Endothelial deletion of murine Jag1 leads to valve calcification and congenital heart defects associated with Alagille syndrome

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SUMMARY
The Notch signaling pathway is an important contributor to the development and homeostasis of the cardiovascular system. Not surprisingly, mutations in Notch receptors and ligands have been linked to a variety of hereditary diseases that impact both the heart and the vasculature. In particular, mutations in the gene encoding the human Notch ligand jagged 1 result in a multisystem autosomal dominant disorder called Alagille syndrome, which includes tetralogy of Fallot among its more severe cardiac pathologies. Jagged 1 is expressed throughout the developing embryo, particularly in endothelial cells. Here, we demonstrate that endothelial-specific deletion of Jag1 leads to cardiovascular defects in both embryonic and adult mice that are reminiscent of those in Alagille syndrome. Mutant mice display right ventricular hypertrophy, overriding aorta, ventricular septal defects, coronary vessel abnormalities and valve defects. Examination of mid-gestational embryos revealed that the loss of Jag1, similar to the loss of Notch1, disrupts endothelial-to-mesenchymal transition during endocardial cushion formation. Furthermore, adult mutant mice exhibit cardiac valve calcifications associated with abnormal matrix remodeling and induction of bone morphogenesis. This work shows that the endothelium is responsible for the wide spectrum of cardiac phenotypes displayed in Alagille Syndrome and it demonstrates a crucial role for Jag1 in valve morphogenesis.

KEY WORDS: Aortic valves, Cardiac valves, EndMT, Notch, Mouse

INTRODUCTION
As the first functional organ during embryonic development, the heart is formed through a complex series of multi-lineage cell interactions that coordinate proliferation, migration and differentiation (Olson, 2006; Srivastava, 2006). The requirement for a highly regulated balance of heterotypic signaling events is evident after one or more genes are lost, leading to dire consequences for cardiac development. In fact, congenital heart malformations account for nearly one-third of all major congenital defects, which remain the leading cause of infant mortality in the United States (Mathews and MacDorman, 2011; van der Linde et al., 2011). Thus, a concrete understanding of the regulatory circuitry of interactions during cardiac development is essential. Over the last two decades, significant strides have been made towards accomplishing this goal; however, the picture is far from complete.

Several congenital vascular diseases are associated with mutations in the Notch signaling pathway, including Alagille syndrome (AGS), cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) and calcific aortic valve disease (Gridley, 2010; High and Epstein, 2008; MacGrogan et al., 2011; Nemir and Pedrazzini, 2008; Penton et al., 2012). The initial link between the Notch ligand jagged 1 (Jag1) and heart development was established in 1997, with the simultaneous discovery by two groups that AGS was caused by mutations in the human JAG1 gene (Penton et al., 2012). AGS is an autosomal dominant multisystem disorder that leads to paucity of intrahepatic bile ducts, as well as cardiac, skeletal, renal, facial and ocular abnormalities. Subsequently, it was found that although the majority of AGS patients had mutations in JAG1, a small subset of individuals harbored mutations in the gene encoding the NOTCH2 receptor (McDaniell et al., 2006).

Over the last decade, many investigations have focused on mapping JAG1 mutations and determining its expression patterns to understand better the function of this gene in the context of the disease (Bauer et al., 2010; Cronnier et al., 2000; Eldadah et al., 2001; Gridley, 2010; High and Epstein, 2008; Jones et al., 2000; Krantz et al., 1999; Loomes et al., 1999; McDaniel et al., 2006; Raas-Rothschild et al., 2002; Rutenberg et al., 2006). Elegant studies using cell-specific loss of function have revealed the involvement of Jag1 in the closure of the ductus arteriosus and in several aspects of cardiac morphogenesis (Feng et al., 2010; High et al., 2009; Manderfield et al., 2012). Particularly during heart development, Jag1 is expressed in the coronary arteries, secondary heart field and neural crest cells (de la Pompa and Epstein, 2012; Penton et al., 2012). Inactivation of the gene within these compartments yields cardiac outflow and aortic arch artery defects resulting in lethality by embryonic day (E) 10.5 (High et al., 2009;
Manderfield et al., 2012). The defects, however, do not recapitulate those displayed by patients with AGS or aortic valve disease. It is likely that the severe cardiac abnormalities displayed by these murine models might also result in lethality in humans.

To examine the contribution of endothelial Jag1 at later developmental time points than those evaluated previously, we used a *VE-Cadherin-Cre*-mediated excision of the gene. The resultant Jag1 mutant mice displayed a spectrum of cardiac defects that were highly reminiscent of those present in AGS, including ventricular septal defects (VSD), overriding aorta, right ventricular hypertrophy, valve malformations and stenosis. Through a combination of genetic, histological and organotypic ex vivo studies, we showed that the loss of Jag1 in the endothelium leads to a blockage of endothelial-to-mesenchymal transformation (EndMT), an essential step in formation of the atrioventricular (AV) valves and septum intermedium. In addition, at later stages of heart development, Jag1 is required for proper valve remodeling. Absence of Jag1 results in myxomatous valves owing to the accumulation of uncleaved versican. Furthermore, 50% of mutant mice developed valve calcifications with accumulation of bone between 5 and 13 months of age. These findings indicate that the cardiac defects manifested in AGS and other valve-specific defects are largely endothelial derived and are rooted in defective Jag1 signaling during early embryonic development.

**MATERIALS AND METHODS**

**Animals**

*VE-Cadherin-Cre* (Alva et al., 2006), *Jag1^floxed^* (Mancini et al., 2005) and *Notch1^floxed^* (Radtke et al., 1999) mice were previously described and bred for six generations on a C57BL/6J background. These mice were also crossed to R26R (Soriano, 1999) or TNR1 Notch reporter mice (Duncan et al., 2005). Embryos were collected using timed matings. PCR analysis on genomic DNA from embryos or tails was used for genotyping.

**Tissue collection and imaging**

Standard protocols for staining, immunochemistry and imaging were used with paraformaldehyde- or glutaraldehyde-fixed tissues (Alva et al., 2006). β-Galactosidase staining was performed as previously described (Alva et al., 2006). Standard protocols for Hematoxylin and Eosin (H&E), Nuclear Fast Red, Alizarin Red S and von Kossa staining were used with paraformaldehyde- or glutaraldehyde-fixed tissues (Alva et al., 2006). Standard protocols for staining, immunocytochemistry and imaging were carried out as previously described (Chen et al., 2010). Immunoprecipitation and western blot analysis were carried out as described (Zovein et al., 2010). For non-fluorescent immunostaining, tissues were incubated with primary antibodies and biotinylated secondary antibodies (Jackson ImmunoResearch Laboratories), and treated with Vectastain ABC Kit reagents (Vector Labs) followed by DAB substrate (Vector Labs). Alexa Fluor-conjugated (Invitrogen) or FITC-conjugated (Jackson ImmunoResearch Laboratories) fluorescent secondary antibodies were used and DAPI (Invitrogen; 1:1000) or TOPRO-3 (Molecular Probes; 1:300) were used for counterstaining. Tissues were analyzed using a Zeiss LSM multichannel microscope (Carl Zeiss AG) and image processing was carried out using Adobe Photoshop CS5 (Adobe Systems).

**Cardiac physiology and imaging**

Mice were anesthetized with 2% isofluorane, and heart rate and body temperature were monitored during Doppler and 2D imaging. For microcomputed tomography (μCT) imaging, hearts were perfused with 1× PBS, fixed with 4% paraformaldehyde, stained with a contrast agent, and scanned at 10 μm isometric voxel resolution at 200 μm/s exposure time (volumetric μCT scanner μCT40 from ScanCo Medical, Numira Biosciences, Salt Lake City, UT, USA; Virtual Histology). Doppler and 2D imaging of the parasternal long-axis and short axis were performed by echocardiography using a Vevo 770 Imaging System (Visual Sonics, Toronto, Canada) that was equipped with the RMV-707B transducer (30 MHz 12.7 mm focal length), with the placement of the Doppler probe at 65° with coronal plane as described (Liao et al., 2001).

**Cardiac explants**

E9.5 atrioventricular cardiac cushions (20-28 somites) were dissected and explanted as described (Camenisch et al., 2002; Runyan and Markwald, 1983). N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT; 10 μM or 50 μM, diluted in DMSO) or 0.2% DMSO (controls) was added to wild-type explants. Media, with/without DAPT and DMSO, was changed daily. Explants were photographed in 12-hour increments. Samples were stained with anti-α-SMA, anti-PECAM1 and TOPRO-3 and imaged. For treatment with y-secretase inhibitors, wild-type explants were adhered to type I collagen gels for a minimum of 6 hours before adding DAPT or DMSO. Two independent observers counted total nuclei embedded within the collagen matrix in selected areas and the percentage of transformed mesenchymal cells (αSMA⁺, PECAM1⁻ expression). P was calculated using Student’s two-tailed t-test for paired comparisons and one-way ANOVA with the Bonferroni correction was applied when analyzing multiple (three) groups.

**Quantitative real time PCR**

Postnatal day (P) 10 mouse outflow tract (OFT) and AV valves were isolated under a dissecting microscope. Total RNA from OFT and AV valves was purified using the RNeasy Mini Kit (QIAGEN, Valencia, CA, USA). Complementary DNA synthesis from 15-25 ng of total RNA was performed using SuperScript III (Invitrogen). Quantitative RT-PCR was then performed on a DNA Engine Opticon 2 Real-Time Cycler PCR (MJ Research; Biorad, Hercules, CA, USA) using the RT² SYBR Green qPCR Master Mix (SA Biosciences; QIAGEN). Primer sequences are listed in supplementary material Table S1. Results were analyzed with MJ Opticon Monitor Analysis software Version 3.1 (MJ Research; Biorad). Each reaction was run in duplicate and normalized with the housekeeping gene *Hprt*. Data are presented relative to mean ΔCT of wild-type animals. *P* was calculated using the Student-Fisher test.

**FACS, western blot analysis and immunoprecipitation**

Endothelial cells were fluorescence-activated cell sorted (FACS) from E12.5, E13.5, E15.5, E17.5 and P10 *Jag1*^WT^ and *Jag1*^ECKO^ embryos using anti-PECAM antibodies, as previously described (Zovein et al., 2010). All samples were stained for viability with 7-AAD (BD Pharmingen) and analyzed on a FACSCaliber with the appropriate isotype controls. Endothelial cell protein lysate from *Jag1*^WT^ and *Jag1*^ECKO^ embryos was separated by SDS-PAGE, quantified and subjected to western blot analysis using anti-Jag1 (Santa Cruz Biotechnology) (1:800). Recovery of the immunocomplexes and western blot were carried out as previously described (Chen et al., 2010).

**RESULTS**

**Temporal loss of Jag1 after endothelial-specific deletion**

We have previously reported that an endothelial-specific deletion of *Jag1* using a *VE-Cadherin-Cre* line, termed *Jag1* endothelial cell knockout (*Jag1*<sup>Cre</sup> mice, yielded viable mutants with normal bile duct development, but defective vascular smooth muscle cell recruitment (Hofmann et al., 2010; Scheppke et al., 2012). By contrast, previous reports of *Jag1*-specific endothelial deletion resulted in embryonic lethality at E10.5 (Benedito et al., 2009; High et al., 2008). We suspected that these differences might relate to the timing/efficiency of deletion conveyed by each Cre line. In fact, evaluation of temporal loss of Jag1 in *Jag1*<sup>Cre</sup> mice demonstrated sporadic loss of the protein by E10.5 (Fig. 1A, arrowheads). By P0, expression of Jag1 protein was largely lost in the endothelium of the dorsal aorta (Fig. 1A, arrowheads). However, Jag1-/- was retained within the smooth muscle cells of the vascular wall (Fig. 1A, arrows).
To quantify more rigorously Jag1 changes in J1 ECKO mice, we performed western blot analysis on lysates from endothelial cells isolated at different stages of development (Fig. 1B). By E12.5, ~60% of Jag1 protein was lost, with a more significant decrease soon after at E13.5 (close to 90%), compared with littermate controls (Fig. 1B). The use of the Rosa26;lacZ reporter line (R26R) (Soriano, 1999) further revealed the temporal activity of VE-Cadherin-Cre at the cellular level and confirmed varying levels of Cre recombination at E10.5. Two extreme examples are shown in Fig. 1C. In mice with high recombination (Fig. 1C, upper panel), endothelial cells of major arteries were almost completely β-galactosidase-positive (Fig. 1C, arrows), with veins slightly less so (Fig. 1C, arrowheads). The endothelial cells (Fig. 1C, black arrows) and endothelial-derived mesenchymal cells of the cardiac cushions (Fig. 1C, red arrows) also showed a high degree of recombination. By contrast, mice with low levels of Cre recombination at a similar time point, exhibited only a few β-galactosidase-positive cells in the vessels and cardiac cushions of the heart (Fig. 1C, lower panels, black arrows). In fact, the majority of the arterial and venous endothelium, as well as the endocardium, was β-galactosidase-negative (Fig. 1C, black arrowheads), whereas few β-galactosidase-positive mesenchymal cells (Fig. 1C, red arrows) were present alongside β-galactosidase-negative cells (Fig. 1C, red arrowheads). The variable penetrance of this particular VE-Cadherin-Cre in early development (E10.5), as well as the mixed C57Bl6 F6 background of the mice, explain the wide range of lethality observed in J1 ECKO mice, reconcile these findings with previous groups and, more importantly, provide an opportunity to explore the requirement for Jag1 in endothelial cells at different developmental stages and to determine the relevance of Jag1 deletion.

**Morphology and physiological analysis of adult J1 ECKO hearts revealed cardiac defects**

Although viable J1 ECKO mice were frequently recovered, several pups and adult mice succumbed to sudden death. A closer examination of surviving adult J1 ECKO hearts showed that mutant mice displayed a range of cardiac anomalies (Fig. 2). This was consistent with the mosaic deletion pattern of Jag1 early in development and mimicked the cardiac components of AGS. However, other organs frequently impacted by AGS, including the liver, eye, kidney and vertebrae, were not affected (Hofmann et al., 2010) (data not shown).

Common features in many adult J1 ECKO animals were macroscopic aberrations in heart morphology, including thin or disorganized coronary vessels (Fig. 2A, arrowheads), dilation noted near the apex of the heart (Fig. 2A, arrows) and abnormal positioning of the aorta and pulmonary trunk (not shown). Echocardiographic evaluations of a sampling of adult mice showed that J1 ECKO mice had increased systolic blood velocity with severe regurgitation at the pulmonic valve, compared with Cre-negative
littermates, indicating valve stenosis (Fig. 2B, white arrows). Mutants also exhibited significant increases in right ventricle area and had right atrial and ventricular hypertrophy, noted with the use of cardiac micro-computed tomography (μCT) imaging (Fig. 2B,C). Thickening of the right ventricular wall was probably a compensation due to the increased pressure load of the right ventricle caused by pulmonary outflow tract narrowing or defective valves, common in AGS patients (McElhinney et al., 2002; Otto, 2009). Additionally, μCT imaging revealed greater density and excessive branching of coronary vessels in many of the J1ECKO hearts (Fig. 2D,E), as would be expected for a Notch loss-of-function phenotype, even though the data did not reach significance.

**Embryonic and neonatal cardiac defects in J1ECKO mice**

Evaluation of J1ECKO hearts from embryos and neonates was also consistent with AGS-related cardiac defects, including many hallmarks of tetralogy of Fallot (TOF): VSDs, overriding aorta and right ventricular hypertrophy, as well as valve pathology (Table 1; Fig. 3). Upon gross evaluation, the morphology of neonatal J1ECKO hearts was often striking, with anomalies in the structure (Fig. 3A, arrows) and patterning of the coronary vasculature, including fragility and hemorrhaging (Fig. 3A, arrowheads), as well as outflow tract defects (Fig. 3B, dotted lines). Histological analysis revealed VSDs (Fig. 3C, D, arrows) and large, hyperplastic (myxomatous) valves in J1ECKO mice (Fig. 3C, asterisk). The ventricular septum of mice typically divides the ventricles at E13.5; however, examination of embryonic J1ECKO hearts showed an incomplete septum at E14.5 (Fig. 3D, arrow). Bulges near the valve annulus were seen in some neonatal J1ECKO mice with otherwise normal valve leaflet development (Fig. 3E, arrow). Macroscopic analysis of 46 embryonic or neonatal J1ECKO mice showed that one or more cardiac abnormalities were present in 47.8% of the mice evaluated, with overriding aorta being the most common defect (Table 1).
Defects in endocardial cushion formation in J1ECKO and N1ECKO mice

Cushion development requires a specialized form of epithelial-to-mesenchymal transition (EMT) called endothelial-to-mesenchymal transformation (EndMT), a process that is initiated in the mouse at E9.5 (de la Pompa and Epstein, 2012). The events associated with EndMT include: endocardial cell delamination from a flattened monolayer, transformation of endothelial cells into mesenchymal cells, migration and invasion into the cardiac jelly, and rapid proliferation (Armstrong and Bischoff, 2004; Eisenberg and Markwald, 1995; Person et al., 2005; Schroeder and Listgarten, 2003). Given the phenotype of J1ECKO mice, we sought to examine the hearts of J1ECKO mice in greater detail, and compared them with VE-Cadherin-Cre; Notch1 flox/flox mice (N1ECKO) during cardiac cushion remodeling at E10.5 (Fig. 4).

The AV cushions of J1ECKO hearts at E10.5 showed rounded endocardial-derived cells (Fig. 4B,C, arrowheads) that accumulated at the cushion interface (Fig. 4B,C, red arrows) and less dense, hypoplastic areas present within the cushions (Fig. 4B,C, open arrows). This was in contrast to the high transformation of cushions from VE-Cadherin-Cre;R26R hearts at E10.5 (Fig. 4A). Overall, EndMT was at least temporally delayed in the J1ECKO hearts, with fewer transformed mesenchymal cells present in the AV cushions.

Consistent with published findings on global deletion of Notch1 or pharmacological inactivation of the Notch pathway (Fischer et al., 2007; Timmerman et al., 2004), endothelial deletion of Notch1 in N1ECKO hearts also resulted in EndMT arrest (Fig. 4D) and phenocopied the J1ECKO hearts, with rounded cells present at the cushion interface (Fig. 4D, red arrows) and areas with few

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**Table 1. Macroscopic embryonic and neonatal cardiac defects in J1ECKO mice**

<table>
<thead>
<tr>
<th>Number of animals</th>
<th>Percentage of J1ECKO mice affected</th>
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</thead>
<tbody>
<tr>
<td>J1ECKO animals with at least one cardiac defect</td>
<td>22</td>
</tr>
<tr>
<td>Overriding aorta</td>
<td>14</td>
</tr>
<tr>
<td>Abnormal coronary vessels</td>
<td>6</td>
</tr>
<tr>
<td>Enlarged heart</td>
<td>3</td>
</tr>
<tr>
<td>Enlarged right ventricle</td>
<td>2</td>
</tr>
<tr>
<td>Abnormal outflow tract</td>
<td>1</td>
</tr>
<tr>
<td>Non-obvious macroscopic defect</td>
<td>24</td>
</tr>
<tr>
<td>Total number of J1ECKO animals examined</td>
<td>46</td>
</tr>
</tbody>
</table>

Some form of cardiac defect, including overriding aorta, abnormal coronary vessel patterning, enlarged heart and enlarged right ventricle, was shown in 47.8% of embryonic and neonatal J1ECKO mice from 16 litters. Overriding aorta was the most common macroscopic defect affecting these mutant mice.

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**Fig. 3. AGS-related cardiac defects in embryonic and neonatal J1ECKO mice.** (A-C) Light microscopy of the back (A) and front (B) of neonatal J1WT and J1ECKO hearts (n>20). (A) Compared with J1WT mice, hearts of P0 J1ECKO mice displayed a variety of cardiac defects, such as abnormal morphology and increased atrial and ventricular size (A, arrows), in addition to dilated, hemorrhaging cardiac vessels (A, arrowheads). (B) P1 hearts of J1WT and J1ECKO mutants, with corresponding H&E-stained histological sections of the respective hearts below (C). An overriding aorta was noted in the J1ECKO heart (B, arrow). Dotted lines delineate great vessels and highlight outflow tract defects. (C) Histological sections of these J1ECKO hearts, with high magnification images below, revealed ventricular septal defects (arrows) and hypertrophic valves (asterisk). (D) Light microscopy of β-galactosidase-stained histological sections of E14.5 VE-Cadherin-Cre;Rosa26R wild-type and J1ECKO hearts revealed VSDs in the mutant hearts (D, arrow) 24 hours after the septum normally closes, as seen in the wild-type heart (D). (E) Large bulges were occasionally found near the valve annulus between the left atria and ventricle in P5 J1ECKO H&E-stained hearts (E, arrow). ao, aorta; la, left atrium; lv, left ventricle; pa, pulmonary artery; ra, right atrium; rv, right ventricle. Scale bars: 200 μm.
transformed mesenchymal cells (Fig. 4D, open arrows). Similar to the AV cushions, the OFT or semilunar cushions, which form the aortic and pulmonary valves, also showed delayed EndMT in both the J1\textsuperscript{ECKO} and N1\textsuperscript{ECKO} mice (Fig. 4E-H). Some β-galactosidase-negative mesenchymal cells were present in the cardiac jelly of J1\textsuperscript{ECKO} mice, demonstrating that EndMT was able to occur in areas without VE-Cadherin-Cre expression (Fig. 4F,G, arrows). However, in regions in which endocardial cells were β-galactosidase positive, EndMT was disrupted (Fig. 4F-H, open arrows). As mentioned previously, the survival of the J1\textsuperscript{ECKO} mice and the wide range in phenotypes can be explained by the slow penetration of VE-Cadherin-Cre recombination at crucial times during EndMT (Fig. 1B).

**J1\textsuperscript{ECKO} hearts demonstrate impaired EndMT in vitro due to a loss of Notch signaling**

To investigate the cellular events associated with the loss of Jag1, we explanted cardiac cushions from the AV canal of E9.5 J1\textsuperscript{ECKO} mice for 48-72 hours and evaluated EndMT as previously described (Eisenberg and Markwald, 1995; Lakkis and Epstein, 1998). AV explants from J1\textsuperscript{WT} mice had outgrowths of elongated αSMA (Acta2 – Mouse Genome Informatics)-positive cells that invaded the collagen gel, indicative of endocardial cells transforming into mesenchymal cells (Fig. 5A, arrows). Loss of Jag1 in the endothelium resulted in a persistence of Pecam1-positive endocardial cells (Fig. 5A, arrowheads) and αSMA-negative cells (untransformed) that grew out of the explant, but remained on the surface of the collagen gel as a sheet (Fig. 5A, bracket). Less than half of J1\textsuperscript{ECKO} endocardial cells were transformed (Fig. 5B). Thus, the loss of only endothelial Jag1 resembled blockade of all Notch reporter signaling via the γ-secretase inhibitor N-[N-(3,5-difluorophenacetyl)-l-alanyl]-s-phenylglycine t-butyl ester (DAPT) in wild-type cushions (Fig. 5A,C) (Timmerman et al., 2004). EndMT disruption was noted in all explants from J1\textsuperscript{ECKO} embryos (7/7 mice), indicating a crucial role for endothelial Jag1 early in this process.

Jag1 can function as an antagonistic ligand to Delta-like 4 (Dll4) in the endothelium (Benedito et al., 2009). Thus, it was important to understand whether absence of Jag1 in the endocardium resulted in blockade or activation of Notch signaling. Using a Notch GFP reporter mouse line crossed to the J1\textsuperscript{ECKO} mice, we found that removal of Jag1 in the endoderm led to a loss of Notch signaling in the endocardial cushion interface and within transformed mesenchymal cells (Fig. 5D). J1\textsuperscript{ECKO} endocardial cells (GFP+), retained Pecam1 expression and were stacked several layers thick, whereas the cardiac jelly remained less densely populated (Fig. 5D, open arrow). By contrast, Notch reporter expression in vessels of J1\textsuperscript{ECKO} mice remained similar to littermate controls (Fig. 5E, arrows). This indicates that the cardiac endothelium of J1\textsuperscript{ECKO} mice display a loss-of-function phenotype, whereas other endothelial populations probably have compensation from additional Notch ligands, such as Dll4.

As the majority of J1\textsuperscript{ECKO} mice survive to adulthood, it suggests an eventual compensation for the loss of Jag1, a modifier effect from the genetic background, or incomplete deletion when EndMT is occurring. However, this fortuitous feature resembles the phenotype of the cardiac defects in human AGS disease and provides a broader picture of the multiple effects of Jag1 at later stages of cardiovascular development.

**Jag1 is required for valve morphogenesis**

The cardiac cushion defects present in J1\textsuperscript{ECKO} and N1\textsuperscript{ECKO} hearts, as well as the link to calcific aortic valve disease in humans and animal models, prompted a more detailed evaluation of the valves in surviving J1\textsuperscript{ECKO} mice (Acharya et al., 2011; Garg et al., 2005; Nigam and Srivastava, 2009). As valve leaflets mature, they initially swell up and are composed of layers of extracellular matrix proteins, which are then remodeled and form tapered and thin structures (Hinton and Yutzey, 2011). However, valves of J1\textsuperscript{ECKO} hearts exhibited defective remodeling, as they remained myxomatous, or club-shaped (Fig. 3; Fig. 6).
To investigate the mechanism behind the abnormal valve thickening in J1ECKO hearts, we first determined whether there were differences in cell number (Fig. 6C,D). In neonatal animals (P0), no significant changes in the number of cells were found in J1ECKO valve leaflets compared with J1 WT. By P10, a point at which the valve thickening was more pronounced in J1 ECKO mice, nuclear density was lower in J1ECKO compared with J1WT valves (Fig. 6C). We found decreased cellularity in the central region of the valve leaflet (spongiosa) in P10 J1ECKO animals (Fig. 6D). These data suggested that the thickening of heart valves in J1ECKO animals is likely to be due to changes in the extracellular matrix (ECM), rather than in cell density.

The composition, stoichiometry and organization of ECM proteins are essential for heart valve morphogenesis and function (Armstrong and Bischoff, 2004). As the spongiosa zone of the valves is rich in versican (Kern et al., 2006; Yamamura et al., 1997), we performed immunodetection analysis of the GAG $\beta$ isoform on heart cross-sections at P10. In all animals examined, versican levels, indicated by a pseudocolor scale, were increased in the valve leaflet of J1ECKO mice compared with J1 WT animals (Fig. 7A).

To elucidate further the molecular events accounting for the increase in versican in J1 ECKO mice, we evaluated transcript expression profiles of OFT and AV valves at P10 (Fig. 7). As expected, Jag1 expression was decreased in both OFT and AV valve leaflets in J1 ECKO animals (by ~70%). By contrast, Notch1 and Dll4 were equivalent in J1 ECKO and J1 WT (Fig. 7B). Interestingly, mRNA expression of relevant versican isoforms...
(V0/V2 and V1) revealed no significant variability in levels associated with Jag1 deletion (Fig. 7C). This observation indicates that increased versican in J1ECKO valves cannot be accounted for by overexpression of the proteoglycan. We then focused on alterations in post-translational processing of versican by evaluating relevant proteases and their inhibitors. The metalloproteases Adamts1 and Adamts9 are known to cleave versican physiologically, and Timp3 inhibits both proteases (Rodríguez-Manzaneque et al., 2002; Sandy et al., 2001). All three molecules are expressed in the heart at the pertinent time points (Kern et al., 2007; Thai and Iruela-Arispe, 2002). In J1ECKO mice, Timp3 showed a consistent, yet low increase in expression compared with J1WT. Adamts1 was not statistically different between mutant and controls. By contrast, Adamts9 expression was significantly reduced in both OFT and AV valves of J1ECKO mice (Fig. 7D). Thus, it appears that the accumulation of versican in J1ECKO valves is likely to be due to limited versican turnover and deficient valve remodeling.

To confirm these findings, we performed immunostaining of valves with antibodies that recognize a neoeptope sequence of versican (DPEAAE) generated after cleavage by matrix metalloproteases, including Adamts (Sandy et al., 2001; Somerville et al., 2003). Cleaved versican in J1WT valves was weak, but was detected throughout the leaflet. By contrast, processed versican was mainly noted in the peripheral areas of J1ECKO valves (Fig. 7E). Thus, Jag1 deletion in the endothelium results in decreased Adamts9 and defects in versican degradation, leading to arrested valve remodeling and myxomatous valves.

**Cartilage nodules and calcification in adult J1ECKO valves**

Given that sudden lethality was observed in adult J1ECKO mice and that Notch1 mutations are linked to aortic valve calcification (Garg et al., 2005), we further examined valves in mutant and control littermates at several adult stages (5-13 months). Histological analysis revealed increased OFT valve leaflet width (Fig. 8A, bracket).
Interestingly, we also detected the presence of cartilage nodules (red arrowheads) and bone (black arrowheads) in the annulus (Fig. 8A, dotted circle) and/or in the body of aortic valve leaflets (Fig. 8A). Even though the animals exhibited no outward signs of disease, we found a 50% frequency (5/10 J1ECKO mice, 0/10 controls) of chondrogenic nodules or bone tissue in J1ECKO animals, whereas no such abnormalities were noted in Cre-negative littermates. Von Kossa and Alizarin Red staining of heart sections confirmed bone dysplasia and high levels of calcium deposition in the valves of adult J1ECKO animals (Fig. 8B, black arrowhead; supplementary material Fig. S1). Calcium deposits were more evident in the annulus, rather than in the leaflet of the valves (Fig. 8B).

To determine the cellular origin of the abnormal tissue, we performed lineage analysis of VE-Cad-Cre;R26R animals. Although it is well accepted that valve fibroblasts originate from the endothelium, lineage analysis in older adult mice has not been performed. Both the annulus and valve leaflets were largely positive for Cre activity, denoted by β-galactosidase staining (Fig. 8C, red arrows). Thus, the majority of cells located in both the leaflets and the annulus are derived from an endothelial progenitor and in J1ECKO mice are, therefore, Jag1 deficient. These data strongly support that, in addition to its role in early heart development, Jag1 signaling is required postnatally to prevent valve calcification (Fig. 9).

**DISCUSSION**

The results of this study demonstrate a crucial role for Jag1 in the endocardium during heart development and provide a new clinically relevant model for the human cardiac phenotypes associated with AGS and valve disease. Loss of Jag1 or Notch1 in the endothelium using VE-Cadherin-Cre, led to disruption of EndMT in the AV canal and OFT cushions. In addition, 50% of the mutants that survived to adulthood exhibited valve defects and calcification. Together, these data indicate that endothelial Jag1 affects distinct temporal stages of heart development through diverse and non-overlapping mechanisms.

The inherent complexity of a multisystem syndrome such as AGS makes the identification of the cells responsible and the treatment of the disease problematic. Other endothelial-specific Jag1 knockout mice succumb to lethality by E10.5, similar to global inactivation of Jag1 (Benedito et al., 2009; High et al., 2008; Xue et al., 1999). Although these endothelial cell Jag1-deficient mice demonstrated pericardial edema and defects in smooth muscle cell recruitment, major cardiac defects, such as those associated with AGS, were not found in those models owing to the early lethality (Nemir and Pedrazzini, 2008). Although much has been done to develop a mouse model that faithfully reproduces AGS, introduction of the human mutations in mice do not yield the clinical features of AGS. Specifically, mice with loss of heterozygosity or harboring missense mutations of Jag1 exhibit only a few characteristics of AGS with very mild severity or weak penetration (Vrijens et al., 2006; Xue et al., 1999). Only doubly heterozygous Jag1;Notch2 mice reconstitute a more human-like AGS model that shows both major hepatic and cardiac defects (McCright et al., 2002). As a result of its later deletion, the J1ECKO model presented here recapitulates, exclusively, many of the...
cardiac defects of AGS, avoiding potential complications caused by liver dysfunction. In fact, as an autosomal dominant disorder, the phenotype displayed in human AGS appears to be mimicked in mice by progressive loss of function when it is manifested after gestation (E9.5-14.5), Jag1 is crucial for EndMT with essential contributions to morphogenesis of the interventricular septum and AV, aortic and pulmonary valves. In addition, Jag1 regulates valve remodeling postnatally by affecting ECM dynamics and by preventing valve calcification.

before the coronary plexus starts forming at E11.5 (Benedito et al., 2009; High et al., 2008; Xue et al., 1999). Because the coronary vessels originate primarily from VE-Cadherin-positive endothelial sprouts of the sinus venosus, with a secondary source coming from the endocardium, and because Jag1 is known to be expressed in both areas, our data suggest a role for Jag1 in the proper formation and integrity of the coronary vasculature (de la Pompa and Epstein, 2012; Penton et al., 2012; Red-Horse et al., 2010). The increased vessel branching demonstrated by μCT imaging was fairly unexpected and intriguing, as inducible loss of endothelial Jag1 led to decreased branching in postnatal retinas (Benedito et al., 2009).

Additionally, recent studies demonstrate a requirement of endothelial Jag1 for mural cell development and differentiation, which warrants a thorough examination of these vessels and their smooth muscle and pericyte coverage (High et al., 2008; Liu et al., 2009; Scheppke et al., 2012).

The contribution of the Notch receptor and its downstream targets to EndMT has long been acknowledged; however, the Notch ligand and the specific cell type responsible for these events had not been definitively identified (de la Pompa and Epstein, 2012). Although Dll4 mutants display mild EndMT defects in the outflow tract cushions (Trindade et al., 2008), the intensity and features displayed with the absence of Jag1 clearly indicate that this is the most relevant ligand in EndMT. Interestingly, Jag1 has been associated with EMT in multiple systems. For example, in cultured epithelial cells from normal tissues and breast cancer lines, as well as human microvascular endothelial cells, Jag1 has been implicated in the cascade of signaling required for EMT (Leong et al., 2007; Noseda et al., 2004; Zavadil et al., 2004). In the secondary heart field, Notch and Jag1 regulate Fgf8 expression in a non-cell-autonomous manner during OFT EndMT (High et al., 2009).

The present study also offers insights into the role of Jag1 in valve remodeling. Mutations in NOTCH1 have been previously associated with congenital aortic valve malformations and calcification (de la Pompa and Epstein, 2012; Garg et al., 2005; MacGrogan et al., 2011). In addition, there is accumulating evidence that Notch signaling inhibits aortic valve calcification (Acharya et al., 2011; Garg et al., 2005; Nigam and Srivastava, 2009). Nonetheless, the specific contribution of Notch signaling to valve organization and ECM remodeling was unclear. Our findings indicate that Jag1 is a crucial Notch ligand in valves and its
inactivation results in a high incidence of valve calcification. Furthermore, we found an imbalance in versican cleavage that could be explained by reduced expression in \textit{Adams9}, the gene encoding a relevant protease for versican processing and turnover. Interestingly, mice heterozygous for \textit{Adams9} exhibit reduced versican cleavage, myxomatous valves, and development of cartilaginous valve nodules (Kern et al., 2010). The adequate balance of matrix proteins in valve remodeling is essential to its morphogenesis and function. Mice lacking a specific subset of ECM molecules succumb to lethality associated with valve disease (for a review, see Hinton and Yutzey, 2011).

The findings presented here indicate that, at least in the valves, Jag1 is an important regulator of ECM organization that is crucial for valve compliance. As determining the factors involved in congenital heart malformations and valve disease is of high clinical relevance, these findings should provide new avenues of investigation, while answering several long-standing questions about the role of Jag1 in heart development and disease.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

Supplementary material available online at [http://dev.biologists.orglookup/suppl-doi:10.1242dev.084871-V1-DC1](http://dev.biologists.orglookup/suppl-doi:10.1242dev.084871-V1-DC1)

References


**Fig. S1. Jagged1 deletion in endothelial cells is associated with calcification of adult heart valves.** Adult J1\textsuperscript{WT} and J1\textsuperscript{ECKO} OFT valve serial cross-sections, following those presented in Fig. 8A,B, were stained with Alizarin Red S. Red precipitate detection confirms the presence of calcium deposits in the valve annulus of the J1\textsuperscript{ECKO} animals (black arrows), whereas no deposits were observed in the J1\textsuperscript{WT} mouse valves. Scale bar: 100 μm.

**Table S1.** Primer sequences for mouse cDNA amplification

<table>
<thead>
<tr>
<th>Target cDNA</th>
<th>Forward sequence (5’-3’)</th>
<th>Reverse sequence (5’-3’)</th>
</tr>
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<tbody>
<tr>
<td>Adamts1</td>
<td>TGACTGCTCCAGGACTTGTG</td>
<td>TTGTAGGCAGCACACTGCTC</td>
</tr>
<tr>
<td>Adamts9</td>
<td>CTTCTACACCAACCCCTGGA</td>
<td>GTCCTGGATGCGAGGTTCATT</td>
</tr>
<tr>
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<tr>
<td>Hprt</td>
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<td>CGAGAGGTCTCTTTTCACCAGC</td>
</tr>
<tr>
<td>Jag1</td>
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</tr>
<tr>
<td>Notch1</td>
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<tr>
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<tr>
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