Analysis of the *Id2* promoter demonstrated that conduction-system-specific expression of *Id2* is dependent on *Nkx2-5* and *Tbx5*. These data define a molecular pathway, including *Nkx2-5*, *Tbx5*, and *Id2*, that is required for ventricular conduction system development.

RESULTS

Isolation of Ventricular Conduction System Tissue

We characterized postnatal expression of the conduction system marker *minK:lacZ* (Kupershmidt et al., 1999) to define maturation of the ventricular conduction system. The left bundle branch of adult mice (age > 12 weeks) was consolidated into thin discrete bands on the surface of the interventricular septum; however, in juvenile mice (age 19 days) this structure covered the entire basal portion of the left ventricular septal surface (Figure 1A versus 1B). Transverse sections of the interventricular septum revealed that left bundle branch cells, expressing nuclear *minK:lacZ,* were tightly adherent to one another and segregated from underlying nonconduction myocytes (Figure 1C).

Presumptive left bundle branches—discrete, thin membranes of tissue—were dissected intact from surrounding myocardium of the left ventricular septum of juvenile wild-type and $minK^{lacZ/+}$ mouse hearts (Figures 1D and 1E). To confirm that these tissues were comprised of conduction system cells, isolated specimens from $minK^{lacZ/+}$ mice were stained for β-galactosidase expression and DAPI. β-galactosidase expression was observed in approximately 90% of cells (Figure 1F), indicating a high degree of conduction system enrichment.

Transcriptional Profiling of the Ventricular Cardiac Conduction System

Left bundle branches from 60 19-day-old wild-type SvEv mice were pooled for SAGE library construction (Velculescu et al., 1995). For comparison, a SAGE library was also constructed from the left ventricle of two 19-day-old SvEv mice. More than 60,000 SAGE tags were sequenced from each library, unique tags were annotated, and total tag counts between the libraries were normalized.

To verify that starting material for the left bundle branch library was enriched in conduction system cells, tags corresponding to previously defined molecular markers of the conduction system were assessed (Figures 1G and 1H). Tags corresponding to the *minK* gene, expression of which guided microdissections, were specific for the conduction system library: 11/60,000 tags were in the left bundle branch library compared to 0/60,000 tags in the left ventricular library ($p < 0.001$). Tags corresponding to *α-3 Na^+,K(+)-ATPase* and *Anf (*atrial natriuretic factor), genes shown by in situ hybridization to have increased expression in the conduction system (Zeller et al., 1987; Zahler et al., 1992), were also significantly more abundant in the left bundle branch library than in the left ventricle library (Figure 1G). We concluded that the left bundle branch SAGE library was significantly enriched in transcripts from conduction system cells.

The left bundle branch conduction system transcriptome was further evaluated by assessment of transcripts with significantly different levels of expression from the left ventricle SAGE library ($p < 0.02$). Interrogation of SAGE libraries identified 13 genes encoding proteins of the myocyte sarcomere complex (Figure 1H). Each myocyte sarcomere gene was represented by lower tag counts in the left bundle branch library compared to the left ventricle library (Figure 1H).

Id2 Expression in the Ventricular Cardiac Conduction System

The left ventricular bundle SAGE library was informative for identifying the expression of genes with previously unknown roles in the conduction system. SAGE tags corresponding to *Id2* (Figure 1G), a member of a gene family encoding helix-loop-helix-containing transcriptional repressors (*Id1–Id4*), were specifically represented in the left bundle branch library (6/60,000 tags) and were absent (0/60,000 tags) from the left ventricle library ($p < 0.02$).