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Cartilage link protein 1 (Crtl1), an extracellular matrix component playing an important role in heart development

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Abstract

To expand our insight into cardiac development, a comparative DNA microarray analysis was performed using tissues from the atrioventricular junction (AVJ) and ventricular chambers of mouse hearts at embryonic day (ED) 10.5–11.0. This comparison revealed differential expression of approximately 200 genes, including cartilage link protein 1 (Crtl1). Crtl1 stabilizes the interaction between hyaluronan (HA) and versican, two extracellular matrix components essential for cardiac development. Immunohistochemical studies showed that, initially, Crtl1, versican, and HA are co-expressed in the endocardial lining of the heart, and in the endocardially derived mesenchyme of the AVJ and outflow tract (OFT). At later stages, this co-expression becomes restricted to discrete populations of endocardially derived mesenchyme. Histological analysis of the Crtl1-deficient mouse revealed a spectrum of cardiac malformations, including AV septal and myocardial defects, while expression studies showed a significant reduction in versican levels. Subsequent analysis of the *hdf* mouse, which carries an insertional mutation in the versican gene (*CSPG2*), demonstrated that haploinsufficient versican mice display septal defects resembling those seen in Crtl1$^{-/-}$ embryos, suggesting that reduced versican expression may contribute to a subset of the cardiac abnormalities observed in the Crtl1$^{-/-}$ mouse. Combined, these findings establish an important role for Crtl1 in heart development.

Keywords: Cartilage link protein 1; Versican; Hyaluronan; Atrioventricular cushions; Atrioventricular septal defects; Ventricular septal defects; Thin myocardium

Introduction

In the early steps of heart development, the fusion of two populations of precardiac mesoderm leads to the formation of a primitive cardiac tube. In this primitive heart, the myocardial and endocardial cell layers are separated by the cardiac jelly, an acellular, and extracellular matrix-rich (ECM), space (Markwald et al., 1977; Moorman and Christoffels, 2003; Wessels and Sedmera, 2003). As the heart tube begins to loop, the cardiac jelly accumulates in the atrioventricular (AV) junction and the outflow tract (OFT), a process that is followed by local epithelial-to-mesenchymal transformation (EMT) of the endocardial lining. Combined, these events result in the formation of the mesenchymal cushions in the AVJ and OFT (Krug et al., 1985; Markwald et al., 1977). In addition to the endocardially derived mesenchyme, the respective cushions also receive mesenchymal contributions from epicardially derived cells (Dettman et al., 1998; Manner, 1999; Perez-Pomares et al., 2002) and cardiac neural crest-derived cells (Danielian et al., 1998; Nakamura et al., 2006).

The AV cushions are involved in the formation of the mitral and tricuspid valves (Wessels et al., 1996) and contribute,

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together with the dorsal mesenchymal protrusion (DMP), to the formation of the AV mesenchymal complex, which plays a crucial role in cardiac septation (Snarr et al., 2007; Wessels et al., 1996, 2000). As many congenital heart malformations involve the derivatives of the AV mesenchyme, elucidation of the mechanisms that govern its development is fundamental to understanding the etiology of congenital heart disease. Over the years, numerous *in vitro* and *in vivo* studies have increased our general understanding of the cellular and molecular mechanisms in the respective phases of cushion development. This has led to the general consensus that endocardial cushion formation is a process that relies on a balanced interaction between myocardially and endocardially/mesenchymally expressed genes, including transcription factors, growth factors, and ECM components (Eisenberg and Markwald, 1995; Lincoln et al., 2006; Schroeder et al., 2003).

To expand our insight into the development of the AV junction, and to create a database of differentially expressed genes at a critical stage of cushion development, we performed a DNA microarray analysis on AV and ventricular tissues of the mouse heart at embryonic day (ED) 10.5–11.0. In this paper, we focus on one of the genes, cartilage link protein 1 (*Crtl1*), also known as "hyaluronan and proteoglycan link protein 1 (HAPLN1)". In non-cardiac tissues, Crtl1 is best known for stabilizing the interaction between HA and proteoglycans such as versican and aggrecan (Binette et al., 1994; Matsumoto et al., 2006), and has recently been described to be expressed in AV cushion mesenchyme by others (Lincoln et al., 2007). The data presented here demonstrate that Crtl1 plays an important role in cardiac development, as perturbation of Crtl1 expression leads to malformations in the valvuloseptal complex as well as in abnormalities of myocardial development.

Materials and methods

DNA microarray analysis

AV canal and ventricular tissues (VT) from eighty embryonic day (ED) 10.5–11.0 C57BL6 mouse hearts were isolated in RNase-free PBS, snap-frozen in liquid nitrogen, homogenized, and total RNA was isolated using the Qiagen RNeasy Mini Kit. Total RNA quality was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) with the RNA 6000 Pico LabChip kit (Agilent Technologies). RNA was precipitated using GenElute Linear Polyacrylamide (Sigma), treated with Promega RQ1 RNase-Free DNase, and purified using the Bio-Rad Aurum Total RNA Mini Kit and then precipitated for a final time. Total RNA was amplified and converted into biotin-labeled RNA using the RiboAmp® HS RNA Amplification Kit (Arcturus Bioscience, Inc., Mountain View, CA). Labeled cRNA was fragmented and then evaluated by Bioanalyzer to ensure appropriate size distribution (~35–200 nt). Each cRNA sample was divided and hybridized to two Mouse Expression 430A GeneChips (Affymetrix, Santa Clara, CA). Hybridization, post-hybridization washing, fluorescence staining, and scanning were performed at the MUSC Proteogenomics Facility (http://proteogenomics.musc.edu/) using Affymetrix instrumentation in accordance with Affymetrix protocols. The resulting hybridization data files have been deposited in the MUSC DNA Microarray database (Argraves PMID 14668234; http://proteogenomics.musc.edu/quickSite/musc_madb.php?page=home&act=manage) and can be accessed with the target identifiers _1093467013.966284, _1093467031.924285, _1093467386.772829 and _1093467564.815494. SOFT matrix data and raw hybridization data have also been deposited in the NCBI GEO database (series accession number GSE4903) in accordance with MIAME convention (Brazma: PMID 11726920).

Analysis of DNA microarray data

Absolute detection calls were calculated for all genes (probe sets) using Affymetrix MAS5.0 software. Hybridization data were normalized using the Bioconductor (Gentleman, PMID 15461798) implementation of Robust Multi-chip Average (Irizarry, PMID 12925520). Differentially expressed genes were defined by the following criteria: (1) the gene was expressed in either the AV or the ventricular region (VT) (i.e., it received "present" calls for both iterations of either AV or VT samples), and (2) the normalized hybridization values for the gene were greater than 2-fold different between AV and VT samples.

Mice

Heterozygous Crtl1 breeder pairs (B6.129-*Hapln1*^{tm1Nid}/Ucd; stock #: MMRRC:000041-UCD) were obtained from the Mutant Mouse Regional Resource Centers (MMRRC). Crtl1 mice were genotyped essentially as described before (Czipri et al., 2003) using the following primer sets: LP12 (5′ taa tga cct ttc ctg tct ctc c 3′) and LP13 (5′ ccc aaa acc cgt agt tcc 3′), generating a PCR product of 283 bp in wild-type alleles, and Neo1013 (5′ gga tcg gcc att gaa caa g 3′) and Neo1014 (5′ cac cat gat att cgg caa gc 3′) generating a product of 600 bp, indicating the cloning vector inserted in the *Crtl1* gene to create the "knockout gene" (Watanabe and Yamada, 1999). *Hdf* mice were genotyped as described previously (Yamamura et al., 1997).

Histology and Immunohistochemistry

Specimens from Crtl1$^{+/-}$/Crtl1$^{+/-}$ matings were collected and staged according to Theiler (1989). Embryos were fixed in 4% paraformaldehyde (PFA) for 4 h. Tissue processing, hematoxylin/eosin staining, and immunohistochemistry were performed as previously described (Waller and Wessels, 2000). The following monoclonal antibodies were used: 9/30/8-A-4 (Developmental Studies Hybridoma Bank), recognizing Crtl1 (Caterson et al., 1985; Neame et al., 1986), A2172 (Sigma) recognizing sarcomeric actin, and sc-56 (Santa Cruz) recognizing PCNA. The polyclonal antibody AB1033, recognizing the beta GAG domain of versican (Snow et al., 2005), was purchased from Chemicon International. Hyaluronan was detected using biotinylated hyaluronan-binding protein (HABP) (Seikagaku Corporation, catalog number 400763) as described previously (Kern et al., 2006, 2007). For fluorescent detection of the primary antibodies, Alexa Fluor 568 (A-11011 goat anti-rabbit IgG from Molecular Probes) and Alexa Fluor 488 (A-11001 Goat anti-mouse IgG from Molecular Probes) were used. HABP binding was visualized with Streptavidin–fluorescein RPN 1232 from Amersham Life Science. Immunofluorescently stained sections were imaged using the Leica TCS SP2 AOBS confocal microscope system. For colometric visualization of primary antibody binding, rabbit anti-mouse (Sigma A9044) and goat anti-rabbit (Sigma A0545) peroxidase conjugated antibodies were used in combination with the Metal Enhanced Diaminobenzidine (DAB) Substrate Kit (Pierce, Rockford, IL; product number 34065).

Tie2-cre β-galactosidase staining and AMIRA reconstruction

β-Galactosidase staining was performed as described before (Snarr et al., 2007). Embryos were isolated and fixed in ice-cold 4% PFA. Embryos were then washed in ice-cold PBS and incubated in permeabilization buffer (0.02% sodium-deoxycholate, 0.01% NP-40, 1× PBS) overnight. Expression was visualized by incubating embryos in X-gal stain solution overnight at 37 °C (5 μM K-ferricyanide, 5 μM K-ferrocyanide, 2 μM $MgCl_2$, 1 mg/ml X-gal, 1× PBS). The tissue was post-fixed in 4% PFA and processed for histochemistry as described above. Amira three-dimensional reconstruction was performed as described previously (Snarr et al., 2007).

In situ hybridization

Whole-mount *in situ* hybridization was performed as previously reported (Norris et al., 2005). Probes were designed using specific primers for Crtl1 mRNA (CRTL1 forward: 5′ tggaccaggacgcagtgatt; CRTL1 reverse: 5′ gcagcggtcatagcccagaa). Total RNA was isolated from wild-type ED10.5 C57BL6 mouse embryos using the Qiagen RNeasy MiniKit. Specific cDNA