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Notch signaling plays a key role in cardiac cell differentiation

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Abstract

Results from lineage tracing studies indicate that precursor cells in the ventricles give rise to both cardiac muscle and conduction cells. Cardiac conduction cells are specialized cells responsible for orchestrating the rhythmic contractions of the heart. Here, we show that Notch signaling plays an important role in the differentiation of cardiac muscle and conduction cell lineages in the ventricles. *Notch1* expression coincides with a conduction marker, HNK-1, at early stages. Misexpression of constitutively active Notch1 (NIC) in early heart tubes in chick exhibited multiple effects on cardiac cell differentiation. Cells expressing NIC had a significant decrease in expression of cardiac muscle markers, but an increase in expression of conduction cell markers, HNK-1, and SNAP-25. However, the expression of the conduction marker *connexin 40* was inhibited. Loss-of-function study, using a dominant-negative form of Suppressor-of-Hairless, further supports that Notch1 signaling is important for the differentiation of these cardiac cell types. Functional studies show that the expression of constitutively active Notch1 resulted in abnormalities in ventricular conduction pathway patterns.

Keywords: Cell differentiation; Notch pathway; Cardiomyocyte; Ventricles; Molecular mechanism

1. Introduction

The cardiac conduction system is a specialized tissue responsible for setting, maintaining, and coordinating the rhythmic contractions of the heart (Gourdie et al., 1999; Moorman et al., 1998; Myers and Fishman, 2003). Precisely timed electrical impulses are generated at the sinoatrial node, spread through the atrial myocytes, and are received at the atrioventricular node. This impulse is then rapidly propagated along the His bundles and its branches, spreading into the ventricular muscle via the Purkinje fiber network. Although much progress has been made in the understanding of heart development (Eisenberg and Markwald, 2004; Srivastava and Olson, 2000), the mechanism underlying the development of the cardiac conduction system is only partially understood.

Cardiac conduction cells are distinguished by their unique gene expression pattern. Antibodies to Leu-7 (HNK-1) have been used widely to delineate the developing conduction system in mammals and in chicken (Chuck and Watanabe, 1997; Gorza et al., 1988; Moorman et al., 1998; Nakagawa et al., 1993; Verberne et al., 2000). The HNK-1 antibody recognizes a complex sulfate-3-glucuronyl carbohydrate moiety, which is present on a series of molecules involved in cell adhesion and extracellular matrix interaction. The antibody to SNAP-25 protein, a component of the SNARE complex, has also been used to mark the elements of the ventricular conduction system in chick (Verberne et al., 2000). Among the gap junction protein connexins, Connexin40 (Cx40) or the chicken homolog Cx42, has been used as a marker for the conduction system in many species (Bastide et al., 1993; Delorme et al., 1997; Gourdie et al., 1993b; Gros et al., 1994).

Retroviral lineage analyses have provided compelling evidence that conduction cells are derived from precursor cells in the heart, sharing a common lineage with working cardiomyocytes (Cheng et al., 1999; Gourdie et al., 1995).

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Clonally related cells are found in both cardiac muscle cells and in the conduction system. Following microinjection of replication-defective retrovirus into the cardiac neural crest, however, no virally tagged cells could be traced into the Purkinje fiber lineage, excluding contribution from the neural crest. It has been demonstrated that the selection of conduction cells within myocardial clones occurs as a result of paracrine signals from endocardial cells and endothelial cells from coronary arteries, including endothelin-1 (ET-1) in chick and Neuregulin-1 in mouse (Hall et al., 2004; Kanzawa et al., 2002; Rentschler et al., 2002; Takebayashi-Suzuki et al., 2000). Coexpression of preproendothelin (preproET-1) and endothelin converting enzyme (ECE-1) in the embryonic myocardium induced myocytes to express Purkinje fiber markers (Hall et al., 2004; Takebayashi-Suzuki et al., 2000). Addition of neuregulin-1 to embryo cultures of *CCS-lacZ* mice, in which *lacZ* delineates the cardiac conduction system, increased *lacZ* expression (Rentschler et al., 2002). Several transcription factors, including Nkx2.5, Tbx5, and HF-1b have also been shown to play an important role in the development of the cardiac conduction system (Jay et al., 2004; Kondo et al., 2003; Kupershmidt et al., 1999; Moskowitz et al., 2004; Nguyen-Tran et al., 2000; Pashmforoush et al., 2004; Thomas et al., 2001). Mouse mutants deficient in either of the transcription factors *HF-1 b*, *Tbx5* or *Nkx2.5* exhibit defects in the development and function of the conduction system.

The Notch signaling pathway is an evolutionarily conserved mechanism used by metazoans to control cell fate decisions through local cell interactions (Artavanis-Tsakonas et al., 1999). The *notch* gene encodes a single-pass transmembrane protein receptor that interacts with its ligands, Delta and Serrate/Jagged. Upon binding of the ligand, the intracellular domain of Notch (NIC) undergoes proteolytic cleavage, and is translocated to the nucleus. In the nucleus, NIC binds to its major downstream effector, Suppressor-of-Hairless [Su(H)]. Su(H) binds to the regulatory sequences of the Enhancer-of-Split [E(spl)] locus, upregulating the expression of basic helix-loop-helix (bHLH) proteins, which in turn regulate the expression of downstream target genes. Signals transmitted through the Notch receptor, in combination with other cellular factors, influence differentiation of various cell types, in the nervous system, immune system and pancreas (Artavanis-Tsakonas et al., 1999).

The Notch pathway has been previously shown to influence cardiogenesis. In *Xenopus*, it is suggested that the interaction of Notch1 with its ligand Serrate1 apportions myogenic and non-myogenic cell fates within the early heart field (Rones et al., 2000). In mouse, null mutations in both *notch1* and *RBP-J*, the mammalian homolog of Suppressor-of-Hairless, leads to embryonic lethality and pericardial edema (Oka et al., 1995; Swiatek et al., 1994). The absence of RBP-J in mouse ES cells causes an increase in cardiac muscle development suggesting that Notch/RBP-J signaling is required for the specification of cell fates within the heart field by suppressing cardiomyogenesis (Schroeder et al., 2003). Recently, mutations in Notch1 in humans have been shown to cause aortic valve defects and activation of Notch1 in mouse leads to abnormal cardiogenesis characterized by deformities of the ventricles and atrioventricular canal (Garg et al., 2005; Watanabe et al., 2006). Additionally, mutations in various Notch signaling pathway genes, including *Jagged1*, *mind bomb 1*, *Hesr1*/*Hey1*, and *Hesr2*/*Hey2*, result in cardiac defects, such as pericardial edema, atrial and ventricular septal defects, cardiac cushion, and valve defects (Donovan et al., 2002; Fischer et al., 2004; Gessler et al., 2002; Kokubo et al., 2005; Kokubo et al., 2004; Koo et al., 2005; McCright et al., 2002; Sakata et al., 2002).

Here, we demonstrate a role for Notch1 in the differentiation of cardiac cell types in the ventricles. *notch1* mRNA transcripts are expressed in the ventricular conduction cell lineage at early stages. Forced expression of constitutively active Notch1 in progenitor cells inhibits muscle marker expression but promotes expression of conduction marker HNK-1 and SNAP-25. Cells expressing constitutively active Notch were localized predominantly in the trabeculae where conduction cells are concentrated, and not in the future myocardial compact zone. Loss-of-function study further demonstrate the requirement for Notch in this lineage decision. By optical mapping, we have further shown that expression of constitutively active Notch1 resulted in abnormal conduction patterns in the heart consistent with a defect in cardiac cell differentiation.

2. Results

To study the mechanism of cardiac cell differentiation, we analyzed the expression of the *notch1* gene by in situ hybridization. At embryonic days 6 (E6), in situ hybridization on heart sections showed that *notch1* was expressed in the ventricles and the atria, concentrated in a subset of cells in the trabecular myocardium, and atrioventricular canal (Figs. 1A, B, and data not shown). Some very weak signals were also detected in the endocardium (Fig. 1B). To determine which cardiac cell type in the myocardium expressed *notch1* mRNA, we performed in situ hybridization on heart sections using the chick *notch1* probe, followed by immunostaining with markers for different cardiac cell types. From E3 to E6, the *notch1* in situ signals in the ventricles were largely associated with staining by HNK-1 (Fig. 1C). HNK-1 antibody recognizes a complex carbohydrate moiety on the cell surface and has been used extensively as a marker for the ventricular conduction system in many species including chick, rat, rabbit and human (Aoyama et al., 1993; Aoyama et al., 1995; Chuck and Watanabe, 1997; Gorza et al., 1988; Ikeda et al., 1990; Luider et al., 1993; Nakagawa et al., 1993; Nakamura et al., 1994; Sakai et al., 1994; Verberne et al., 2000), and overlaps with another conduction system marker, Cx40 (Supplementary Fig. 1S). As in situ signals are localized in the cytoplasm whereas HNK-1 staining is found on the plasma membrane, the association of the expression