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Connexin45 contributes to global cardiovascular development by establishing myocardial impulse propagation

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ABSTRACT

Among gap junction-encoding genes, the loss of *connexin* (*Cx*) *45* most profoundly obstructs embryogenesis through an endocardial cushion defect and conduction block. However, the interdependence of these defects is not known, and the details of conduction block have not been elucidated. Here, we examined mouse embryos with a region-specific deletion of *Cx45* in the myocardium (*CA-Cre*; $Cx45^{flox/flox}$) or endothelium (*Tie2-Cre*; $Cx45^{flox/flox}$). Although the deletion of *Cx45* in the myocardium was heterogeneous, the *CA-Cre*; $Cx45^{flox/flox}$ embryos were lethal at the same stage as the constitutive *Cx45*-deficient ($Cx45^{-/-}$) embryos. We determined the onset and patterns of their conduction block through point-tracking in video recordings of embryonic heart contractions. An incomplete conduction block at the atrioventricular canal appeared at embryonic day (E) 8.5 and was predominant around the lethal E9.5 stage in both the $Cx45^{-/-}$ and *CA-Cre*; $Cx45^{flox/flox}$ embryos. Although the $Cx45^{-/-}$ hearts showed a consistently severe reduction in atrioventricular conduction velocity, the *CA-Cre*; $Cx45^{flox/flox}$ hearts had delay times within the normal range and showed frequent retrograde conduction. As previously reported, the $Cx45^{-/-}$ endocardial cushion was consistently defective, and nuclear factor of activated T-cells cytoplasmic (NFATc)1 within the endocardium showed inactive cytoplasmic distribution. In *CA-Cre*; $Cx45^{flox/flox}$, however, the endocardial cushion was partially formed, with active NFATc1 within the endocardium. There was no developmental abnormality in the *Tie2-Cre*; $Cx45^{flox/flox}$ embryos. These results indicate that myocardial dysfunction is responsible for most of the reported defects in $Cx45^{-/-}$, which are alleviated by sporadic Cx45 expression in the *CA-Cre*; $Cx45^{flox/flox}$ myocardium.

1. Introduction

Gap junctions are intercellular channels that are permeable to molecules smaller than approximately 1 kDa. In mice and humans, approximately 20 connexin (Cx) isoforms constitute gap junction channels through which cells communicate to maintain homeostasis, such as that required for proper organogenesis and coordinated excitation between neurons (Nishii et al., 2014). Among Cxs, Cx45 gap junction channels have unique characteristics of low unitary conductance, strong voltage dependence, selective permeability, and pH sensitivity (Barrio et al., 1997; Elfgang et al., 1995; Moreno et al., 1995; Peracchia, 2004; Veenstra et al., 1994). In the early heart, slow conduction is required for peristaltic contractions. Based on its localization within the mature conduction system and pervasiveness in the early heart, Cx45 expression is presumed to be involved in slow impulse propagation between cardiac myocytes (CMs) (Alcoléa et al., 1999; Giovannone et al., 2012; Jansen et al., 2010; Kumai et al., 2000). Significant delays in conduction are observed in artificial *Cx43*-deficient ($Cx43^{-/-}$) ventricular myocyte strands that have Cx45 as the only functional gap junction protein (Beauchamp et al., 2004; Beauchamp et al., 2012). Cx45 can form heteromeric or heterotypic channels with other Cxs, and this diversity confers a range of channel properties (Gemel et al., 2008; Martinez et al., 2002; Rackauskas et al., 2007a; Rackauskas et al., 2007b). In the adult heart, a twofold increase in Cx45 expression alters the permeability of ventricular CM, thereby increasing susceptibility to ventricular tachyarrhythmias (Betsuyaku et al., 2006). In contrast, *Cx45* deletion in adult CMs reduces conductivity in the atrioventricular (AV) node,

Abbreviations: AV, atrioventricular; CA, cardiac α-actin; CM, cardiac myocyte; cTnT, cardiac troponin T; Cx, connexin; E, embryonic day; EMT, epithelial–mesenchymal transformation; NFATc, nuclear factor of activated T-cells cytoplasmic; TRP, transient receptor potential; VEGF, vascular endothelial growth factor; WT, wild-type.

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where Cx30.2 is also downregulated (Frank et al., 2012). In individual subsets of CMs, Cx45 is usually coexpressed with one or more of Cx30, Cx30.2, Cx40, Cx43, and Cx46 (Bao et al., 2011; Chi et al., 2010; Giovannone et al., 2012; Gros et al., 2010; Jansen et al., 2010; Johnson et al., 2002; Kreuzberg et al., 2005; Kreuzberg et al., 2006). Thus, the *in vivo* function of Cx45 depends on its Cx partners and the region of expression. The coexpression of Cx43 and Cx45 is probably most prevalent in the developing embryo; it is initially detected at compaction, which is the first evidence of the junctional complex, at the 8-cell stage, and then later in other organs at varying levels and stoichiometry (Nishii et al., in press; Nishii et al., 2001). The defects in *Cx45*-deficient ($Cx45^{-/-}$) embryos may therefore be mitigated by compensatory expression of Cx43.

The cardiovascular system is a closed circuit in which cardiac development and vascular development occur interdependently. Primitive CMs start beating at the 3-somite stage on embryonic day (E) 8.25 (Nishii and Shibata, 2006). Unidirectional blood flow through the AV canal is established around the 20-somite stage at E9.25 and is correlated with the development of the valvular endocardial cushion arising from the epithelial–mesenchymal transformation (EMT) of the endocardial endothelium. At this stage, Cx45 is the only Cx detected in CMs (Alcoléa et al., 1999), and $Cx45^{-/-}$ embryos fail to develop past this point owing to defects in cardiac cushion formation, AV conduction, and vasculogenesis (Krüger et al., 2000; Kumai et al., 2000). Nuclear factor of activated T-cells cytoplasmic (NFATc)1 translocates into the nucleus upon sustained elevation of $[Ca^{2+}]_i$ (Hogan et al., 2003). It was first discovered in T lymphocytes as one of four isoforms (NFATc1/2/3/4), but broader roles have been found in its regulation of the production of a large number of growth factors, cytokines, and cell–cell interaction molecules essential for the morphogenesis and functional development of many cell types and organs (Wu et al., 2007). In $Cx45^{-/-}$ embryos, NFATc1 remains inactive within the endocardium, and TGF-β signaling is downregulated throughout the cardiovascular system. Cx45 mediates the transfer of Ca^{2+} and inositol-1,4,5-trisphosphate, which may be required for NFATc1 activation within the endocardium (Hogan et al., 2003). NFATc1-deficient ($NFATc1^{-/-}$) mice show defects in valvular morphogenesis (de la Pompa et al., 1998; Ranger et al., 1998). Because NFATc1 is inactivated in the endocardium in $Cx45^{-/-}$, the cushion defect has been assumed to be of endocardial origin (Nishii et al., 2001). However, during E8.5–10.5, EMT is normal in $NFATc1^{-/-}$, whereas it is completely disrupted by the loss of Cx45 (Chang et al., 2004; Kumai et al., 2000).

The endocardial cushion is thought to be formed by interactions between the myocardium and endocardium (Combs and Yutzey, 2009a; de Vlaming et al., 2012; Kirby, 2002; Kruithof et al., 2012; Nishii et al., 2001). Myocardial vascular endothelial growth factor (VEGF) activates endocardial NFATc1 (Combs and Yutzey, 2009b) and is critical for cardiovascular development. However, VEGF overexpression has adverse effects on cushion formation (Combs and Yutzey, 2009b; Dor et al., 2001; Miquerol et al., 2000). These findings imply that any myocardial dysfunction in early cardiogenesis can cause EMT defects. We have previously reported that mice deficient in cardiac troponin T ($cTnT^{-/-}$), whose expression is confined to thin filaments in CMs, where it regulates Ca^{2+} sensitivity, die at approximately the same stage (E10.5) because of the absence of a heartbeat (Nishii et al., 2008). Thus, $cTnT^{-/-}$ hearts show cardiac cushion and EMT defects of myocardial origin (Bartman et al., 2004; Nishii et al., 2008).

In this study, the role of Cx45 was independently analyzed in the myocardium and endothelium. A novel image-processing method is described, in which myocardial function can be calculated through point-tracking in video recordings of embryonic heart contractions. These analyses revealed that Cx45, and not Cx43, is essential for impulse propagation within early embryonic CMs. Many other abnormalities within the $Cx45^{-/-}$ embryo are probably of myocardial origin.

2. Results

2.1. Region-specific deletion of Cx45 reveals its essential role in the myocardium

To examine the region-specific function of Cx45, mice with a "floxed" *Cx45* locus were generated, in which the *Cx45*-coding exon was deleted in the presence of Cre recombinase and was replaced by *nls-lacZ*, which served as a reporter (Fig. S1). Because all inner cell mass-derived embryonic tissues express *Cx45* before E10.5, recombined cells in which Cre-mediated excision of *Cx45* had occurred were marked by X-gal staining (Kumai et al., 2000). *Cx45* was deleted in the myocardium and endothelium in the cardiac α-actin (*CA-Cre*; $Cx45^{flox/flox}$) and *Tie2* (*Tie2-Cre*; $Cx45^{flox/flox}$) promoter-driven Cre lines, respectively (Koni et al., 2001; Miwa et al., 2000).

CA-Cre; $Cx45^{flox/flox}$ mice were obtained from crosses of *CA-Cre*; $Cx45^{+/flox}$ males with $Cx45^{flox/flox}$ females (Table 1) and were observed at the expected Mendelian ratio of 1/4 from E8.5 to E11.5 ($P = 0.08$; χ^2-test). However, except for one embryo with severe conduction block, the hearts of all *CA-Cre*; $Cx45^{flox/flox}$ mice stopped beating by E10.5 (Table 2). In contrast, the *Tie2-Cre*; $Cx45^{flox/flox}$ mice were healthy and fertile. Strains of $Cx45^{+/+}$ (WT), $Cx45^{+/-}$, $Cx45^{flox/flox}$, *CA-Cre*; $Cx45^{+/flox}$, and *Tie2-Cre*; $Cx45^{+/flox}$ were also normal with regard to their development and growth after birth, and therefore, X-gal staining of their samples was included to render the extent of Cre expression within the normal tissue (Figs. 1, 2). The *CA-Cre*; $Cx45^{flox/flox}$ embryos showed an AV conduction block where not all of the atrial contractions were transmitted to the ventricle, similar to that in $Cx45^{-/-}$ hearts (Supplementary Movies 1–3). This type of block was easily identified in videos and corresponded to second- or third-degree AV conduction block in the more developed heart as diagnosed by electrocardiogram, although diagnostic criteria for abnormal embryonic heart contractions do not exist. The initial heartbeat was normal in both strains around E8.25 (3–8-somite stage); the AV conduction block appeared between E8.5 and E9.5 (9–20-somite stage), with all mutant embryos at later stages showing the defect (Fig. 1E). The onset of the AV conduction block was delayed in *CA-Cre*; $Cx45^{flox/flox}$, and X-gal staining of the heart revealed many X-gal-negative cells, distributed in a manner similar to that of colonies of myocardial clones (Figs. 1F–H, 2) (Meilhac et al., 2004). In contrast, in serial sections, complete endothelial recombination in the AV canal was observed in *Tie2-Cre*; $Cx45^{flox/flox}$ (Fig. 2D), and these embryos reached adulthood.

2.2. EMT defects are related to the degree of myocardial dysfunction

As previously reported, $Cx45^{-/-}$ embryos had an endocardial cushion defect caused by EMT failure (Fig. 2A) (Kumai et al., 2000). Mesenchymal cells were rarely observed in the AV canal region, and the development of the myocardium was perturbed. Similar histological observations were made in $Cx43^{-/-}$ $Cx45^{-/-}$ embryos, although tissues in the compound mutants were generally more hypoplastic (Fig. 2B). The amount of cushion mesenchyme in *CA-Cre*; $Cx45^{flox/flox}$ embryos was variable, as were heart shape and myocardial wall thickness (Figs. 1, 2A, C). *CA-Cre*; $Cx45^{flox/flox}$ hearts had most of the abnormalities of $Cx45^{-/-}$, but the degree of severity was milder. In contrast, there were no abnormalities in *Tie2-Cre*; $Cx45^{flox/flox}$ embryos, which had an

Table 1
Genotype distribution from crosses of *CA-Cre*; $Cx45^{+/flox}$ males with $Cx45^{flox/flox}$ females.

	Genotype			
	$Cx45^{+/flox}$	$Cx45^{flox/flox}$	*CA-Cre*; $Cx45^{+/flox}$	*CA-Cre*; $Cx45^{flox/flox}$
E8.5	11	13	14	11
E9.5	16	26	28	38
E10.5	4	3	7	9
E11.5	11	7	7	6